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Investigating a potential mechanism of Cd resistance in *Chironomus riparius* larvae using kinetic analysis of calcium and cadmium uptake

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ABSTRACT

The uptake kinetics of waterborne Ca and Cd, both independently and in combination, were examined in *C. riparius* larvae, which are extremely Cd tolerant. Larvae exposed to Ca (100–2500 μmol L^{-1}), exhibited classic Michaelis–Menten saturation kinetics for Ca influx, measured using ^{45}Ca as a radio-tracer. The maximum rate of Ca influx (J_{max}^{Ca}) was 0.39 μmol g^{-1} h^{-1}, and the Ca concentration where the carrier reached half saturation (K_M^{Ca}) was 289 μmol L^{-1}. Cd influx was measured using ^{109}Cd as a radio-tracer in larvae exposed to Cd (0–1400 μmol L^{-1}) while the Ca concentration was set to the K_M^{Ca}. This revealed a J_{max}^{Cd} (2.26 μmol g^{-1} h^{-1}) which was nearly 6-fold higher that of Ca. This unusually high capacity for Cd uptake is in accordance with the huge tissue Cd burdens that chironomid larvae are able to accumulate during high level exposures. The apparent K_M^{Cd} (1133 μmol Cd L^{-1}), when recalculated to account for the background Ca level, was still high (567 μmol Cd L^{-1}), suggesting that this organism has a low affinity for Cd relative to most aquatic animals, indeed lower or comparable to its affinity for Ca. In consequence, even well above environmentally relevant Cd exposures, *C. riparius* does not accumulate Cd at the expense of Ca, thereby avoiding internal hypocalcaemia, in contrast to most other organisms which are much more sensitive to Cd. However, Ca influx was significantly reduced when 1200 μmol Cd L^{-1} was added to Ca exposures (96–2410 μmol L^{-1}). Michaelis–Menten analysis revealed a similar J_{max}^{Ca} in Cd-exposed and control larvae (i.e. exposed only to Ca), but that the apparent K_M^{Ca} was many-fold higher in larvae which were simultaneously exposed to Ca and Cd. Conversely, increasing Ca concentrations (96–2410 μmol L^{-1}) progressively inhibited Cd uptake from a Cd exposure concentration (1200 μmol L^{-1}), providing additional support for a common transport system. These results suggest that the interaction of Cd and Ca in *C. riparius* is one of simple competitive interaction, and that the unusual Cd transport kinetics (low affinity, high capacity) relative to fairly standard Ca transport kinetics help explain the unusual tolerance that this organism has to acute Cd exposure.

1. Introduction

The aquatic larvae of midge flies, also known as chironomids, are tolerant to a range of adverse environmental conditions including near anoxia (Irving et al., 2004) and elevated metal contamination (Wentsel et al., 1978; Krantzberg and Stokes, 1989; Postma et al., 1996). The incredible Cd resistance of late instar *Chironomus riparius* larvae was demonstrated in an earlier study (Gillis and Wood, 2008) where the acute waterborne Cd LC50 (48 h exposures) of over 1 g L^{-1} (~10 mmol L^{-1}) illustrated that this organism is one of the most, if not the most, Cd-tolerant aquatic organisms studied (see also Béchard et al., 2008). The mechanism of acute Cd toxicity appears to be interference with whole-body calcium regulation resulting in hypocalcaemia, but this interference only occurs at industrial-type concentrations. Furthermore, during such exposures, Cd accumulates in the whole body of the organism to levels (>50 μmol g^{-1}) as much as 5-fold greater than normal whole-body Ca concentration (10–15 μmol g^{-1}). This raises interesting questions about the interaction of Ca and Cd uptake in this animal.

The mechanism of acute Cd toxicity has been well investigated in fish (reviewed by Wood, 2001) but less so in aquatic invertebrates. In freshwater fish, Verbost et al. (1987, 1988, 1989) provided evidence that Cd enters gill cells though apical Ca channels and then blocks the basolateral Ca-transporting ATPase pump so that active Ca influx becomes impaired. Overall, the nature of this interaction between Cd and Ca transport appears to be simple competitive inhibition (Chang et al., 1997, 1998; Niyogi and Wood, 2004). The high toxicity of Cd to freshwater fish, with acute LC50s usually in the

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low microgram per liter range (~nmol L^{-1}) range is explained by the fact that the affinity of the transport sites for Cd is 3–4 orders of magnitude greater (i.e. much lower K_M) than for Ca, even though the maximum transport capacity (J_{max}) of these sites may be much greater for Ca than for Cd. Thus fish die of hypocalcaemia, even though body Cd burdens remain very low (Hollis et al., 1999; Niyogi and Wood, 2004).

From the few studies which have investigated Ca and Cd interactions in chironomid larvae it appears that the mechanism of acute Cd toxicity may be at least qualitatively similar to that of fish. Craig et al. (1999) found that both elevated environmental Ca levels and Ca channel blockers reduced the uptake of Cd and therefore concluded that Cd is accumulated through Ca channels in *C. staegeri* larvae. Similarly, Bervoets et al. (1995) investigated the effect of Ca and salinity on Cd uptake in *C. riparius* larvae. They reported a significant decrease in Cd uptake when either the salinity or the Ca concentration of the exposure water was increased. Krantzberg and Stokes (1989) reported that chironomid larvae from a metal-contaminated lake were more efficient at 'sequestering' Ca than those from a reference lake. They suggested that this could result in metal resistance in larvae from metal-contaminated lakes. Although there is evidence of a Ca *versus* Cd interaction in chironomids, to our knowledge no study has directly compared the uptake kinetics of these two metals to confirm their interactions. Such an investigation may explain or reveal how the animals can withstand such extreme Cd exposure, and yet at the same time accumulate such high levels of Cd in the body.

The objective of the current study was to use a radio-tracer approach to assess the uptake kinetics of Ca and Cd independently and to look at the interactions between the two metals. Our principal hypothesis was that there would be a competitive inhibition of Ca transport by Cd, as in fish, and that the inhibition would occur at very high levels of Cd exposure, comparable to those of Ca. We further hypothesized that Michaelis–Menten kinetic analysis of concentration-dependence would reveal a Cd uptake system with a very low affinity (i.e. high K_M) compared to that which has been shown in many other aquatic animals, perhaps even in the same range as that of the Ca uptake system. In addition, based on the large body accumulations of Cd that we and others have observed in chironomids (Postma et al., 1996; Gillis et al., 2002; Gillis and Wood, 2008), we also hypothesized that the Cd system would have a high transport capacity (J_{max}), possibly even greater than that of the Ca system. To this end, radio-tracers were employed to determine the unidirectional uptake kinetics of the two metals separately, using Michaelis–Menten analyses. The kinetic analysis of Ca uptake was repeated in the presence of a concentration of Cd which approximated the apparent Cd K_M (affinity constant or concentration providing 50% of the maximal Cd uptake). The unidirectional influx rate of Cd at this concentration was also determined in exposures at a range of Ca concentrations. The results cast light on the unusual tolerance of this animal to acute Cd toxicity. It should be noted that, due to the high levels of Cd (mM) used in these exposures, any resistance mechanism revealed by this study may only be applicable to acute Cd exposure and not necessarily relevant to low level (i.e. nM) chronic exposure.

