

**Dietary iron alters waterborne-copper induced gene expression in  
softwater acclimated zebrafish (*Danio rerio*)**

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## **Abstract**

Metals like iron (Fe) and copper (Cu) function as integral components in many biological reactions and in excess, these essential metals are toxic, and organisms must control metal acquisition and excretion. We examined the effects of chronic waterborne Cu exposure and the interactive effects of elevated dietary Fe on gene expression and tissue metal accumulation in zebrafish. Softwater acclimated zebrafish exposed to 8µg/L Cu, with and without supplementation of a diet high in Fe (560 vs 140 mg Fe/kg food) for 21d demonstrated a significant reduction in liver and gut Cu load relative to waterborne Cu exposure alone. Gene expression levels for divalent metal transport (DMT)-1, copper transporter (CTR)-1 and the basolateral metal transporter ATP7A in the gills and gut increased when compared to controls, but the various combinations of Cu and high Fe diet revealed altered levels of expression. Further examination of the basolateral Fe transporter, ferroportin, showed responses to waterborne Cu exposure in the gut, and a significant increase with Fe treatment alone in the liver. Additionally, we examined metallothionein 1 & 2 (MT1 & MT2), which indicated that MT2 is more responsive to Cu load. To explore the relationship between transcription and protein function, we examined both CTR-1 protein levels and gill apical uptake of radiolabelled Cu<sup>64</sup>, which demonstrated decreased Cu uptake and protein abundance in the elevated Cu treatments. This study shows that high dietary Fe can significantly alter the genetic expression pattern of Cu transporters at the level of the gill, liver, and gastrointestinal tract.

## 22    **Introduction**

23            Trace elements such as copper (Cu), and iron (Fe) are essential micronutrients for  
24    all organisms due to their high redox potential, and importance as cofactors for a variety  
25    of metabolic proteins, such as cytochrome C oxidase and haemoglobin (45). However,  
26    due to increased anthropogenic activities, exogenous concentrations of these trace  
27    elements are tending to increase in natural ecosystems, which can be harmful, if not fatal,  
28    to aquatic organisms. Due to their persistence in the aquatic environment, it is important  
29    to examine the chronic biological impacts of these metals, especially in tropical water  
30    systems, where focus has been lacking. Zebrafish, a tropical species, have become an  
31    excellent model to study the physiological and genetic impact of increased metal  
32    contamination due to their publicly available genome and ability to tolerate softwater,  
33    which is key in examining metal impacts without interference of other cations  
34    (12,13,49,55,56). Furthermore, zebrafish are an endemic species to the Indian  
35    subcontinent, and may be found in waters that can contain Fe and Cu at levels 100 and 15  
36    times greater, respectively, than those dictated by Indian environmental protection rules  
37    (Fe: 3mg/L vs 326mg/L; Cu: 3mg/L vs 48mg/L; 57) or United States EPA regulations  
38    (66) . It is essential to understand the impact metals can have on tropical species in such  
39    situations to assess the potential risk of morbidity and mortality which would lead to a  
40    population decline.

41            The primary uptake pathway of trace metals in fish is the diet, although  
42    considerable evidence suggests that Cu and Fe can also be taken up by the gills  
43    (10,23,28). Several transport mechanisms associated with Cu and Fe uptake are found in  
44    the gill, liver and gastrointestinal tract of zebrafish. Although principally characterized as

45 a Fe transporter, divalent metal transporter-1 (DMT-1) appears to function as a carrier for  
46 most divalent metal ions across the apical surface of the cell (24). Its expression has been  
47 detected in both the gills and gastrointestinal tract of zebrafish (11,31). Ferroportin, a  
48 known Fe exporter, transports Fe from the cell into circulation and has been characterized  
49 in both mammalian and fish models (1,17). Interestingly, in macrophages, ferroportin  
50 gene expression has been shown to increase in a dose- dependent manner upon increased  
51 Cu exposure, which stimulates the release of Fe (9). Other transporters involved are  
52 specifically related to Cu: the apical copper transporter-1 (CTR-1) and the basolateral Cu-  
53 ATPase (ATP7A or Menkes gene). CTR-1 is ubiquitously expressed in all tissues in both  
54 mammals and other vertebrates, although the largest concentration of CTR-1 in mammals  
55 is found in the small intestine, where 90% of total body Cu is absorbed (61). In zebrafish,  
56 the CTR-1 gene was first cloned and characterized in studies focusing on embryonic  
57 development and the importance of Cu for growth (39). It was found that altering  
58 ambient levels of Cu inversely affected CTR-1 transcripts, indicating CTR-1 expression  
59 changes are crucial for normal zebrafish development (39). However, the CTR-1 gene  
60 has not been examined under elevated waterborne Cu in adult zebrafish. Craig et al (13)  
61 found that zebrafish had fluctuations in transcript levels of CTR-1 in the gill during  
62 softwater acclimation, indicating that waterborne Cu is required for normal physiological  
63 homeostasis. ATP7A ('Menkes' protein) is an essential Cu transport protein involved in  
64 both the packaging and transport of cellular Cu into the plasma. Essentially, ATP7A can  
65 transfer Cu to the Golgi apparatus to be incorporated into copper-dependent enzymes  
66 such as lysyl oxidase or allow for direct secretion of Cu into the circulation (see review  
67 by 33). Characterization of this transporter is well established in mammals, due to its

importance in human copper deficiency diseases such as Menkes disease (36). To date, no characterization of ATP7A expression has been performed in adult zebrafish exposed to elevated waterborne Cu. Changes in expression of this protein may be important for the regulation of basolateral Cu transport with changing environmental Cu exposure.

As metals accumulate within cells above ambient levels, several mechanisms are invoked to prevent cellular damage, particularly metal chaperone proteins such as metallothioneins (MT). MTs are metal-binding proteins of low molecular mass that play essential biological roles in metal homeostasis, cytoprotection and detoxification (27). Furthermore, they are increasingly used as biomarkers of metal pollution in the environment (14,30,51). Two different isoforms of MTs have been cloned and characterized in zebrafish, MT1 and MT2 and are found in the majority of tissues (7,21). These isoforms are particularly sensitive to cadmium (Cd) and Cu, but they are known to bind to various other metals (27). However, the relative responsiveness of the two isoforms has not been studied previously in zebrafish. It is of particular interest to examine which isoform is most sensitive to Cu, as this may aid in determining biomarkers of Cu toxicity.

