Evidence of common cadmium and copper uptake routes in zebrafish *Danio rerio*

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Abstract

Cadmium and copper accumulation in gills of zebrafish was measured during a 48h exposure to 0.025µM $^{106}$Cd and 0.05 or 0.5µM $^{65}$Cu as a single metal or their mixtures. The gill transcript levels of genes involved in the transport of Cu (CTR1 and ATP7a), Na (NHE-2), Ca (ECaC), divalent metals (DMT1) and Zn (ZIP8) were compared between the treatments at 24 and 48h. Cd uptake was significantly suppressed in the presence of Cu, but Cu uptake was unaffected by Cd. The decrease in Cd accumulation rates in the presence of Cu was associated with an increase in transcript abundance of ECaC at 24h and DMT1 at 48h, both known as routes for Cd uptake. This indicates that the reduced Cd uptake is due to a direct interaction between the Cu and Cd at these uptake sites. Fish exposed to 0.5µM $^{65}$Cu show an increase in gill ATP7a transcript abundance suggesting that Cu is removed from the gill and is transferred to other organs for detoxification. A reduction in gill CTR1 transcript abundance during the Cu-Cd exposure may be a regulatory mechanism to reduce Cu loading if there are other routes of Cu uptake such as ECaC and DMT1.
Introduction

Understanding the environmental toxicology of metals is essential for setting adequate site specific water quality criteria. In natural environments, aquatic organisms are constantly exposed to a variety of metals via the water and diet. Some of trace metals, such as Cu, Fe, and Zn are essential for the health of most organisms because they are integral components of enzymes and other molecular complexes.¹ Intracellular concentrations of these metals are tightly regulated by complex uptake/excretion mechanisms. Other metals, such as Cd and Pb, are toxic to living organisms even at low exposure concentrations and tend to accumulate in the body.² Due to anthropogenic activities metals are present in surface waters as mixtures and often at elevated concentrations. Single metal toxicity studies that are currently used to derive water quality standards do not accurately represent the processes occurring in complex natural environments. Consequently, current standards may over- or underestimate toxicity.

Some essential-nonessential metal- interactions, such as Cd-Ca and Cu-Na are well studied in fish.³,⁴ In particular, Cd is known to enter gill tissue via Ca²⁺ channels present on the apical cell membrane.⁵ Even at low concentrations Cd compete with Ca ions for the uptake sites causing acute hypocalcaemia.³ The uptake of Cu is known to interfere with Na uptake resulting in the impairment of branchial Na influxes.⁴ Based on a current understanding of metal transport across the cell membrane, Cd and Cu do not share common uptake routes. Nevertheless, the presence of Cd-Cu interactions has been demonstrated for several aquatic
organisms during aqueous and dietary exposures even at low concentrations.6-7 Studies show that proteins responsible for the transport of essential metals, such as Fe and Zn, may also be involved in the uptake of non-essential elements.8-10 Cooper et al11 proposed that in addition to a well characterized Ca uptake pathway, Cd may be taken up via a divalent metal transporter (DMT1), a protein which delivers Fe to the cell. Dalton et al10 showed that a mouse zinc importer ZIP8 also transports Cd. Other studies indicate the existence of Cd-Cu, Cd-Pb or Pb-Zn interactions, which cannot be explained by simple competition for binding sites and most likely involve unknown common uptake route7, 12 or alterations in intracellular compartmentalization/efflux processes.13

To better understand the relationship between Cd and Cu uptake, this study utilises stable isotopes and molecular approaches to investigate the accumulation of these metals in gills of adult zebrafish *Danio rerio*. The stable isotope technique enables simultaneous exposure of organisms to several metals and allows separate detection of newly added and background metals in the exposure medium and organisms.14-16 This feature is particularly important for studying uptake processes of essential metals, such as Cu, when small amounts of newly accumulated metals are masked by a naturally high metal content of the tissue. Zebrafish were exposed to 106Cd and 65Cu as single metals and their mixtures in medium hard OECD water at environmentally relevant metal concentrations. The metal burdens of zebrafish gills were followed in time for 48h to obtain metal uptake rates. The transcript abundance of genes involved in transport of Cu (CTR1, ATP7a), Na (NHE-2), Ca (ECaC), divalent metals (DMT1) and Zn (ZIP8) were compared between the treatments at 24 and 48h of exposure. By comparing the metal uptake profiles with changes in
relative transcript abundance levels, a further aim was to identify potential transporters involved in Cu and/or Cd uptake during a metal mixture exposure and suggest a possible uptake pathway.