2. Materials and methods

2.1. Chironomid cultures

Culture methods are described in detail in Gillis and Wood (2008). Briefly, a continuous culture of *C. riparius* was maintained in 10 L glass aquaria using silica sand as substrate and dechlorinated Hamilton tap water (Lake Ontario) as the overlying culture water. The ionic composition of this water in mmol L^{-1} was $[Na^+] = 0.6$, $[Cl^-] = 0.8$, $[Ca^{2+}] = 1.8$, $[K^+] = 0.4$, $[Mg^{2+}] = 0.5$, $[Cd] < 5.0 \times 10^{-7}$. Water hardness was approximately 140 mg L^{-1} (as $CaCO_3$ equivalents), and pH 7.8–8.0. The cultures were aerated, and held at 21 ± 2 °C under a 16:8 h light:dark photoperiod regime. New culture tanks were initiated with first instar larvae and fed crushed Nutrafin™ fish flakes *ad libitum*.

2.2. Calcium influx experiment

Exposures were conducted in 250 mL glass beakers and held under the same conditions (temperature, light, etc.) as the cultures except that no substrate or food was added to any of the exposures. A calcium-free, reconstituted water was used as a 'base' water in the exposures. Composition was as follows: $[NaCl] = 0.65$, $[K_2SO_4] = 0.05$, $[MgSO_4] = 0.25$, $[NaHCO_3] = 1.0$ mmol L^{-1}. Ca was added to this water using an aqueous stock of reagent grade $Ca(NO_3)_2$ (Sigma Chemicals). In order to determine the short-term influx of Ca, chironomid larvae were exposed to a range of Ca concentrations (nominally 100–2500 μmol L^{-1}) using ^{45}Ca (as $CaCl_2$, PerkinElmer) as a radio-tracer. A working stock of 52 μCi ^{45}Ca/μmol Ca was used to create the Ca exposures.

Twenty-four hours prior to use in an exposure, the larvae were transferred to clean culture water (dechlorinated Hamilton tap water) in order to purge their gut contents. Upon initiation of the exposure, ten 3rd–4th instar larvae were added to each treatment (i.e. Ca concentration). Water samples (10 mL) were taken at the beginning of the exposure to determine the concentration of dissolved (filtered through an Acrodisk™ 0.45 μm in-line-syringe-tip filter) Ca and the radioactivity of each treatment (analytical details described below). Treatments were initiated at 15 min intervals. Staggered starting times were necessary to maintain constant exposure duration due to the time required to process samples following exposure. After 5 h of exposure, the larvae were transferred to a rinse solution of 'cold' Ca for five minutes in order to remove any loosely bound radioisotope. The concentration of Ca (as $Ca(NO_3)_2$) used in the 'cold displacement' rinse was 10-fold higher than the highest Ca exposure concentration (i.e. 25 mmol L^{-1}). Following rinsing, each larva was individually blotted dry on filter paper and weighed to the nearest 0.01 mg.

This Ca influx experiment was repeated twice, once as a separate exposure series and again as part of the Ca/Cd influx experiment (described below). Six Ca concentrations (treatments) were used in each run. The actual (i.e. measured) concentrations of Ca in the first run were 108, 182, 347, 655, 1289, and 2584 μmol L^{-1} and the actual Ca concentrations in the second run of this exposure were 96, 171, 319 578, 1164, and 2410 μmol L^{-1}. The results were not significantly different and the two runs were combined.

2.3. Cadmium influx experiment

Experimental details (gut clearing, exposure vessels, water samples, etc.) were as described above for the Ca influx experiment. In this exposure the concentration of Cd was varied (nominally 0–1500 μmol L^{-1}) and the concentration of Ca was set to 290 μmol L^{-1}, selected to approximate the Ca K_M value determined by Michaelis–Menten analysis of the Ca influx experiment (see description of kinetic analysis below). Seven concentrations of Cd (i.e. treatments) were produced by adding incremental amounts of a $Cd(NO_3)_2$ (Reagent grade, Fisher Scientific) solution which had been labeled with the radio-tracer ^{109}Cd (as $CdCl_2$, PerkinElmer). A working stock of 51 μCi ^{109}Cd/μmol Cd was used to create the Cd exposures.

The actual (i.e. measured) Cd concentrations were 0, 60, 120, 230, 470, 930, and 1420 μmol L^{-1}. Exposure duration and weighing procedures were as described above, with the exception that Cd (at 15 mmol L^{-1} as $Cd(NO_3)_2$) was used in place of Ca in the 'cold displacement' rinse.

2.4. Calcium/cadmium influx interactions

The interaction of Cd on Ca uptake was determined using a two-part concurrent experiment. In the first part, the concentration of Ca in the exposure water was varied as above using incremental additions of Ca and ^{45}Ca (actual Ca concentrations 96, 171, 319 578, 1164, and 2410 μmol L^{-1}). In the second part, a separate set of larvae were exposed to the same range of Ca concentrations as in the first part but, in addition, they were simultaneously exposed to a constant concentration of Cd as $Cd(NO_3)_2$ (measured 1200 μmol L^{-1}). This Cd concentration was chosen to approximate the apparent Cd K_M value determined by Michaelis–Menten analysis of the Cd influx experiment (see description of kinetic analysis below) which had been performed at a Ca concentration of 290 μM, the true Ca K_M value. Our goal was to ensure that a sufficiently high concentration of Cd was used such that clear inhibition of Ca uptake would be seen if it were present. The interaction of Ca on Cd uptake was studied by measuring Cd uptake in the larvae exposed to 1200 μmol L^{-1} Cd across the range of Ca exposures (96–2410 μmol L^{-1}).

2.5. Metal analysis

The concentrations of dissolved (filtered <0.45 μm) Cd and Ca in each exposure solution were determined by flame atomic absorption spectrophotometry (FAAS) using a Varian 220FS SpectraAA (Varian Techtron, Mulgrave, Victoria, Australia). Method blanks (5) and Fisher Scientific calibration standards (every 25 samples) were included in every run. A maximum of 5% difference between duplicates was accepted.

^{109}Cd radioactivity (as counts per minute (cpm)) in the exposure solutions and the individual larvae was determined using a gamma counter (Auto-Gamma 5000 series, Canberra Packard, Canada). Tests demonstrated that ^{109}Cd counting efficiency (gamma) was constant for all samples.