There is a wealth of data on single metal exposures in fish (4,5,8) including zebrafish (12). However, it is more relevant, both environmentally and physiologically, to examine exposure to metal mixtures. We chose to examine the interactive effects of Fe and Cu since in a pilot study, we found that chronic exposure to 15µg/L waterborne Cu caused a surprising increase in expression of DMT-1 in the gills, which was contrary to our predictions of zebrafish maintaining homeostatic control over Cu. There was also a significant reduction in the Fe load in the liver indicating a potential competition between

91 Cu and Fe, though interpretation was confounded by some mortality (unpublished  
92 observations). Many studies have shown that the best characterized link between Cu and  
93 Fe is the Cu-derived protein ceruloplasmin, which has been shown to act as a serum  
94 ferrioxidase, and is required for Fe mobilization and metabolism in Fe storage tissues (see  
95 review 62). There is a link between decreased total body Cu and decreased ceruloplasmin  
96 levels, which in turn leads to an impairment of iron metabolism resulting in anaemia  
97 (47,52). Furthermore, under conditions of excess Cu in mammals, haemolytic anaemia  
98 occurs due to Cu interruption of glycolysis in erythrocytes, which denatures the  
99 haemoglobin (20). However, this may not occur in fish since their erythrocytes have a  
100 greater aerobic capacity (19). If excess Cu in zebrafish leads to altered Fe homeostasis,  
101 then supplementing the fishes' diet with Fe should alleviate any Cu-induced anemia.

102 In the present study, we employed a level of chronic waterborne Cu exposure (8  
103  $\mu\text{g/L}$ ) that is environmentally relevant for softwater environments (66), examining its  
104 effects on gill, liver, and gut expression of CTR-1, DMT-1, ATP7A, ferroportin, MT1  
105 and MT2. To further characterize the expressional pattern of Cu uptake, we examined  
106 how a Fe supplemented diet can alter the gene expression of these transcripts at the gills  
107 and the liver. Several studies on fish have demonstrated how a metal- or ion-amended  
108 diet can affect uptake of non-essential ions from both the water and food. For example,  
109 dietary Ca or Fe can affect branchial cadmium uptake (2,11 in trout and zebrafish,  
110 respectively). The goal of this study was to identify the transcription profile of Cu  
111 transporters in the gill, liver and gut, of softwater acclimated zebrafish chronically (21d)  
112 exposed to moderate levels of waterborne Cu and to determine how dietary Fe can affect  
113 both the transcription profile and gill apical uptake of an essential micronutrient.

114 Additionally, as a functional test, short-term uptake of radiolabelled Cu ( $^{64}\text{Cu}$ ) was  
115 employed to complement this data to clarify the relationship between transcript levels,  
116 protein levels, and functional Cu transport activity.

## 118 **Materials & Methods**

### 120 *Fish Care*

121 Adult zebrafish of mixed sex (*Danio rerio*) were purchased from a local pet fish  
122 distributor (DAP International, Canada) and acclimated to soft-water over a 7 day period  
123 in an aerated 40L aquarium as described previously (10; Table 2). After acclimation,  
124 zebrafish were housed in multiple 3L self-cleaning AHAB tanks racked in a soft-water  
125 recirculating stand-alone AHAB filtration system (Aquatic Habitats, Apopka, FL). Fish  
126 were fed twice daily with a commercial tropical fish food (Topfin, Phoenix, AZ) and  
127 maintained on a 12-h light, 12-h dark photoperiod regime until experiments began. All  
128 procedures used were approved by the McMaster University Animal Research Ethics  
129 Board, and conform to the principles of the Canadian Council for Animal Care.

### 131 *Exposure to waterborne Cu and Fe diet*

132 Zebrafish (n=224; 40-48 per treatment) were weighed and placed in 8L aerated,  
133 flow through soft-water tanks set to 25ml/min. There were four experimental treatments:  
134 0  $\mu\text{g Cu/L}$ (Ctrl), 0  $\mu\text{g Cu/L}$  (Ctrl+Fe diet), 8  $\mu\text{g Cu/L}$ , or 8  $\mu\text{g Cu/L}$  + Fe diet. For the  
135 two elevated Cu treatments, Mariotte bottles were used to dose tanks with a concentrated  
136 Cu solution made from  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  dissolved in 0.05%  $\text{HNO}_3$ , nitric acid, which had no  
137 measurable impact on water pH. Fish were fed 2% body weight once per day of

commercial tropical fish food or a diet supplemented with Fe. The high iron diet was formulated by the addition of 240mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  to 40g of powdered tropical food. Food and Fe was mixed with the addition of a small amount of water, spread on a baking sheet and dried at 35°C for 1h, and then collected and re-powdered. A sample of the normal diet and elevated Fe diet was taken and dissolved in  $\text{HNO}_3$  and measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z, Varian, Palo Alto, CA) to verify Fe concentrations. The normal diet contained ~ 140 mg Fe/kg food, whereas the high Fe diet contained ~560 mg Fe/kg food. Tanks were monitored daily for mortality and cleaned of any food or waste that had accumulated. Daily, a 10ml water sample was taken from each tank, filtered through a 0.45µm filtration disc (Pall Corporation, East Hills, NY), added to a plastic tube containing 100µl  $\text{HNO}_3$  and kept at 4°C for analysis of Cu and Fe concentrations. At the end of the exposure period, fish were quickly euthanized by cephalic concussion and sampled for gill, gut, and liver tissue which were immediately frozen in liquid  $\text{N}_2$  for further analysis of Cu and Fe burdens and gene expression. Additional gill samples were taken for western blotting.

#### *Water and tissue ion levels*

All tissues (and food) were first digested in 1ml of 1N  $\text{HNO}_3$  for 48 h at 60°C. Digests were diluted 10× and dissolved Cu levels were measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z; Varian, Palo Alto, CA) and compared to a 40 µg/L Cu standard (Fisher Scientific, Ottawa, ON). Water Cu levels were read without dilution. Water Fe levels were measured by graphite furnace and compared to a diluted 1mg/L Fe standard (Fisher Scientific). Both tissue digests



(Na<sup>+</sup>, Mg<sup>2+</sup>, Fe<sup>2+</sup> and Ca<sup>2+</sup>) and water ion composition (Na<sup>+</sup>, Mg<sup>2+</sup>, and Ca<sup>2+</sup>), were measured by flame atomic absorption spectroscopy (Spectra AA 22 0FS, Varian, Palo Alto, CA) after 10× dilutions were made with 1% HNO<sub>3</sub> (Na<sup>+</sup>) or 0.5% LaCl<sub>3</sub>/1% HNO<sub>3</sub> (Mg<sup>2+</sup>, Ca<sup>2+</sup>, Fe<sup>2+</sup>), and verified using certified Na<sup>+</sup>, Mg<sup>2+</sup>, Fe<sup>2+</sup> and Ca<sup>2+</sup> standards (1 mg/L diluted in 1% HNO<sub>3</sub> or 0.5% LaCl<sub>3</sub>/1% HNO<sub>3</sub>; Fisher Scientific).