**Experimental Section**

**Test Organisms**

Adult zebrafish, *Danio rerio*, (~ 1 g) were obtained from a local supplier and maintained in 150 L glass tank containing 100–150 fish per aquarium at 26± 1 °C under a 12 h light/12 h dark regime. Fish were fed with the commercial tropical fish food twice a day on a 1% body mass daily ration. The aquaria were equipped with trickling filters and constantly aerated. The water was checked routinely for NH$_4^+$, NO$_2^-$, and NO$_3^-$. The medium was renewed if the concentration of any of these ions exceeded 5, 2, or 200 μM, respectively. The rearing and test media of the aquaria (pH=7) were prepared according to the OECD guidelines for testing chemicals and contained 2mM CaCl$_2$·2H$_2$O, 0.5 mM NaHCO$_3$, 0.5 mM MgSO$_4$·7H$_2$O and 0.077 mM KCl dissolved in deionised water. Fish were acclimatized to the test waters for at least 20 days. Feeding was stopped 12h prior to the start of experiments to allow sufficient time for gut depuration and no food was provided during the experiment to avoid metal leaching into the exposure solution.

**Metal exposure, sample collection and measurement**

Twenty four h prior to conducting the experiments 5L polypropylene aquaria were filled with freshly prepared test medium and spiked with individual metals (0.03 and 0.025 μM $^{106}$Cd, 0.05 and 0.5 μM $^{65}$Cu) or their mixtures ( 0.025 μM $^{106}$Cd - 0.05 μM $^{65}$Cu and 0.025 μM $^{106}$Cd - 0.5 μM $^{65}$Cu) to achieve metal equilibration in the...
solution. These concentrations are in the range of the UK Environmental Quality Standards (Cd EQS 0.045 µM and Cu EQS 0.08 to 0.625 µM)\textsuperscript{17}. Measured total metal concentrations in the exposure media were within 100-104% (\textsuperscript{106}Cd) and 90-112% (\textsuperscript{65}Cu) of the nominal values (Supporting Information Table S1). No \textsuperscript{106}Cd was detected in Cu-only exposure solutions. Organisms were randomly distributed among experimental and control containers with five aquaria per condition and seven fish per aquarium. At 4, 24, 30 and 48h five fish were randomly sampled for metal analysis. Fish were allowed to swim in fresh test water without metal additions for 10 to 15 min to wash off any metals weakly bounded to the surface, thereafter they were blotted dry and killed by approved UK Home Office Schedule 1 procedure. The gills of each fish were carefully dissected, dried to a constant weight at 60°C, weighed and digested with 200 µL of 69% nitric acid (Ultrapur, Merck) at room temperature for 48h, which was enough to completely digest soft gill tissue. Water samples (10mL) were collected from each aquarium at the beginning and at the end of experiments, acidified with 100 µL of 69% nitric acid and kept for total metal measurements. Gill digests, water samples and procedural blanks were spiked with Y as an internal standard and analysed with ICP-MS (Perkin Elmer Sciex Elan 6100 DRC, 3 replicates per sample). The instrument was recalibrated every 25 samples to account for any signal drift. Isotope concentrations in the samples (µg/L) were determined from calibrations curves constructed for each isotope of interest. Gill metal loadings were calculated on a µmole-kgdw\textsuperscript{-1} basis using measured concentrations of \textsuperscript{106}Cd and \textsuperscript{65}Cu isotopes and dry weights of the tissue samples. In parallel, at 24 and 48h additional five fish were randomly sampled for transcript abundance analysis. Each fish was dissected on a cold surface, the gills were immediately placed in an Eppendorf tube containing 0.5 mL ice-cold TRI-reagent\textsuperscript{®},
homogenized and stored at -80 °C until the preparation of total RNA. All surfaces and the instruments used at the sample collection and disruption stages were treated with RNaseOut™ to minimise RNA degradation.