In order to determine ^{45}Ca radioactivity, water samples were analyzed directly by liquid scintillation counting (Tri-Carb 2900TR, PerkinElmer), whereas larvae were first subjected to acid digestion (described below). Water samples (0.5 mL), or larval digests (1 mL), were added to 5 mL of an acid-compatible liquid scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT, USA). Samples were held for 24 h in the dark to eliminate chemiluminescence. All ^{45}Ca cpm (beta) were corrected to constant counting efficiency using a quench curve constructed from the materials of interest.

The specific activity of each exposure solution (either Ca or Cd) was calculated by dividing the cpm per liter in the dissolved phase by the measured concentration of dissolved Ca or Cd (μmol L^{-1}). The concentration of newly accumulated Ca (in the ^{45}Ca exposures) and Cd (in the ^{109}Cd exposures) was calculated by the following formula:

$$(ab^{-1}) \cdot c^{-1} \quad (1)$$

where a is the radioactivity (cpm) of the larval tissue sample, b is the specific activity of the exposure solution (cpm/μmol Cd or Ca) and c is the wet weight (g) of the tissue sample. Dividing this concentration by time (h) yielded influx rates in micromoles per gram wet weight per hour.

When required for ^{45}Ca analysis, larvae tissues were digested in 2.0 mL Cryovials™. Concentrated metals grade nitric acid was added to each sample (10 μL/mg wet tissue) and they were incubated at 60 °C for 6 days. Hydrogen peroxide (30%) was added (4 μL/mg wet tissue) before the samples were held at room temp for 24 h (after Croteau et al., 2002) to complete the digestion. Digested tissue samples were then brought up to a final volume of 1.5 mL with 1% nitric acid.

2.6. Kinetic analysis

Non-linear regression analyses of the relationships between Ca uptake rates and environmental concentrations, and between Cd uptake rates and environmental concentrations, were performed with a hyperbolic curve fit (Sigma Plot version 10.0) in order to fit the Michaelis–Menten equation:

$$J_{in} = \frac{J_{max} \times [X]}{([X] + K_M)} \quad (2)$$

where J_{in} is the unidirectional influx rate, $[X]$ is the concentration of substrate, J_{max} is the maximum transport rate when the system is saturated, and K_M is the concentration of substrate which provides a J_{in} of half of the J_{max} value.

As the influx of Cd was measured in the presence of Ca (Ca was set to K_M^{Ca} at 290 μmol L^{-1}) it is not appropriate to directly compare the true K_M of Ca (obtained in the absence of Cd) with the apparent K_M of Cd (obtained in the presence of Ca). Therefore in order to account for this presence of Ca at its true K_M concentration during the kinetic determination, the K_M^{Cd} measured in this experiment was divided by 2 in accordance with classic competitive inhibition theory.

In order to quantify the inhibitory effect of Cd exposure on Ca uptake and *vice versa*, the K_i, or the dissociation constant for the inhibition, was calculated for each. Firstly, to quantify the inhibition of Cd on Ca uptake the following formulae were applied:

$$K_{M\,apparent} = K_M \times \left(\frac{1 + [Inhibitor]}{K_i}\right) \quad (3)$$

where [Inhibitor] is the concentration of Cd added to the Ca exposures (1200 μmol L^{-1}). Since the K_M for Ca was determined in the presence ($K_{M\,apparent}^{Ca}$) and absence of Cd (true K_M^{Ca}; see above and Fig. 3), Eq. (3) can be rearranged as:

$$K_i^{Cd} = \frac{[Inhibitor]}{[(K_{M\,apparent}^{Ca}/K_M^{Ca}) - 1.0)]} \quad (4)$$

Secondly, to quantify the inhibition of Ca on Cd uptake the following formula (after Cheng and Prusoff, 1973) was used:

$$K_i^{Ca} = \frac{IC_{50}}{(1 + [Substrate]/K_M^{Cd})} \quad (5)$$

where IC_{50} is the concentration of the inhibitor (Ca) where Cd uptake is inhibited by 50% (see Fig. 4).

The observed rate of Cd influx across a range of Ca concentrations (96–2410 μmol L^{-1}) was compared to the predicted rate of Cd influx (J_{in}^{Cd}) across that range based on Michaelis–Menten theory of simple competitive inhibition. Eq. (3) was employed to determine a predicted K_M^{Cd} and then using that calculated K_M^{Cd} and Eq. (2), a predicted J_{in} or Cd influx rate was determined. The predicted values are presented as a line in Fig. 4 along with the observed influx values.

2.7. Statistical analysis

Influx rates are reported as means ($n = 10$) and are given with standard errors (SE). Significant differences ($p < 0.05$) in influx rates (J_{in}) between Ca or Cd exposure concentrations were determined using ANOVA followed by Tukey's multiple comparisons

test. Michaelis–Menten constants are reported with standard errors (SE). Student's *t*-tests were used to compare the Michaelis–Menten constants (K_M and J_{max}) between two treatments. SPSS version 13.0 and the 'Regression Wizard' function of Sigma Plot version 10.0 were used for statistical analyses.

3. Results

3.1. Kinetics of calcium influx

C. riparius larvae exposed to a range of Ca concentrations (in the absence of Cd) exhibited typical Michaelis–Menten saturation kinetics for Ca influx rate (J_{in}^{Ca}). J_{in}^{Ca} increased with the concentration of Ca in the exposure water until it reached a maximum (J_{max}^{Ca}) of 0.388 (SE 0.056) μmol Ca g^{-1} h^{-1} (Fig. 1). The K_M^{Ca}, or the Ca concentration at which the influx reached half saturation, was 289 (SE 135) μmol Ca L^{-1}.

3.2. Kinetics of cadmium influx

C. riparius larvae exposed to a range of Cd concentrations (while the Ca concentration was set to 290 μmol L^{-1} to approximate the Ca K_M), also exhibited a saturation relationship for Cd influx rate (J_{in}^{Cd}) (Fig. 2). However, the J_{in}^{Cd} did not begin to level off until the exposure approached 1000 μmol L^{-1} Cd. Michaelis–Menten kinetic analysis revealed that maximum rate of Cd influx ($J_{max}^{Cd} = 2.26$ (SE 0.92) μmol Cd g^{-1} h^{-1}) was 5.8 times that of Ca, and that the apparent affinity for Cd accumulation was 1133 μmol Cd L^{-1}. However, assuming that Ca is a competitive inhibitor, since the kinetic curve was run at the true K_M^{Ca} (289 μmol L^{-1}), this value can be divided by 2 to yield the true $K_M^{Cd} = 567$ (SE 418) μmol Cd L^{-1}. The affinity of the transport system for Cd therefore appears to be about 2-fold lower (i.e. 2-fold higher K_M) than for Ca. However, only the difference in J_{max} values was significant, reflecting variability in the responses.