#### *Quantification of mRNA by real-time RT-PCR.*

Total RNA from the gill, gut and liver tissues were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified immediately by UV spectrophotometry at 260 nm, and RNA purity verified by the 260/280 nm ratio (Fisher Scientific, Nanodrop ND-1000, Wilmington, Delaware). First strand cDNA was synthesized from 1 µg of total RNA treated with DNase I (Invitrogen, Calsbad, CA) and reverse transcribed to cDNA using SuperScript II RNase H- reverse transcriptase (Invitrogen, Calsbad, CA). mRNA expression was quantified in duplicate on a Stratagene MX3000P real-time PCR machine using SYBR green with ROX as reference dye (Bio-Rad, Mississauga, ON). Each reaction contained 12.5 µl SYBR green mix, 1 µL of each forward and reverse primer (5 µM), 5.5 µL RNase/DNase free H<sub>2</sub>O, and 5 µL of 5× diluted cDNA template. Cycling conditions were as follows: 3 min initial denaturation at 95°C, 40 cycles of 95°C for 15 sec, 60°C for 45sec, 72°C for 30 sec. This was followed by a melting curve analysis to verify the specificity of the PCR products within and between tissues. To account for differences in amplification efficiency between different cDNAs, standard curves were constructed for each target gene using serial dilutions of

stock gill, gut and liver cDNA. To account for differences in cDNA production and loading differences, all samples were normalized to the expression level of the house-keeping gene EF1 $\alpha$ , which did not change over the course of the experimental treatments. Gene expression data were calculated using the  $2^{-\Delta\Delta Ct}$  method (38). Both DNase- and RNase-free water and non-reverse transcribed RNA were assayed on each plate to ensure there was no contamination present in reagents or primers used. Primers were designed using Primer3 (59). Target genes of interest are as follows: Menkes transporter (ATP7a), copper transporter-1 (CTR-1), divalent metal transporter-1 (DMT-1), elongation factor-1 alpha (EF1 $\alpha$ ), ferroportin, metallothionein 1 (MT1), and metallothionein 2 (MT2). Primers and accession numbers can be found in Table 1.

#### *Apical gill uptake of $^{64}\text{Cu}$*

Following final sampling on day 21 (i.e. at the end of the exposure period), an acute (20min) Cu gill uptake study was performed on fish remaining from each of the experimental treatments (n=8 per treatment).  $^{64}\text{Cu}$  was prepared from dried  $\text{Cu}(\text{NO}_3)_2$  (300 $\mu\text{g}$ ) and irradiated at the McMaster nuclear reactor to achieve a radioactivity level of 0.6mCi (half-life = 12.9h). After irradiation, the  $\text{Cu}(\text{NO}_3)_2$  was dissolved in 0.1mM  $\text{HNO}_3$  (400 $\mu\text{l}$ ), 0.01mM  $\text{NaHCO}_3$  (400 $\mu\text{l}$ ) and Nanopure water (1.7ml). The resuspended  $^{64}\text{Cu}$  stock was added to 1.5L tanks containing aerated softwater 30 min prior to the addition of fish. Water samples (10ml) were taken in duplicate at the beginning and end of the exposure for the measurement of dissolved and radioactive Cu. After exposure, fish were removed, rinsed in a concentrated solution of Cu to remove any loosely bound radioisotope by displacement, and were terminally anesthetised with an overdose of MS-

222 (1g/L). Previous tests have validated this procedure (23). The gills were excised from the body, and both gills and carcass were blotted and weighed prior to gamma counting. The gamma radioactivities of  $^{64}\text{Cu}$  of the gills (representative of apical uptake; 23) and carcass were measured on a Minaxi-  $\gamma$  Auto gamma 5530 counter (Canberra Packard, Mississauga, ON) using energy windows of 433-2000 KeV for  $^{64}\text{Cu}$ .  $^{64}\text{Cu}$  was corrected for decay to a common reference time due to a short half-life (12.9h).

#### *Western blot of gill CTR-1*

Whole gill arches were homogenized in buffer (100 mM imidazole, 5mM EDTA, 200 mM sucrose, and 0.1% deoxycholate, pH 7.6), and centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was collected and diluted to 30  $\mu\text{g}$  of protein in 4 $\times$  loading buffer (48 mM Tris-HCl pH 6.8, 4% glycerol, 3.2% SDS, 600 mM  $\beta$ -mercaptoethanol, 1.6% bromophenol blue). Samples were denatured in boiling water for 5 minutes, and loaded onto a 7.5% SDS-polyacrylamide gel. Proteins were separated by electrophoresis for 1h at 150V. Samples were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Mississauga, ON), and blots were incubated overnight at 4°C in 7.5% skim-milk + PBST (10mM phosphate buffer, 0.09% NaCl, 0.05% Tween-20, pH 7.5, PBST). Blots were washed 3 $\times$  for 5min in PBST and incubated at room temperature with the primary antibody diluted in 5% skim-milk + PBST. The primary antibody (1:200) used was a human CTR-1 antibody (ab30907; Abcam, Cambridge, MA) which was designed on a highly conserved region of the hCTR protein. The % amino acid homology of the conserved region between the hCTR-1 (accession # NM\_001855) and the zebrafish CTR-1 (accession # NM\_205717) was 90%. Tubulin was used due to

availability and good reactivity with zebrafish tissues and did not change with our treatments (13). Membranes were washed  $3 \times 5$  min in PBST, and incubated for 1 h at room temperature with an HRP-conjugated anti-rabbit IgG (CTR-1, 1:25,000; PerkinElmer, Boston MA) or anti-mouse IgG (tubulin, 1:50,000). After  $3 \times 5$  min washes with PBST, proteins were visualized with a Western Lightning chemiluminescence kit, following the manufacturer's protocol (PerkinElmer). Blots and band density analysis were completed on a ChemiImager (AlphaInnotech Corporation, San Leandro, CA), which used pixel density to quantify band intensity. Bands were normalized to tubulin, and expressed as a ratio of the control.

#### *Statistical Analysis*

Statistical analysis was performed using Sigma Stat (SPSS Inc, Chicago, MI). In particular, a one-way ANOVA and a Student-Newman Keuls post-hoc test was used to test for significance for all pairwise treatments ( $p < 0.05$ ). All data have been expressed as mean  $\pm$  SEM.

## **Results**

#### *Water ion composition & fish weights*

Water ion analysis verified that all experiments were conducted in a soft-water environment (hardness as  $\text{CaCO}_3$  equivalents =  $6.9 \pm 0.3$  mg/L; Table 2). This reduced the protective effects which would be exerted by ions normally present in hard water against metal toxicity. Statistical analysis revealed that a significant difference exists only when comparing Cu concentrations to that of controls (Table 2). The elevated Fe diet in

experiment 2 did not cause any change in waterborne Fe levels (Table 2). Zebrafish were weighed prior to and after exposure to all treatments, with the control and control + Fe diet increasing in weight after 21d, and all other treatments decreasing in weight, by up to 17% for fish exposed to 8 µg /L Cu alone (Table 3). There were no mortalities in any of the treatments.