**RNA extraction and RT-PCR analysis**

Homogenised gills samples were defrosted and incubated in TRI-reagent® for 5 minutes at room temperature and total RNA was isolated from gills according to manufacturer instructions. Briefly, nucleic acids were extracted with 100 µL of chloroform and precipitated with 250 µL isopropanol. The pellet was washed twice with ice cold 75% EtOH, resuspended in 20 µL RNAase free water and DNAse treated with Turbo DNA-free kit (Ambion®). An aliquot of each extract was used to assess purity of RNA preparation and its concentration using Nanodrop ND-100 spectrophotometer. All extracts with 260/280 ratio between 1.95 and 2.2 were considered acceptable and RNA integrity was additionally evaluated by running samples on 1% agarose TAE gel. Samples with 260/280 ratios below 1.95 were precipitated with EtOH/CH$_3$COONa overnight and re-suspended in RNAase free water. cDNA was synthesised from 2 µg of total RNA (20 µL final volume) using RNA-to-cDNA Ecodry™ premix kit from Clontech. Subsequently, 2 µL of cDNA template (1:40 dilution, 10 µL final reaction volume) was amplified in triplicates with 5 µL SYBR *Premix Ex Taq™* II Master Mix (Clontec), 0.4 µmole-L$^{-1}$ each primer pair (IDT) and ROX (1:50 final dilution) as a reference dye using ABI Prism 7900 HT RT-PCR sequence detection system. Initial denaturation step (95°C for 30s) was followed by 40 cycles of amplifying stage (95°C for 5s, 55°C for 34s). None-template (with no cDNA added) and reverse transcriptase negative (with reverse transcriptase omitted during cDNA synthesis) control samples were included in each PCR run to
ensure there was no amplification due to genomic contamination. The primers for each gene (Table 1) were designed using NCBI software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer amplification efficiencies were determined using calibration curves generated from a serial dilution of a pooled cDNA sample and varied between 101 and 115 %. After 40 cycles a dissociation curve was run and showed that each primer pair produced a single amplicon indicating absence of primer dimers formation or non-specific amplification. Transcript abundance levels were determined from relative standard curves prepared by serial dilutions of pooled cDNA samples and normalised to transcript abundance levels of a reference gene EF1 α in the same sample (β-actin, EF1α, ubiquitin and ribosomal protein L13a were evaluated as potential references genes and EF1α was the most stable transcript in our experimental conditions). The changes in relative transcript abundance levels in treatment groups were then expressed as fold change (FC) to that in the control (non-exposed) group.

**Statistics**

The statistical significance of the changes in transcript abundance levels (based on fold changes, FC±SD, p<0.05) and branchial metal uptake rates was assessed by one-way ANOVA analysis followed by a Holm-Sidak Post-hoc test.

**Results and discussions**

**Cd and Cu accumulation**

The $^{106}$Cd branchial accumulation followed time-dependent linear uptake kinetics in all experimental conditions (Supporting Information Figure S1). The
obtained uptake rates were comparable with those reported in the previous study performed at similar conditions. Addition of $^{65}$Cu to the exposure medium had a significant dose dependent effect on reducing branchial $^{106}$Cd accumulation (Figure 1). Previous studies demonstrated similar effects in zebrafish and daphnia where Cu suppressed Cd uptake, but the underlying mechanism was unclear.$^{7,18}$

The observed linear Cd accumulation profile suggest that the elimination component to the total Cd gill burdens was likely very low. Other studies report low Cd elimination rates constants in gills of yellow perch and rainbow trout in the range of 0.024-0.4 d$^{-1}$. Thus, the decline in Cd accumulation in the presence of Cu was likely due to inhibited Cd uptake into the cell caused either by Cd-Cu competition at entry sites on the apical membrane or Cu-induced decrease in the population of Cd-binding and/or uptake sites.