3.3. The influence of cadmium on the kinetics of calcium influx

The presence of 1200 μmol Cd L^{-1} resulted in a significant depression in the kinetics of Ca influx (Fig. 3). For example, a 90% reduction in J_{in}^{Ca} (0.015 μmol g^{-1} h^{-1} *vs.* 0.15 μmol g^{-1} h^{-1}) was observed at the 96 μmol Ca L^{-1} exposure concentration. The inhibitory effect of Cd on Ca influx remained nearly constant (80–90% reduction) in all Ca exposures of under 1000 μmol L^{-1}. The rate of Ca influx recovered to half of the control rate (0.18 μmol g^{-1} h^{-1} *vs.* 0.38 μmol g^{-1} h^{-1}) in the 2410 μmol Ca L^{-1} exposure, the highest concentration of Ca used (Fig. 3).

Michaelis–Menten kinetic analysis of Ca influx in these data sets revealed a J_{max}^{Ca} of 0.389 μmol (SE 0.027) g^{-1} h^{-1} in the control larvae (i.e. those not exposed to Cd) and a similar J_{max}^{Ca} of 0.560 (SE 0.095) μmol g^{-1} h^{-1} in the larvae which were exposed to 1200 μmol Cd L^{-1}. These J_{max}^{Ca} values were not significantly different. However, there was a large (30-fold) and significant difference in Ca affinity between the control chironomids (true K_M^{Ca} = 163 (SE 44) μmol Ca L^{-1}) and those exposed to 1200 μmol Cd L^{-1} ($K_{M\,apparent}^{Ca} = 4957$ (SE 1148) μmol Ca L^{-1}). This pattern is indicative of simple competitive inhibition, but the change in K_M^{Ca} was greater than anticipated. If the K_i^{Cd} were actually the same as the

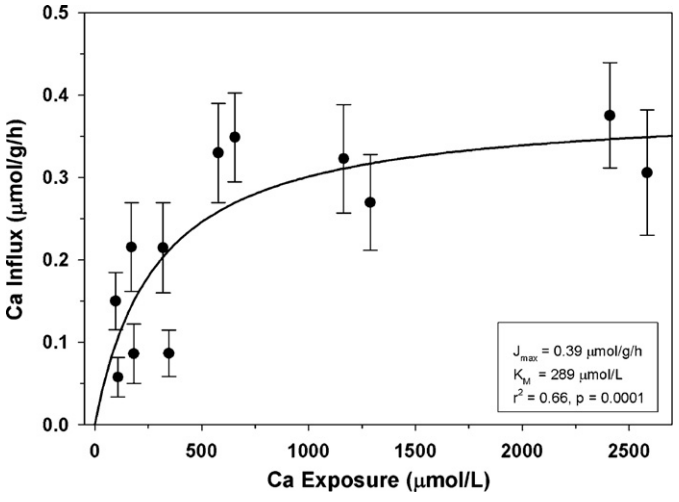

Fig. 1. Short-term calcium influx (μmol Ca g^{-1} wet weight h^{-1}) in *C. riparius* larvae (3rd–4th instar) exposed to a range of Ca (96–2584 μmol L^{-1}) concentrations for 5 h using ^{45}Ca as a radio-tracer. Error bars represent standard errors (n = 10 replicates per treatment).

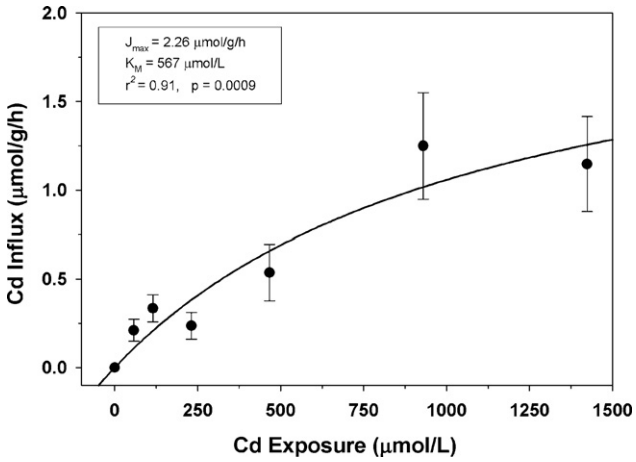

Fig. 2. Short-term cadmium influx (μmol Cd g^{-1} wet weight h^{-1}) in *C. riparius* larvae (3rd–4th instar) exposed to a range of Cd (0–1420 μmol L^{-1}) concentrations for 5 h using ^{109}Cd as a radio-tracer. Calcium concentration in the exposure water was 290 μmol L^{-1} in all treatments. Error bars represent standard errors (n = 10 replicates per treatment).

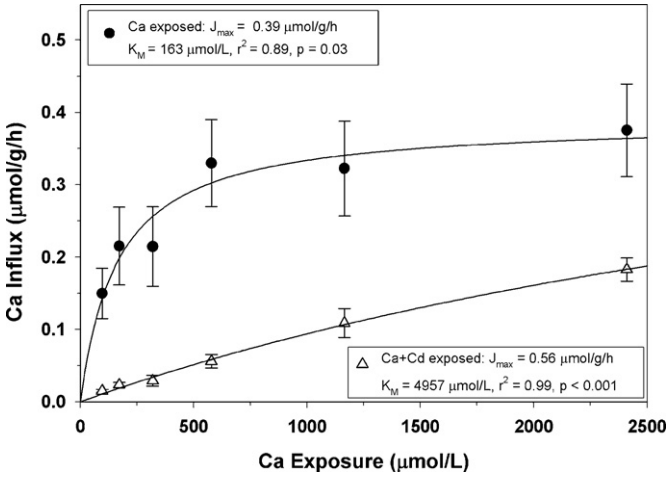

Fig. 3. Short-term calcium influx (μmol Ca g^{-1} wet weight h^{-1}) in a two-part parallel experiment with *C. riparius* larvae (3rd–4th instar) using ^{45}Ca as a radio-tracer. Solid black circles represent Ca influx in larvae that were exposed (5 h) to only Ca (96–2410 μmol L^{-1}). Grey triangles represent Ca influx in larvae that were simultaneously to Ca and Cd (1200 μmol Cd L^{-1}). Error bars represent standard errors (n = 10 replicates per treatment).