#### *Exposure to waterborne Cu and Fe diet*

Significant increases in Cu load were found in all tissues examined in zebrafish exposed to 8 µg/L Cu (Fig 1). Interestingly, there was a significant accumulation of Cu in the gills of zebrafish fed the high Fe diet only, which was further exacerbated in fish exposed to 8µg/L Cu + high Fe diet (Fig 1A). There was a tendency towards a decrease in Fe tissue burden in both the gut and gill of fish exposed to 8µg/L Cu alone ( $p=0.06$  &  $p=0.07$ , respectively), and surprisingly a significant decrease in liver Fe of zebrafish exposed to 8 µg/L Cu + high Fe diet (Fig 2). There was little change in expression of CTR-1 except in zebrafish treated with 8µg/L Cu + high Fe diet where there was a 13- and 7-fold significant increase in CTR-1 gene expression in both the gill and gut (Figure 3A, B). Likewise, we saw a 5-fold increase in DMT-1 gene expression in the gill under the same conditions (Fig 4A), but no changes were detected in the gut or liver. However, DMT-1 expression did increase in both the gill and gut of zebrafish fed a high Fe diet in the absence of additional Cu (Fig 4 A,B). Interestingly, DMT-1 was upregulated in the liver when zebrafish were exposed to 8µg/L Cu (Fig 4C), as was ATP7A (Fig 5C). Zebrafish exposed to 8µg/L Cu + high Fe diet had significant increases in ATP7A expression in both the gill and gut. There was also a significant increase in gut ATP7A expression, but no change in the gill with Cu alone(Fig 5 A,B). Ferroportin expression

increased significantly in the gut and gill of fish exposed to 8 µg/L Cu alone but the gut was not responsive to other treatments (Fig 6A, B). Furthermore, we saw a 2.5 - and 4-fold increase in gene expression of ferroportin in the gill and liver of fish fed a high Fe diet in the absence of Cu. However, there were no increases in ferroportin gene expression in liver of fish exposed to 8 µg/L Cu + high Fe Diet (Fig 6C). When we compared the expression profiles of MT1 versus MT2 in the gill and liver, we found that MT2 had a greater response to Cu than MT1, but MT1 did significantly increase when fish were exposed to both 8 µg/L Cu + Fe diet (Fig. 7A,C). Neither MT1 nor MT2 showed changes in mRNA expression in the gut with any of the experimental treatments (Fig. 7B).

We examined CTR-1 protein expression changes using western blotting. Band intensities were normalized to tubulin and expressed as a ratio of the control. We saw a significant decrease in protein expression of CTR-1 in zebrafish exposed to 8µg/L Cu only. Feeding zebrafish a high Fe diet resulted in a partial reversal of the Cu-induced decline in CTR-1 protein expression(Fig 8). Functional measurements of Cu transport revealed a significant decrease in the gill apical and whole body uptake of <sup>64</sup>Cu in zebrafish exposed for 21d of 8µg/L Cu, and only a decrease in the whole body uptake of zebrafish exposed to 8µg/L Cu + high Fe diet (Fig. 9).

## **Discussion**

This study shows that both chronic waterborne Cu exposure and a high Fe diet can on their own significantly alter the genetic expression pattern of Cu transporters at the level of the gill, liver, and gastrointestinal tract, but that there are also unique

interaction effects. With a moderate Cu exposure (8µg/L), in confirmation of an initial pilot study at a higher Cu level (15 µg/L, unpublished), we found there was a decrease in tissue Fe levels in the gill and gut (Fig 2A,B). This prompted us to expose zebrafish to an elevated Fe diet, since we assumed Cu could have a competitive effect with Fe due to a shared uptake pathway (DMT-1). With the addition of Fe to the diet, we did not see any changes in Fe tissue levels, despite an increased Cu load in the gills, gut and liver (Fig 1). Upon examination of the gene expression profile, we found 8 µg/L Cu + Fe diet significantly increased expression of CTR-1 in the gill and gut (Fig 3A,B). Additional increases in DMT-1 were found in the gill and liver with waterborne Cu, with the Fe diet exacerbating the increase in gill DMT-1 expression over the 8µg/l Cu exposure (Fig 4A, C). Interestingly, we found that a high Fe diet without waterborne Cu significantly increased DMT-1 expression in the gut (Fig 4B). With respect to the basolateral Cu transporter ATP7A, we found that moderate Cu exposure increased expression levels in the gut and liver, and with the addition of an Fe diet, we saw increased expression in the gills and gut (Fig 5). Contrary to our gene expression profile, the protein expression of CTR-1 and the apical uptake of Cu in the gills tell a different story. Protein expression actually decreased concurrent with a decrease in Cu uptake. This may hint at an increased protein turnover rate under stressful conditions (Fig 6, 7).

Copper homeostasis in fish is tightly regulated, and as in higher vertebrates, such as mammals, excess Cu is accumulated in the liver and excreted in the bile (22). Likewise in zebrafish, we saw an elevated Cu load in the liver of fish exposed to increased waterborne Cu (Fig 1C). However, we also saw increases in Cu load in the gut under these conditions (Fig 1B). Waterborne Cu has two possible modes of uptake, either

through the gills or the gut, and excess Cu is excreted through the bile (22). Increased Cu load in the gut may be primarily due to increased biliary excretion of Cu. Furthermore, at 8µg/L waterborne Cu, we saw elevated Cu load in the gills. Grosell & Wood (23) demonstrated that gill Cu load rapidly peaks with waterborne Cu exposure after the first 3hr, and then gradually reaches equilibrium, which supports our findings at a moderate Cu exposure.

In our initial pilot experiment of exposing zebrafish to 15µg/L Cu (unpublished), we found evidence that fish exhibited significantly lower tissue Fe load in both the gill and liver. Although there is little prior evidence in fish to suggest Cu would have such an effect, there is the likelihood that high waterborne Cu can out-compete Fe for apical uptake at the gill, since they can use the same transporter, DMT-1 (24). With zebrafish exposed to both moderate waterborne Cu (8 µg/L) and Fe diet, we were able to reverse the diminished Fe tissue levels, although a high Fe diet significantly increased the Cu load in the gills both with and without waterborne Cu (Fig 1A). Potentially, this is an adaptive response to an elevated Fe load, as there are Cu-essential transmembrane ferroxidases, like hephaestin, which function in addition to ferroportin, to translocate Fe across the basolateral membranes into the general circulation, although this protein has not as yet been localized in the zebrafish gill (68).

In an effort to identify potential genetic endpoints of chronic waterborne toxicity, we examined both metal sequestering proteins and several key transporters thought to regulate the uptake of Cu at both the level of the gills and gut. Furthermore, we examined gene expression changes for these transporters in the liver, to identify any compensatory responses to toxicity and characterize detoxification methods. Upon examination of the



346 two known MT isoforms, we found that MT2 was more responsive to Cu exposure than  
347 MT1 in both gill and liver tissue (Fig 7A,C), where there is the highest accumulation of  
348 Cu (Fig. 1A, C). Previous studies examining increased zinc exposure demonstrated that  
349 MT-1 is more responsive to excessive Zn in rats (40), whereas MT-2 has demonstrated  
350 specificity to Cu in blue crabs, although this has yet been identified in fish species (63) .  
351 We additionally saw a 9-fold increase in expression of MT1 in the liver of zebrafish  
352 exposed to both 8µg/L Cu + high Fe diet (Fig 7C). Aside from a role in metal  
353 sequestration, MTs have been shown to scavenge both hydroxyl radicals and superoxides  
354 in mouse and rat liver cells (46). Free cytoplasmic  $\text{Cu}^{2+}$  and  $\text{Fe}^{2+}$  are highly reactive ions  
355 and can result in the production of superoxide hydroxyl radicals if left unchecked. In the  
356 liver of zebrafish, there is an excessive build-up of Cu which may promote increased  
357 ROS production (12), and this may be further exacerbated by Fe flux through the liver.  
358 However, the bottom line is that MT2 has been identified as a Cu related biomarker,  
359 opposite to the current thinking of examining all forms of MTs.