The copper uptake was expressed as a $^{65}$Cu/$^{63}$Cu molar ratio within the gills of a single fish, where $^{63}$Cu served as an internal measurement correction factor. The amount of $^{63}$Cu in gill tissue remained fairly constant throughout the experiment (Supporting Information Figure S2), and therefore any changes in $^{65}$Cu/$^{63}$Cu ratio were solely due to increase in $^{65}$Cu content of the gill tissue. The accumulation of $^{65}$Cu also followed linear kinetics over the 48hrs (Supporting Information Figure S3), but was not affected by cadmium (Figure 2).

**Transcript abundance and Cd uptake pathways**

Exposure of freshwater fish to metals perturbs ion homeostasis that may ultimately lead to death.$^{5,21}$ Consequently, to avert cellular toxicity and maintain ion homeostasis the gills of fish have to respond in a dynamic manner to changes in
water chemistry by altering gene expression patterns of cation and anion transport
proteins, as well as metal binding proteins. More specifically, Cd is known to
interfere with homeostasis of Ca, Fe and Zn and in some fish species has also been
reported to affect Na balance.

Cd does not have any known physiological function in fish and no Cd-specific
transporting system has been identified in fish gills. The mechanism of Cd entry is
currently described as mimicking or replacing a number of essential metals in a
competitive process on the apical site of cell membrane and may involve low-
capacity high-affinity sites. In rainbow trout gills, Cd ions are known to compete
with essential Ca ions for binding sites at epithelial calcium channels (ECaC) and
use this pathway as an effective uptake route. In conditions of Fe deficiency Cd
uptake by zebrafish gills has been proposed to be via DMT1. Recently, evidence
emerged of possible involvement of Zn transporting proteins (ZIP8 and 14) in Cd
traffic in mammalian systems and intestinal Cd uptake in rainbow trout is likely to
be mediated by Zn and Fe transporting proteins, presumable ZIPs and DMT1.

In our study fish exposed to the metal mixtures showed a significant 4.7
(0.05µM Cu-0.025µM Cd treatment) and 5 fold (0.5µM Cu-0.025µM Cd treatment)
upregulation of ECaC transcript abundance compared to the control at 24hrs (Figure
3). Alsop and Wood showed that in addition to Cd perturbing Ca homeostasis, a 5h
exposure of zebrafish to Cu also inhibited Ca uptake. Thus, an increase in ECaC
expression maybe a compensatory mechanism that increase Ca uptake and rectify
any Ca loss in the presence of both Cd and Cu. At 48h the expression level of ECaC
gene returned to the control value, while upregulation of mRNA levels of DMT1
transporter was observed in 0.025µM Cd and 0.05µM Cu-0.025µM Cd treatment
compared to controls. An increase in DMT1 expression has been associated with reduced iron status in fish\(^{11}\), and though iron levels were not measured in the current study suggests that Cd perturbs iron homeostasis, as previously observed in rainbow trout.\(^{33}\) In contrast, the transcript abundance of ZIP8 was significantly decreased in fish exposed to the 0.5\(\mu\)M Cu-0.025\(\mu\)M Cd compared to the Cd and Cu only exposures at 24 and 48hrs, respectively (Figure 3).

ECaC and DMT are the most likely uptake routes of Cd into the gill\(^{5,27}\), and thus it is counter intuitive to observe a large increase in transcript abundance of both transporters in the metal mixture exposures and a concomitant reduction in Cd uptake. This implies direct competition between Cu and Cd for uptake at these sites. It has been shown that DMT1 is able to transport Cu in a number of systems, but this has not been verified in fish.\(^{34-36}\) There is no direct evidence for Cu transport via ECaC, but it has been suggested that Cu epithelial transport maybe via a Ca pathway in zebrafish\(^{32}\). Alternatively, the decrease of ZIP 8 transcript levels in combined Cd-Cu treatments may partially account for the reduction in Cd transport, if Cd uptake occurs via this transporter as suggested in mice.\(^{10}\) The alterations in gene expression provide details of transcript abundance and potential alterations in cation uptake during metal exposures. But, further characterisation of the cation uptake profiles for the different transport proteins present on the gill is required to identify metal substrate specificity.