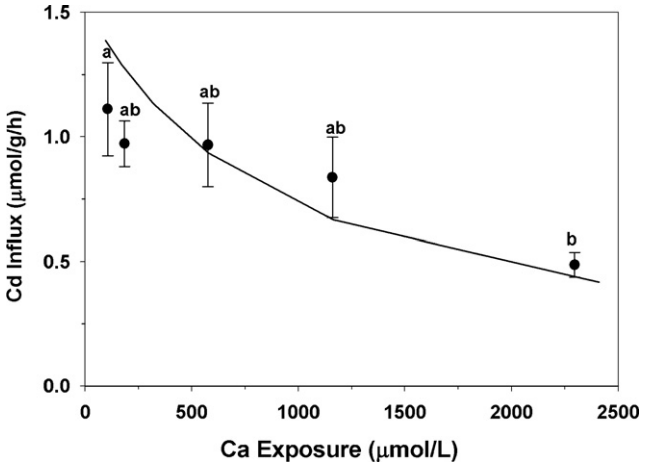

Fig. 4. Short-term cadmium influx (μmol Cd g^{-1} wet weight h^{-1}) in *C. riparius* larvae (3rd–4th instar) exposed to 1200 μmol Cd L^{-1} across a range of Ca concentrations (96–2410 μmol L^{-1}). Error bars represent standard errors (n = 10 replicates per treatment). Data points labeled with the same letter are not significantly different from each other (Tukey's multiple range test). The solid line represents the predicted rate of Cd influx assuming simple competitive inhibition (see text for details).

true K_M^{Cd} as expected in a simple competitive system, then from Eq. (4), we can calculate that the $K_{M\,apparent}^{Ca}$ should have been about 515 μmol Ca L^{-1} rather than 4957 μmol Ca L^{-1} – i.e a 3-fold rather than 30-fold increase. Conversely, application of Eq. (3) using $K_{M\,apparent}^{Ca}$ = 4957 μmol Ca L^{-1} yields a K_i^{Cd} value of 41 μmol Cd L^{-1}. This is unexpectedly low (>10-fold difference) relative to the true K_M^{Cd} = 567 μmol Cd L^{-1} calculated from Fig. 2 (see Section 4).

3.4. The influence of calcium on cadmium influx

The interaction of Ca on Cd uptake was studied by measuring Cd uptake in the larvae exposed to 1200 μmol Cd L^{-1} across a range of Ca exposures (96–2410 μmol L^{-1}). Overall, J_{in}^{Cd} tended to fall as external Ca concentration increased (r = 0.43, P < 0.02). Although J_{in}^{Cd} changed only slightly across Ca concentrations from 96 to 1164 μmol L^{-1}, at the highest exposure concentration (2410 μmol Ca L^{-1}) there was a significant 50% decrease (compared to lowest concentration) in Cd influx. Applying Eq. (5), K_i^{Ca} is calculated to be 770 μmol Ca L^{-1}. This value may be compared to the true K_M^{Ca} = 289 μmol Ca L^{-1} calculated from the data of Fig. 1, a difference of less than 3-fold.

In a simple competitive system, true K_M^{Ca} and K_i^{Ca} should be the same. It was therefore of interest to substitute the K_M^{Ca} for K_i^{Ca} in Eq. (3) (i.e. to use 289 μmol Ca L^{-1} as K_i^{Ca}) while using 567 μmol Cd L^{-1} as the true K_M^{Cd}. This then yielded a $K_{M\,apparent}^{Cd}$ at each Ca concentration, and in turn, application of this value in the standard Michaelis–Menten equation (Eq. (2)) yielded an estimate of J_{in}^{Cd} at each Ca concentration. This is the basis of the line inserted in Fig. 4, which fits the observed data very well. We therefore conclude that the small discrepancy in K_i^{Ca} *versus* K_M^{Ca} values is of negligible consequence.

4. Discussion

4.1. Calcium and cadmium uptake kinetics

Ca influx experiments demonstrated that *C. riparius* larvae obey classic Michaelis–Menten saturation kinetics, with constants not greatly dissimilar from those in fish (Fig. 1). A comparison of interest is with two fish species acclimated to the same water as used in the present study (Niyogi and Wood, 2004). The maximum Ca uptake rate, or J_{max}^{Ca} of *C. riparius* (0.39 μmol g^{-1} h^{-1}) was only moderately higher than the range (0.1–0.2 μmol g^{-1} h^{-1}) recorded in the two fish, rainbow trout (*Onchorhynchus mykiss*) and yellow perch (*Perca flavescens*). Although acclimation conditions were somewhat different (softer water), the larval *Oreochromis mossambicus* of Chang et al. (1997, 1998) provide a more closely size-matched comparison (4–10 mg) to the chironomids (3–6 mg) of the present study. These workers reported very similar J_{max}^{Ca} values of approximately 0.3 μmol g^{-1} h^{-1} in these tilapia larvae. Interestingly, the K_M^{Ca}, which indicates the affinity of the transport system for Ca (affinity = $1/K_M$) for *C. riparius* (289 μmol L^{-1}) was almost identical to the value in the rainbow trout (244 μmol L^{-1}), but somewhat higher than that in the yellow perch (92 μmol L^{-1}) reported in the Niyogi and Wood (2004) study. Lower K_M^{Ca} values around 20 μmol L^{-1} were recorded in the larval tilapia, perhaps reflecting their acclimation to low Ca water (Chang et al., 1997, 1998).

However a major difference from fish, and indeed from many other aquatic animals, was seen in the dynamics of Cd uptake (Fig. 2). Again, Michaelis–Menten saturation kinetics were observed, but *C. riparius*'s maximum capacity for Cd uptake (J_{max}^{Cd} = 2.3 μmol g^{-1} h^{-1}) was nearly six times higher than its capacity for Ca uptake (J_{max}^{Ca} = 0.39 μmol g^{-1} h^{-1}). By way of comparison, the J_{max}^{Cd} values in the two fish species studied by Niyogi and Wood (2004) were only 0.2–0.4 nmol g^{-1} h^{-1}, while in two aquatic insects *Siphonuris* sp. and *Drunella flavilinea*, comparable values were 0.002 and 0.5 nmol g^{-1} h^{-1}, respectively (Buchwalter and Luoma, 2005). In all cases, note that these rates are in nanomolar rather than micromolar units, indicating a 3–4 order of magnitude higher Cd uptake capacity for *C. riparius*.

Differences in K_M^{Cd} values were also pronounced. The affinity of the transport system for Cd was approximately two times lower (i.e. 2-fold higher K_M = 567 μmol L^{-1}) than for Ca (K_M = 289 μmol L^{-1}), although the difference was not significant – i.e. the two values were in a similar range. In contrast, in the rainbow trout and yellow perch of Niyogi and Wood (2004), the K_M^{Cd} values were in the 30–200 nmol L^{-1} range, whereas in the two mayflies of Buchwalter and Luoma (2005), the K_M^{Cd} values were in the 10–25 nmol L^{-1} range. Again, the difference *versus* other aquatic organisms is 3–4 orders of magnitude (nanomolar *vs.* micromolar), but in the opposite direction – i.e. much lower affinities (higher K_M's) for Cd transport in the chironomids, in a similar range to their affinities for Ca uptake. It should be noted that the Buchwalter and Luoma (2005) mayfly study employed much lower concentrations of waterborne Cd (0–50 nmol range) and thus their corresponding Michaelis–Menten kinetic (e.g. K_M^{Cd}) may describe a different population of high affinity uptake sites more relevant to chronic exposures. In summary, *C. riparius* exhibits 'normal' Ca uptake kinetics, but unique Cd uptake kinetics, in which the affinity is unusually low, comparable to that for Ca, whereas the maximum transport capacity is very high, even greater than that for Ca. These findings confirm two of our original hypotheses (see Section 1).