360 CTR-1 is a high affinity Cu transporter first cloned in zebrafish by Mackenzie et  
361 al. (39). Interestingly, there is no evidence for transcriptional regulation of CTR-1 by Cu  
362 in mammals, although protein levels undergo changes in subcellular location and  
363 stabilization (35,54,65). Our study suggests that CTR-1 is transcriptionally regulated in  
364 the gills and gut of zebrafish by moderate Cu + high Fe diet (Fig 3A, B). Although  
365 increased transcription of CTR-1 in a situation of elevated waterborne Cu seems counter  
366 intuitive, it again could be a compensatory response to promote cellular assimilation and  
367 translocation of essential metals. Cu is transported from the cell into the blood stream  
368 through two potential routes, either direct secretion via ATP7A or incorporation into

ceruloplasmin (44,67). Ceruloplasmin can act as a ferroxidase, oxidising  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ , which allows for Fe delivery to peripheral organs for use or detoxification (26,29). Therefore, to respond to an increased Fe diet, more Cu needs to be absorbed to aid in transport and oxidization of Fe.

DMT-1 has primarily been identified as an Fe transporter, although it does have the potential to transport other divalent metals (24,60), and has been identified in numerous fish species, including zebrafish (18). In *Xenopus* oocytes, DMT-1 appears to transport a diverse range of metals such as  $\text{Pb}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Zn}^{2+}$  (24). Recently, it has been implicated in the dietary uptake of Cu and zinc in rainbow trout intestine (48,50). In similar fashion to CTR-1, we saw a significant increase in DMT-1 expression in the gills and gut of zebrafish exposed to a high Fe diet (Fig. 4A,B), suggesting increased Fe uptake, although we did not see any substantial increase in tissue Fe load (Fig 2A,B). Cooper et al (11) have shown that a low Fe diet results in increased DMT-1 expression as an adaptive response to absorb more Fe from a deficient diet. In our study, we found that DMT-1 expression increased with a high Fe diet in the gut and gills (Fig. 4A,B). There was also significant increase in DMT-1 expression in zebrafish exposed to  $8\mu\text{g/l}$  Cu + high Fe diet in the gills, although there were no changes in expression in the gut in this treatment (Fig 4A,B). Potentially, zebrafish exposed to high waterborne Cu are reducing the uptake of Cu in the gut by decreasing the number of uptake pathways. In the liver, we see a significant increase in DMT-1 only in zebrafish exposed to  $8\mu\text{g/l}$  waterborne Cu (Fig 4C). In fish, Cu is transported through the blood via ceruloplasmin (53) and as seen in mammalian liver cell suspensions, Cu bound to ceruloplasmin is taken up into hepatocytes by a ceruloplasmin receptor (64). However, in

cases of excessive Cu exposure, free Cu ion levels can increase in the blood serum, although this phenomenon has only been identified in mammals (6,15). Although there was no change in CTR-1 expression in the liver (Fig. 3C), the increase in DMT-1 expression (Fig. 4C) may allow for the uptake of excessive Cu ions from the blood serum into the liver hepatocytes for sequestration and excretion.

In a manner similar to DMT-1, ferroportin, a known Fe exporter, is modulated by both Cu and Fe (Fig 6). The function of ferroportin as a Fe exporter has been studied in mammalian and amphibian models, and has shown increased expression levels upon exposure to excessive exogenous Fe and Cu (9,17,42). Likewise, we saw increased expression in the gut when zebrafish were exposed to 8 µg/L Cu alone and a 4-fold increased expression in the liver of zebrafish fed a high Fe diet in the absence of Cu (Fig 6B,C). Furthermore, there were significant increases in ferroportin expression in the gills of zebrafish exposed to either 8 µg/L Cu alone or Fe diet alone (Fig 6A). In *Xenopus* oocytes and mammalian macrophages, excessive Fe results in increased ferroportin expression and protein levels to offload excessive Fe, which can increase levels of reactive oxygen species (9,17,42). However, in our fish, we saw that in the liver, there was an increased ferroportin expression when fish were exposed to a high Fe diet alone, even though there was no accumulation of Fe in the liver (Fig 2C). Indeed, under all experimental conditions there was a depression of Fe levels in the liver, which indicates that the liver functions to offload excessive Fe in these situations, yet we did not see the respective changes in gene expression, except under exposure of a high Fe diet (Fig 6C). Furthermore, we saw that Cu can stimulate ferroportin expression in the gills and gut (Fig. 6A,B) as previously demonstrated (9,17,42). In mammalian macrophages, increased Cu

not only increased expression levels of ferroportin gene and protein, it also produced a dose-dependent excretion of Fe (9). This supports the model that Cu plays an essential role in Fe cycling and transport, and also eludes to ferroportin having the potential to transport Cu and other divalent metals as well.

Cu metabolism and export from the cell rely on important Cu-transporting ATPases. In mammals, there are two types of ATPases: ATP7A (Menkes gene) & ATP7B (Wilson's gene), which are associated with human genetic disorders of the same name (37). In mammals, ATP7A is found through all tissues, except the liver, where only ATP7B is found (32). Barnes et al. (3) demonstrated that ATP7A has high turnover rates and can transport more copper per minute than ATP7B. In fish, little is known about that localization and characterization of Cu-transporting ATPases, although only the ATP7A gene has been identified in the zebrafish (43). Furthermore, although the ATP7B gene has been identified in the zebrafish genome through predicted computational sequencing, there is no published RNA sequence in Genbank, which hints at a functional loss of the ATP7B gene. Our study suggests that ATP7A is important in basolateral transport of Cu out of the cells of the gill and gut under conditions of moderate Cu + high Fe diet (Fig 5A,B). In addition, there were significant increases in ATP7A in the gut and liver under conditions of moderate Cu exposure (Fig 5B,C). Two plausible explanations for the increase in ATP7A are 1) Cu plays a vital role in the oxidation of Fe ions, and this relates to the increased expression of ATP7A, as this transporter also acts to shuttle Cu to the Golgi apparatus to be incorporated into secreted cuproenzymes (44); 2) Increased cellular Cu is known to increase reactive oxygen species, and this results in increased oxidative damage (12,16). Therefore increased basolateral transport of Cu would help remove Cu

from the cell, and in the case of the liver, allow for the excretion of excessive Cu, although this requires further investigation.