**Transcript abundance and Cu uptake pathways**
The process of Cu uptake by gills of freshwater fish is currently described as a facilitated transport via Na\(^+\)-sensitive and Na\(^+\)-insensitive pathways.\(^4\) Both routes exhibit high copper affinity at ambient Cu concentrations below 0.02 \(\mu\)M, which are environmentally relevant.\(^17\) In rainbow trout Na\(^+\)-sensitive pathway dominates Cu uptake at low Na concentrations in water (IC\(_{50}\)=104 \(\mu\)M) and at higher Na concentrations (1-20 mM) presence of Na\(^+\)-insensitive component of branchial Cu uptake was observed.\(^37\) A number of Na\(^+\)/H\(^+\) exchangers have been identified and located to the gills with NHE-2 as a candidate for Na-sensitive component of Cu uptake. Later studies identified a high-affinity high-specificity copper uptake protein encoded by CTR1 gene as the putative Na\(^+\)-insensitive route of Cu entry in zebrafish gills.\(^38,39\)

The results of our studies revealed insignificant changes in transcript levels of NHE-2 indicating that there is unlikely to have been a perturbation to Na balance in these fish, or, if there was a disturbance, then changes to the expression of other unidentified transporters involved in Na acquisition may have occurred (Figure 3). In the 0.5 \(\mu\)M Cu only exposed fish the transcript abundance of ATP7a, was significantly elevated compared to controls and those exposed to 0.05 \(\mu\)M Cu. This suggests that in response to metals loading on the gill the fish increase expression of this basolateral membrane Cu exporter to minimise accumulation in the gill cells and divert metal internally to other organs for detoxification and excretion. An interesting observation is a significant increase in the expression of CTR1 in the 0.5 \(\mu\)M Cu exposed fish compared to those exposed to 0.05 \(\mu\)M Cu. This suggests that in response to elevated metal that fish increase the transcript of an apical metal importer, this would inadvertently increase the potential for the gill to accumulate
metal. This would appear to be a unusual physiological response to exposure to a metal and there is no simple explanation, but increases in the expression of a number of apical metal transporters was observed in the gills of a population of brown trout residing in a river that has a long history of elevated metals.23

The addition of Cd resulted in a significant decrease in gill CTR1 transcript abundance at 48hrs compared to fish from the 0.5 µM Cu only treatment (Figure 3). This decrease might be a regulatory mechanism to prevent excessive Cu from entering the cell via the Cu specific CTR1, if Cu also shares the suggested Cd uptake route via ECaC and/or DMT1 - the two transporters to show elevated transcript abundance in fish treated with Cu and Cd in combination (Figure 3). This response would explain the lack of inhibition of branchial $^{65}$Cu uptake rate on the gills of fish exposed to Cu and Cd (Figure 2), but the significant reduction in $^{106}$ Cd accumulation in similar conditions (Figure 1). Copper is present in natural waters as Cu$^{2+}$, and thus the existence of a divalent Cu ion uptake route is feasible. The divalent metal transporter DMT1, which primary function is Fe$^{2+}$ transport, has the potential to transport a range of divalent metals including Cu$^{35}$. Recent studies demonstrated decreased Cu accumulation in zebrafish gills at elevated waterborne Ca$^{2+}$ during chronic exposure.39 These observations imply a potential shared uptake route between Cu and Ca, e.g. ECaC.