This large capacity for Cd uptake is consistent with the very high levels of Cd that chironomids can accumulate. *C. riparius* larvae exposed to Cd in laboratory tests have accumulated 20–30 μmol Cd g^{-1} (Postma et al., 1996; Gillis et al., 2002) and reports of tissue Cd burdens in field-collected chironomid larvae from metal-contaminated sediments range from 0.5 to 2.6 μmol Cd g^{-1} (Krantzberg and Stokes, 1989; Saki et al., 1995; Bervoets et al., 1998; Gillis et al., 2006). Also, when exposed to extreme (865 mg L^{-1} or 8000 μmol L^{-1}) waterborne Cd, *C. riparius* larvae have been shown to accumulate massive amounts of Cd (50–60 μmol g^{-1}) (Gillis and Wood, 2008). In comparison, back-

ground whole-body Ca concentrations in *C. riparius* larvae are in the range of 10–15 μmol g^{-1} (Gillis and Wood, 2008). The fact that this organism has nearly a 6-fold higher capacity for Cd uptake than it does for Ca would help account for its ability to accumulate Cd tissue burdens that are more than five times the concentration of internal Ca. [Note: In order to allow comparison with the current study, any Cd tissue concentrations reported in the literature on per dry weight basis (μmol Cd g^{-1} of dry tissue) were converted to a per wet weight basis (μmol Cd g^{-1} of wet tissue) based on the assumption that *C. riparius* larvae have approximately 90% water content (Gillis et al., 2002).]

4.2. Effects of cadmium on calcium uptake

Michaelis–Menten analysis clearly demonstrated that Cd inhibited unidirectional Ca uptake in a classical competitive fashion, with unchanged J_{max}^{Ca} but increased K_M^{Ca} (Fig. 3). This finding confirms our principal original hypotheses, specifically that high waterborne Cd is a competitive inhibitor of Ca transport in *C. riparius*, and is in qualitative accord with an abundant literature based mainly on fish studies (see Section 1). It also explains our recent observations that high Cd exposure selectively reduces whole body Ca concentrations while having negligible effects on whole body Na concentrations in *C. riparius* (Gillis and Wood, 2008). In two other arthropods, Rainbow and Black (2005) reported that a much lower level of Cd exposure (455 nmol L^{-1}) inhibited Ca uptake in two crabs (*Carcinus maenas* and *Eriochier sinensis*).

A high concentration of waterborne Cd (1200 μmol L^{-1}) was used in these tests. This was well below the LC50, causing no detectable mortality in 48 h toxicity tests (Gillis and Wood, 2008). This Cd concentration was specifically chosen to approximate the apparent Cd K_M value determined by Michaelis–Menten analysis of the Cd influx experiment which had been performed at a Ca concentration of 290 μM, the true Ca K_M value. Our goal was to ensure that a sufficiently high concentration of Cd was used such that clear inhibition of Ca uptake would be seen if it were present. In this respect the experiment succeeded. However, as demonstrated in the Results, the 30-fold shift in K_M^{Ca} was much greater than the 3-fold shift expected in a simple competitive system, and the calculated K_i^{Cd} value was unexpectedly low. We attribute these discrepancies to the difficulty of curve-fitting to a relationship where inhibition is so intense such that the kinetic curve tends to flatten out. In hindsight, tests at lower Cd concentrations would have been useful.

The reduction in Ca influx caused by 1200 μmol Cd L^{-1} ranged from 50 to 90% depending upon the concentration of Ca in the exposure water. Thus the % inhibitory effect of Cd on Ca influx in *C. riparus* larvae is related to the ambient concentration of Ca, as expected for competitive inhibition. At the much lower levels of waterborne Cd normally present in even highly contaminated environments (generally no more than 15 μg L^{-1}, i.e. nanomolar range (Mebane, 2006)), it is likely that inhibition of Ca uptake would be negligible, and toxicity thereby avoided. This is another benefit of the low affinity transport system.

4.3. Effects of calcium on cadmium uptake

Ca inhibited unidirectional Cd uptake, providing additional support for a common transport system (Fig. 4). In these tests, a range of environmentally relevant Ca concentrations (96–2410 μmol L^{-1}) from below to well above the K_M^{Ca} concentration (289 μmol L^{-1}) were tested against a Cd concentration (1200 μmol L^{-1}). Once the Ca concentration was increased to 2410 μmol L^{-1}, a 50% inhibition of Cd uptake was observed. As a full kinetic analysis was not performed, these data in themselves cannot distinguish whether the inhibition was competitive or non-competitive, but they do confirm the reciprocal nature of the Cd *versus* Ca interaction. Furthermore, it was encouraging that when the assumption of simple competitive inhibition was made, there was less than a 3-fold difference between the K_i^{Ca} of 770 μmol Ca L^{-1} calculated from the IC50 by Eq. (5) and the true K_M^{Ca} of 289 μmol Ca L^{-1} measured in Fig. 1. Furthermore, the relationship of Fig. 4 could be well-predicted by the use of this value as the K_i^{Ca} in Eq. (3), again suggestive of simple competitive inhibition.

Notably, these data also illustrate an important adaptive characteristic of the dual transport system – although environmentally relevant concentrations of Cd will have negligible effect on Ca uptake (see above), environmentally relevant concentrations of Ca can alter Cd uptake. This explains the protective effect of water hardness against both Cd uptake and acute toxicity in *C. riparius* demonstrated by Gillis and Wood (2008). These findings are also in agreement with Craig et al. (1999) who showed that specific antagonists of calcium channels greatly inhibited the uptake of Cd by *C. staegeri* larvae at much lower, environmentally relevant Cd levels (50 nmol L^{-1}). Furthermore, Cd accumulation was reduced by 70% when Ca in the exposures was raised from 100 to 2000 μmol L^{-1}. In a recent study with insect larvae (mayfly, stonefly, and caddisfly), Buchwalter and Luoma (2005) similarly reported a significant reduction in both Cd and Zn uptake when the concentration of Ca in the exposures was increased. In another group of arthropods, the crustaceans, there have been a number of reports of similar Ca *versus* Cd interactions, including Wright (1977) who demonstrated a decrease in haemolymph Cd in the crab *C. maenas* when the external concentration of Ca was increased.