Contrary to our findings that CTR-1 expression remained stable following exposure to 8µg/L Cu and actually increased after treatment with 8µg/L Cu + high Fe diet (Fig. 3A), we found a significant reduction in both apical uptake (in the 8µg/L Cu + high Fe diet exposure) and whole body incorporation of waterborne Cu (in both treatments) and reduced protein expression levels of CTR-1 in the gill (Figs. 8,9). Note also that the expression levels of the other two genes likely involved in Cu uptake at the gills (DMT1, Fig. 4A) and ATP7A (Fig. 5A) remained stable or increased in one or both of these treatments. This is the first study to investigate both the transcription profile and functional uptake of CTR-1, and provides significant insight into predictions of chronic endpoints of Cu toxicity, since increased expression of target genes does not necessarily translate into increased protein levels or increased function.

However, the importance of CTR-1 proteins in Cu transport cannot be disputed, as they are an essential route of Cu uptake for all vertebrates (62). There is obviously some post-transcriptional regulation occurring, that down-regulates the protein abundance under periods of excessive Cu; however, this requires further investigation. Outside of the above explanations for increased transcription rates of our genes of interests, an alternate explanation is that under conditions of environmental stress, there is an increased protein turnover rate, which allows for the faster production of new proteins, and faster degradation of harmful proteins (see review 25). Furthermore, it has been shown that growth rate is sacrificed as protein turnover rate increases in rainbow trout (41). In all of our fish exposed to waterborne Cu, we see a significant reduction in growth

rate compared to those fish under normal circumstances (Table 3). Increased gene expression could in part be explained by increased turnover rates, although this was not directly examined.

## **Perspectives**

Overall, we have demonstrated that alterations in the diet can affect uptake and accumulation of Cu in zebrafish when exposed to elevated waterborne Cu, as well as the gene expression of transport and binding proteins that may be involved. This study provides further evidence that Fe can enhance the uptake of an essential metal Cu (as well as non-essential metals), which has been demonstrated in both fish and mammalian species (11,34). We have also provided evidence that MT2 can be used as a Cu-specific genetic endpoint of chronic exposure, and furthered our knowledge that Cu plays an essential role in Fe cycling and transport by modifying the expression level of ferroportin. Furthermore, this is the first study to examine the CTR-1 protein from both a transcriptional and functional point of view in zebrafish, and highlights the importance of functional studies in future research, because of the disconnect between message, protein, and functional responses seen, and this has been identified in other studies (eg 58). Our results suggest that while transcriptional responses may be of diagnostic importance, they may not relate directly to protein abundance and function.

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**Figure legends:**

**Figure 1:** Copper (Cu) load ( $\mu\text{g/g}$  tissue) in gill (A), gut (B), and liver (C) tissue from soft-water acclimated zebrafish exposed to control + Fe diet,  $8\mu\text{g/L}$  water-borne Cu,  $8\mu\text{g/L}$  water-borne Cu + Fe diet, and  $15\mu\text{g/L}$  water-borne Cu for 21d . Values are presented as means  $\pm$  SEM and treatments that do not share a common letter are significantly different from each other ( $n=7$  for all treatments,  $p<0.05$ ).

**Figure 2:** Iron (Fe) load ( $\mu\text{g/g}$  tissue) in gill (A), gut (B), and liver (C) tissue from soft-water acclimated zebrafish exposed to control + Fe diet,  $8\mu\text{g/L}$  Cu,  $8\mu\text{g/L}$  Cu + Fe diet, and  $15\mu\text{g/L}$  Cu for 21d . Values are presented as means  $\pm$  SEM and treatments that do not share a common letter are significantly different from each other ( $n=7$  for all treatments,  $p<0.05$ ).

**Figure 3:** Gene expression of CTR-1 in the gill (A), gut (B), and liver (C) tissue from soft-water acclimated zebrafish exposed to control + Fe diet,  $8\mu\text{g/L}$  Cu,  $8\mu\text{g/L}$  Cu + Fe diet, and  $15\mu\text{g/L}$  Cu for 21d . Gene expression values were normalized to EF1 $\alpha$ , and are presented as mean  $\pm$  SEM (arbitrary units) and treatments that do not share a common letter are significantly different from each other ( $n=6$  for all treatments,  $p<0.05$ ).

**Figure 4:** Gene expression of Divalent Metal Transporter (DMT-1) in the gill (A), gut (B), and liver (C) tissue from soft-water acclimated zebrafish exposed to control + Fe diet,  $8\mu\text{g/L}$  Cu,  $8\mu\text{g/L}$  Cu + Fe diet, and  $15\mu\text{g/L}$  Cu for 21d . Gene expression values were normalized to EF1 $\alpha$ , and are presented as mean  $\pm$  SEM (arbitrary units) and treatments

that do not share a common letter are significantly different from each other (n=6 for all treatments, p<0.05).

**Figure 5:** Gene expression of Cu<sup>2+</sup> transporting ATPase, alpha polypeptide (ATP7A) in the gill (A), gut (B), and liver (C) tissue from soft-water acclimated zebrafish exposed to control + Fe diet, 8µg/L Cu, 8µg/L Cu + Fe diet, and 15µg/L Cu for 21d. Gene expression values were normalized to EF1α, and are presented as mean ± SEM (arbitrary units) and treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, p<0.05).

**Figure 6:** Gene expression of ferroportin in the gill (A), gut (B), and liver (C) tissue from soft-water acclimated zebrafish exposed to control + Fe diet, 8µg/L Cu, 8µg/L Cu + Fe diet, and 15µg/L Cu for 21d. Gene expression values were normalized to EF1α, and are presented as mean ± SEM (arbitrary units) and treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, p<0.05).

**Figure 7:** Gene expression of metallothionein 1 & 2 (MT1 & MT2) in the gill (A), gut (B), and liver (C) tissue from soft-water acclimated zebrafish exposed to control + Fe diet, 8µg/L Cu, 8µg/L Cu + Fe diet, and 15µg/L Cu for 21d. MT2 expression Gene expression values were normalized to EF1α, and are presented as mean ± SEM (arbitrary units) and treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, p<0.05).

**Figure 8:** (A) Relative protein expression of gill CTR-1 from soft-water acclimated zebrafish exposed to control + Fe diet, 8µg/L Cu, 8µg/L Cu + Fe diet, and 15µg/L Cu for 21d. Tubulin was measured to account for differences in protein loading. (B) Representative western blot picture of CTR-1 (68kDa) and protein normalizer, tubulin (60 kDa). Values are represented as means ± SEM, and treatments that do not share a common letter are significantly different from each other (n=8 for all treatments, p<0.05).