The results from the current study show that there is complex dynamic response in the expression pattern of genes encoding metal ion and cation transport protein in the gills as a result of exposure to Cd and/or Cu alone or as a mixture. The observed temporal variations in relative transcript abundances of the genes studied and the changes in gill Cd uptake rates suggest that Cd enters via ECaC, and that
Cu competes for Cd at these uptake sites, as well as ZIP8. There is a reduction in ECaC transcript abundance at 48h, but the uptake of Cd is linear over the 48hrs and thus the increase in expression of DMT1 at this time point suggests Cd uptake continues likely via this divalent metal ion transport protein. Gill metal load is a key determinant of toxicity and a number of single metal biotic ligand models (BLM) based on this load have been developed to predict site specific acute metal toxicity. Metals are rarely seen in isolation, however, and there is great interest in developing metal mixture BLM models. The current study indicates that gene expression response at the gills to metals may be unexpected and that this may have a significant effect on metal uptake rates. Future research is necessary to identify the promiscuity of these fish gill metal transporters to help identify all potential metal apical entry routes.

Acknowledgement

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References:


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Table 1. PCR primer sequences used for RT-PCR

<table>
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<th>Gene (Accession No)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>CTR1 (NM205717)</td>
<td>GGCTACAAAAATGTGGAGCTG</td>
<td>AACAAGAACACGCCAATGC</td>
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<td>DMT1 (NM001040370)</td>
<td>ATCGAGTCGTCATCCGCTCT</td>
<td>AGCCAGAACACACCCT</td>
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<td>ECAC(NM001001849.1)</td>
<td>GCTGCAGTCAGTGAATA</td>
<td>ACCGACGCTCACCTAAGCT</td>
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<td>ATP7a (BC122285.1)</td>
<td>GGCCTTGCTTTACGGTT TGCC</td>
<td>CTGAAGGCTGAGAAGTGCC</td>
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<td>NHE-2 (NM001114095.1)</td>
<td>CCACCTGCACCTCTTGGAA</td>
<td>GCAGATGGCAAATAGGGGA</td>
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<tr>
<td>ZIP8 (XM_001342527)</td>
<td>TCCCCGCTGCGCTTACACTT</td>
<td>AGTGTCGGGATGCGAGTCCAA</td>
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<tr>
<td>EF1α (NM131263.1)</td>
<td>GTGCTGTGCTGATTGTGCT</td>
<td>TGTATGCGCTGACTTCCTTG</td>
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* – taken from Ho et al. 41
**Figure 1.** Cd accumulation rates in zebrafish gills exposed to 0.003-0.025 µM $^{106}$Cd and 0.05-0.5 µM $^{65}$Cu as single metals or their mixtures. Values represent mean ± SEM from 5 biological replicates and capital letters indicate those treatments that are significantly different from each other (One-way ANOVA analysis followed by a Holm-Sidak Post-hoc test $p<0.05$).
**Figure 2.** Cu accumulation rates in zebrafish gills exposed to 0.003-0.025 µM $^{106}$Cd and 0.05-0.5 µM $^{65}$Cu as single metals or their mixtures. Values represent mean ± SEM from 5 biological replicates and capital letters indicate those treatments that are significantly different from each other (One-way ANOVA analysis followed by a Holm-Sidak Post-hoc test p<0.05).
Figure 3. Temporal variations in transcript abundance of genes for proteins involved in Ca (ECaC), Na (NHE-2), Cu (CTR1, ATP7A), Zn (ZIP8) and divalent metal (DMT1) transport in zebrafish gills during single metal (0.025µM $^{106}$Cd, 0.05µM $^{65}$Cu or 0.5µM $^{65}$Cu) or combined metal (0.05µM $^{65}$Cu +0.025µM $^{106}$Cd or 0.5µM $^{65}$Cu +0.025µM $^{106}$Cd) exposures. The dotted lines correspond to two-fold increase or decrease in relative transcript abundance level (results presented as mean ± SD from 5 biological replicates). Those treatments with different upper case (24h) or lower case (48h) letters are significantly different from each other (One-way ANOVA analysis followed by a Holm-Sidak Post-hoc test p<0.05). Significant differences within treatment (24h vs 48h) is indicated by #.