4.4. Implications for cadmium resistance

In our previous study (Gillis and Wood, 2008), we found that the level of Cd required to elicit a negative response on whole-body Ca was exceptionally high, since in all but the most extreme Cd exposures, *C. riparius* larvae could maintain their baseline level of internal Ca. In fact, the disruption of Ca regulation could be correlated to the onset of toxicity as larvae exposed to Cd at or above the LC50 had roughly one quarter the whole-body Ca content of the unexposed larvae (Gillis and Wood, 2008). The results of the current study elucidate why *C. riparius* larvae are able to withstand extreme Cd exposures without succumbing to hypocalcaemia. The most notable feature of the Michaelis–Menten kinetic analyses was that the affinity of the Ca uptake system for Cd was much lower than what has been shown in other aquatic organisms (Verbost et al., 1987, 1989; Chang et al., 1997, 1998; Niyogi and Wood, 2004; Niyogi et al., 2004). Typically, organisms that are acutely sensitive to Cd will have a higher affinity for Cd than for Ca and will accumulate Cd at the expense of Ca. In contrast, the Cd-tolerant *C. riparius* larvae are able to maintain their internal Ca concentrations while exposed to Cd concentrations several orders of magnitude higher than the LC50s of other more Cd sensitive organisms. However it has been shown that some insects that are chronically sensitive to metals, including Cd, can have low dissolved uptake rates and that differences in efflux kinetics can help explain bioaccumulation and sensitivity differences Rates of Cd uptake measured on a short-term basis may not necessarily be predictive of long-term bioaccumulation patterns in insects for two major reasons. Firstly, insects differ tremendously in their abilities to eliminate metals. Secondly, diet may be a more important route of Cd exposure than the dissolved phase in some circumstances (Buchwalter et al., 2007; Martin et al., 2007).

We have demonstrated (this study and Gillis and Wood, 2008) that when late instar chironomid larvae are exposed to extreme Cd concentrations (hundreds of milligrams per liter) that the mech-

anism of toxicity is hypocalcaemia, but as indicated above, levels such as those used in this study are unlikely to occur in even the most polluted sites. Therefore we would suggest that in the natural environment where levels of Cd rarely reach over a few micrograms per liter, chironomid larvae would be more likely to be chronically exposed to Cd and would not experience acute Cd toxicity in the same manner as Cd-sensitive fish such as trout would.

Chironomids are often one of the dominant organisms in significantly polluted areas (Wentsel et al., 1977; Winner et al., 1980). In fact, the number of Chironomidae genera has been shown to increase as metal contamination increases (Canfield et al., 1994). Chironomids are thought to employ a number of techniques in order to withstand metal exposure. Studies by Krantzberg and Stokes (1989) and Timmermans and Walker (1989) indicate that chironomids do not regulate metal uptake. Furthermore, Postma et al. (1996) suggest that they employ other techniques such as excretion to deal with accumulated metal. Chironomids are also known to induce metallothioneins to detoxify metals (Yamarura et al., 1983; Seidman et al., 1986a; Gillis et al., 2002) and to produce metal-rich granules to store accumulated metals (Seidman et al., 1986a, 1986b). Based on this and other studies, there appear to be a number of factors that allow *C. riparius* larvae to tolerate Cd exposure much better than other aquatic organisms. Firstly, the low affinity for Cd allows *C. riparius* larvae to maintain internal Ca levels even while being exposed to Cd. Secondly, the induction of metallothionein enables the larvae to detoxify the accumulated Cd and prevent, or at least reduce any damaging non-specific binding of Cd to other proteins and enzymes. There may also be a component of Cd excretion involved in the tolerance as Postma et al. (1996) reported that Cd-adapted populations of *C. riparius* have increased Cd excretion efficiency. Therefore the ability of chironomid larvae to survive in metal-contaminated environments likely depends upon a number of physiological adaptations, one of which is the unique setting of the Ca *versus* Cd affinity relationship demonstrated here.

The sensitivity of aquatic insects to metals varies by hundreds of orders of magnitude depending upon insect species, its life stage, and the metal. Considering the ecological importance of aquatic insects there has been very limited study to elucidate the reasons for this high variability in their sensitivity to metals (Cain et al., 2006). Buchwalter and Luoma (2005) reported huge variation in metal (Cd and Zn) uptake rates between various taxa of insect larvae. They found that metal uptake depended upon the number of chloride cells on the gills of the larvae, not on gross morphological features such as gill surface area or larvae size. Cain et al. (2006) reported that the physiological mechanisms that determine metal bioaccumulation and toxicity are not known for most aquatic insects and therefore the mechanisms for ecological change in insect species assemblages are not well understood. In this study we have investigated one of the physiological mechanisms involved in acute Cd toxicity and tolerance in *C. riparius*. We would suggest that in order to fully understand why some species of insects are able to survive in contaminated environments and others cannot, that there is need to investigate mechanisms of tolerance in other resistant species as well the mechanisms that drive the sensitivity of intolerant ones. In this study we have investigated whole-body Cd and Ca uptake kinetics and thus have treated the chironomid larvae somewhat like a 'black box'. Therefore we suggest that there is also a need to investigate these physiological processes on a much finer scale. Craig et al. (1999) presented convincing evidence that a small segment of the chironomid's midgut was the main site of Cd accumulation, but it was not clear whether this was also the main site of initial uptake. There is a need to examine other possible routes of Cd uptake and elimination (e.g. other parts of the gut, Malpighian tubules, anal papillae etc.) in order to fully understand the complex mechanisms that enable this animal to tolerate such extreme metal exposure.

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References

Béchard, K.M., Gillis, P.L., Wood, C.M., 2008. Acute toxicity of waterborne Cd, Cu, Pb, Ni, and Zn to first instar *Chironomus riparius* larvae. Arch. Environ. Contam. Toxicol. 54, 454–459.

Bervoets, L., Blust, R., Verheyen, R., 1995. The uptake of cadmium by the midge larvae *Chironomus riparius* as a function of salinity. Aquat. Toxicol. 33, 227–243.

Bervoets, L., Solis, D., Romero, A.M., Van Damme, P.A., Ollevier, F., 1998. Trace metal levels in chironomid larvae and sediments from a Bolivian River: Impact of mining activities. Ecotoxicol. Environ. Saf. 41, 275–283.

Buchwalter, D.B., Cain, D.J., Clements, W.H., Luoma, S.N., 2007. Using biodynamic models to reconcile differences between laboratory toxicity tests and field biomonitoring with aquatic insects. Environ. Sci. Technol. 41, 4821–4828.

Buchwalter, D.B., Luoma, S.N., 2005. Differences in dissolved cadmium and zinc uptake among stream insects: mechanistic explanations. Environ. Sci. Technol. 39, 498–504.

Cain, D.J., Buchwalter, D.B., Luoma, S.N., 2006. Influence of metal exposure history on the bioaccumulation and subcellular distribution of aqueous cadmium in the insect *Hydropsyche california*. Environ. Toxicol. Chem. 25, 1042–1049.

Canfield, T.J., Kemble, N.E., Brumbaugh, W.G., 1994. Use of benthic invertebrate community structure and the sediment quality triad to evaluate metal-contaminated sediment in the Upper Clark Fork River, Montana. Environ. Toxicol. Chem. 13, 1999–2012.