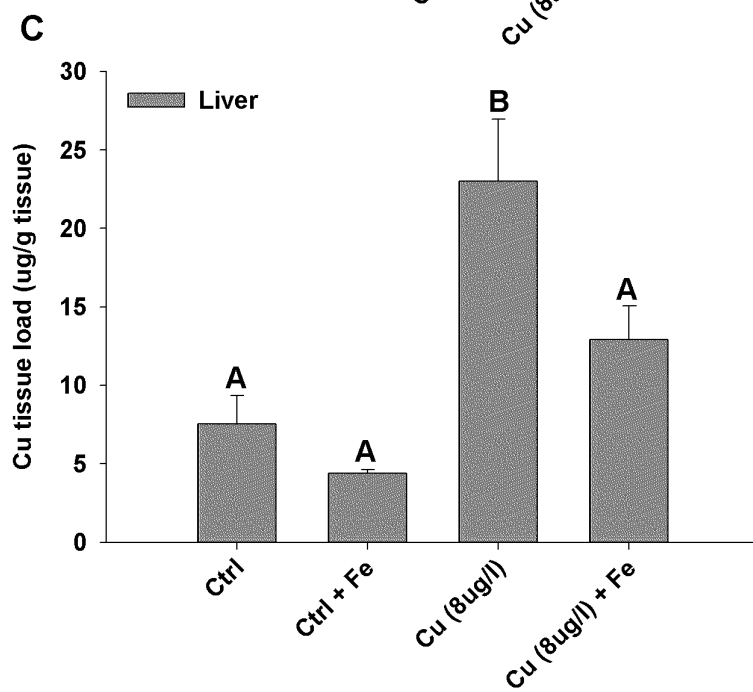
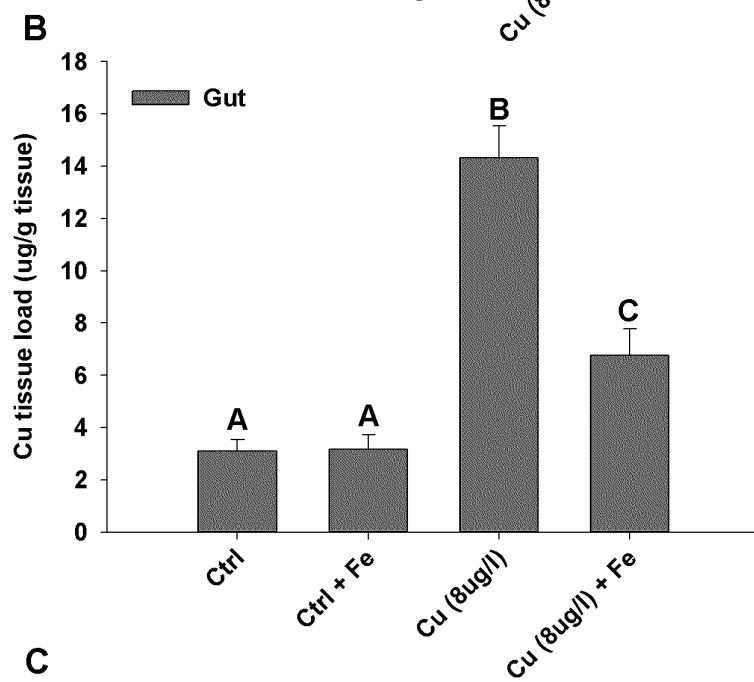
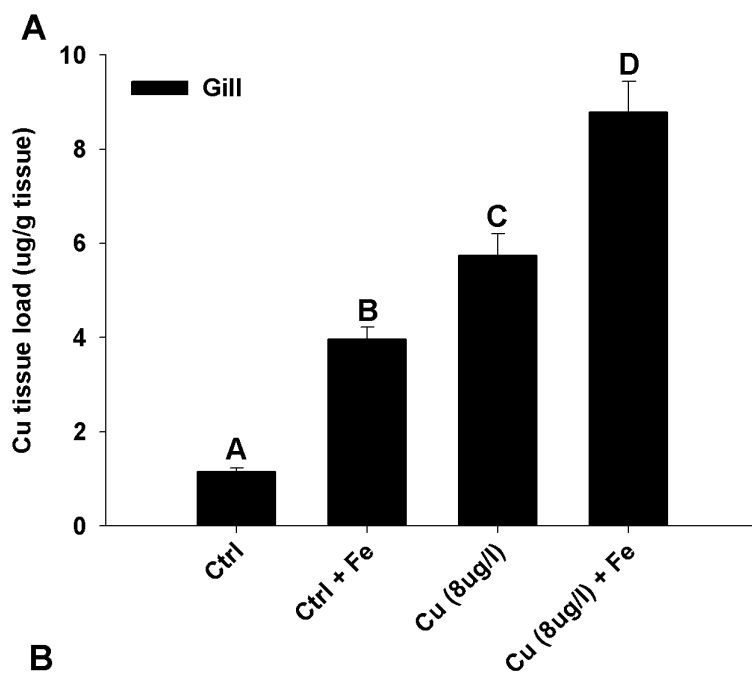
**Figure 9:** Gill apical (A) and whole body (B) uptake rate of <sup>64</sup>Cu (20min uptake exposure) from soft-water acclimated zebrafish exposed to control + Fe diet, 8µg/L Cu, 8µg/L Cu + Fe diet, and 15µg/L Cu for 21d . Gene expression values were normalized to EF1α, and are presented as mean ± SEM (arbitrary units). \* indicates significance from the soft-water control and treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, p<0.05).