Chang, M.H., Lin, H.C., Hwang, P.P., 1997. Effects of cadmium on the kinetics of calcium uptake in developing tilapia larvae, *Oreochromis mossambicus*. Fish Physiol. Biochem. 16, 459–470.

Chang, M.H., Lin, H.C., Hwang, P.P., 1998. Ca^{2+} uptake and Cd^{2+} accumulation in larval tilapia (*Oreochromis mossambicus*) acclimated to waterborne Cd^{2+}. Am. J. Physiol. Regul. Integr. Comp. Physiol. 274, R1570–R1577.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_1) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Craig, A., Hare, L., Tessier, A., 1999. Experimental evidence for cadmium uptake via calcium channels in the aquatic insect *Chironomus staegeri*. Aquat. Toxicol. 44, 255–262.

Croteau, M.N., Hare, L., Campbell, P.G.C., Couillard, Y., 2002. Metallothionein-like protein in the biomonitor *Chaoborus*: occurrence and relationship to ambient metal concentrations in lakes. Environ. Toxicol. Chem. 21, 737–741.

Gillis, P.L., Diener, L.C., Reynoldson, T.B., Dixon, D.G., 2002. Cadmium induced production of a metallothionein-like protein in *Tubifex tubifex* (Oligochaeta) and *Chironomus riparius* (Diptera): correlation with whole body (reproduction and growth) endpoints of toxicity. Environ. Toxicol. Chem. 21, 1836–1844.

Gillis, P.L., Reynoldson, T.B., Dixon, D.G., 2006. Metallothionein-like protein and tissue metal concentrations in invertebrates (Oligochaetes and Chironomids) collected from reference and metal contaminated field sediments. J. Great Lakes Res. 32, 565–577.

Gillis, P.L., Wood, C.M., 2008. The effect of extreme waterborne cadmium exposure on the internal concentrations of cadmium, calcium, and sodium in highly tolerant *Chironomus riparius* larvae. Ecotox. Environ. Saf. 71, 56–64.

Hollis, L., McGeer, J.C., McDonald, D.G., Wood, C.M., 1999. Cadmium accumulation, gill Cd binding, acclimation, and physiological effects during long term sublethal Cd exposure in rainbow trout. Aquat. Toxicol. 46, 101–119.

Irving, E.C., Liber, K., Culp, J.M., 2004. Lethal and sublethal effects of low dissolved oxygen condition on two aquatic invertebrates, *Chironomus tentans* and *Hyalella azteca*. Environ. Toxicol. Chem. 23, 1561–1566.

Krantzberg, G., Stokes, P.M., 1989. Metal regulation, tolerance and body burden in the larvae of the genus *Chironomus*. Can. J. Fish. Aquat. Sci. 19, 389–398.

Martin, C.A., Luoma, S.N., Cain, D.J., Buchwalter, D.B., 2007. Cadmium ecophysiology in seven stonefly (Plecoptera) species: delineating sources and estimating susceptibility. Environ. Sci. Technol. 41, 7171–7177.

Mebane, C.A., 2006. Cadmium risks to freshwater life: Derivation and validation of low-effect criteria values using laboratory and field studies (version 1.1): U.S. Geological Survey Scientific Investigations Report 2006-5245, 130 p.

Niyogi, S., Wood, C.M., 2004. 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. J. Comp. Physiol. B 174, 243–253.

Niyogi, S., Couture, P., Pyle, G., McDonald, D.G., Wood, C.M., 2004. An evaluation of cadmium and calcium gill-binding characteristics in laboratory reared and metal-impacted wild yellow perch (*Perca flavescens*) in comparison to rainbow trout (*Oncorhynchus mykiss*): implications for the Biotic Ligand Model (BLM). Can. J. Fish. Aquat. Sci. 61, 942–953.

Postma, J.F., Van Nugteren, P., Buckert-De Jong, M.B., 1996. Increased cadmium excretion in metal-adapted populations of the midge *Chironomus riparius* (Diptera). Environ. Toxicol. Chem. 15, 332–339.

Rainbow, P.S., Black, W.H., 2005. Cadmium, zinc, and the uptake of calcium by two crabs, *Carcinus maenas* and *Eriocheir sinensis*. Aquat. Toxicol. 72, 45–65.

Saki, M.K., Castleberry, D.T., May, T.W., Bullard, F.N., 1995. Copper, cadmium, and zinc concentrations in aquatic food chains from the Upper Sacramento River (California) and selected tributaries. Arch. Environ. Contam. Toxicol. 29, 484–491.

Seidman, L.A., Bergtrom, G., Remsen, C.C., 1986a. Accumulation of cadmium by the fourth instar larva of the fly *Chironomus thummi*. Tissue Cell 18, 395–405.

Seidman, L.A., Bergtrom, G., Remsen, C.C., 1986b. Structure of the larval midgut of the fly *Chironomus thummi* and its relationship to sites of cadmium sequestration. Tissue Cell 18, 407–418.

Timmermans, K.R., Walker, P.A., 1989. The fate of trace metals during metamorphosis of chironomids (Diptera, Chironomidae). Environ. Pollut. 62, 73–85.

Verbost, P.M., Flik, G., Lock, R.A.C., Wendelaar-Bonga, S.E., 1987. Cadmium inhibition of Ca^{2+} uptake in rainbow trout gills. Am. J. Physiol. Regul. Integr. Comp. Physiol. 253, R216–R221.

Verbost, P.M., Flik, G., Lock, R.A.C., Wendelaar-Bonga, S.E., 1988. Cadmium inhibits plasma membrane calcium transport. J. Membr. Biol. 102, 97–104.

Verbost, P.M., Van Rooij, J., Flik, G., Pang, P.K., 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.

Wentsel, R., McIntosh, A., Anderson, V., 1977. Sediment contamination and benthic macroinvertebrate distribution in a metal-impacted lake. Environ. Pollut. 14, 187–193.

Wentsel, R., McIntosh, A., Anderson, V., 1978. Evidence of resistance to metals in larvae of the midge *Chironomus tentans* in a metal contaminated lake. Bull. Environ. Contam. Toxicol. 20, 451–455.

Winner, R.W., Bossel, M.W., Farrel, M.P., 1980. Insect community structure as an index of heavy-metal pollution in lotic ecosystems. Can. J. Aquat. Sci. 37, 647–655.

Wood, C.M., 2001. Toxic responses of the gill. In: Schlenk, D., Benson, W.H. (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts, vol. 1. Taylor & Francis, London, pp. 1–89.

Wright, D.A., 1977. The effect of calcium on cadmium uptake by the shore crab *Carcinus maenas*. J. Exp. Biol. 67, 137–146.

Yamarura, M., Suzuki, K.T., Hatakeyama, S., Kubota, K., 1983. Tolerance to cadmium and cadmium-binding proteins in the midge larvae, *Chironomus yoshimatsui*. Comp. Biochem. Physiol. C 75, 21–24.