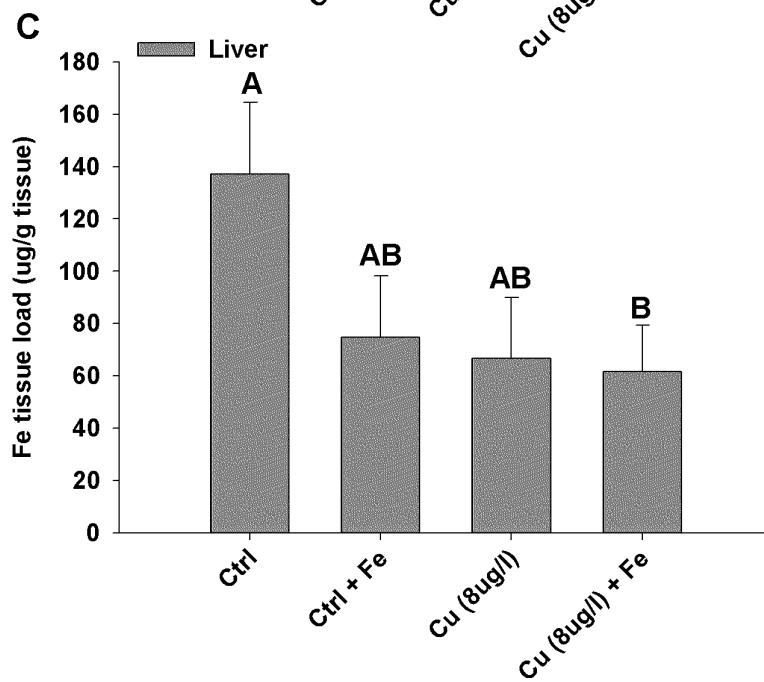
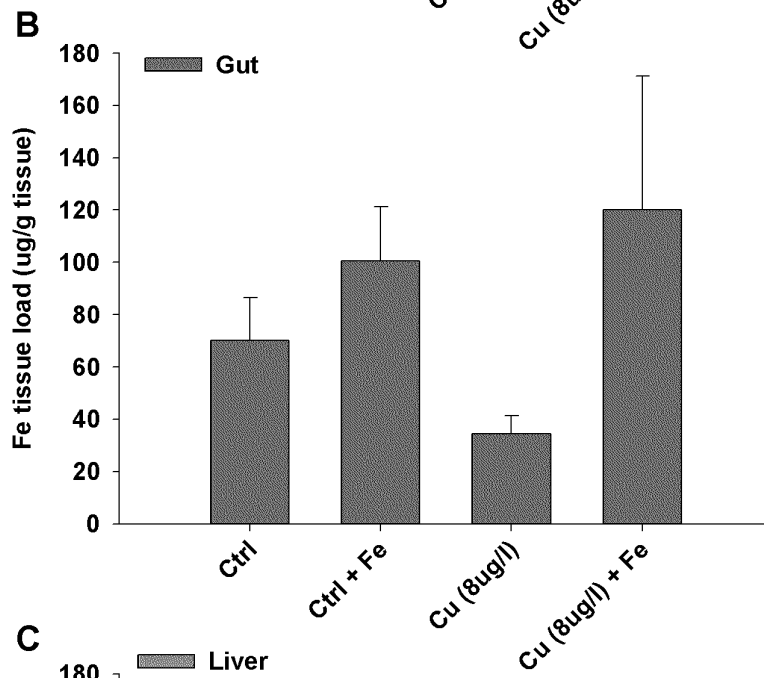
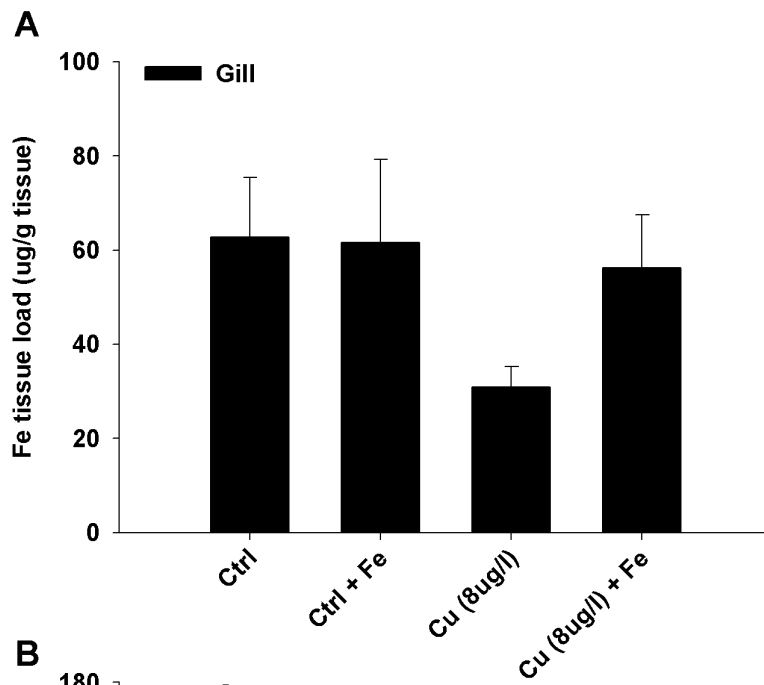
**Table 1:** Forward (F) and reverse (R) primers used for real-time qPCR

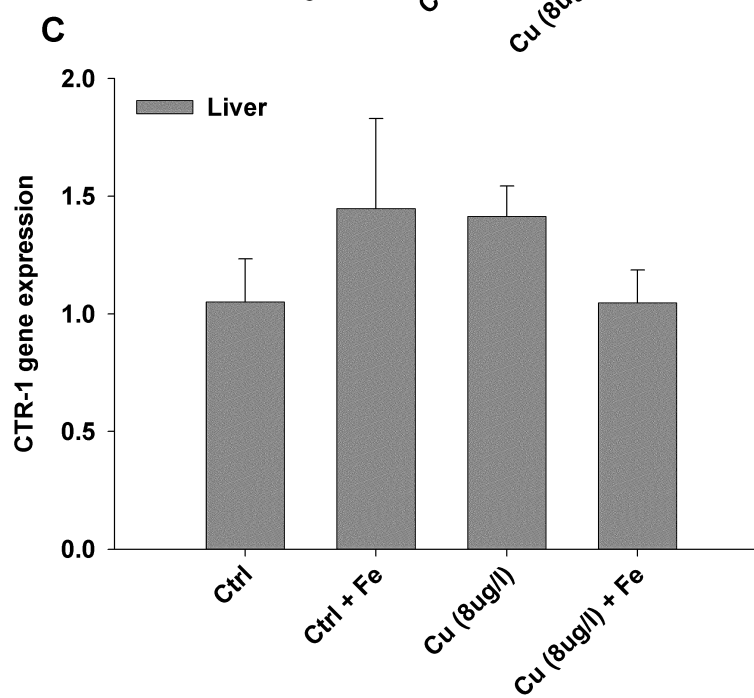
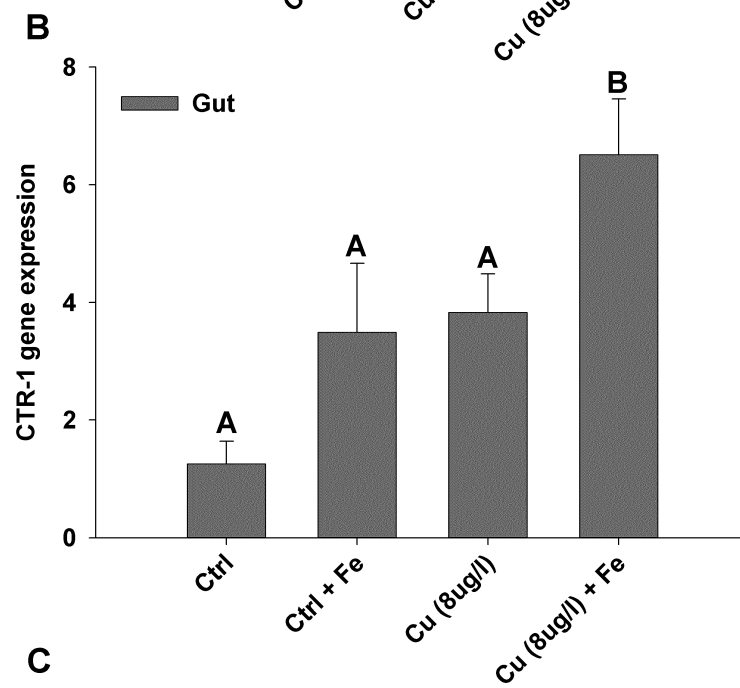
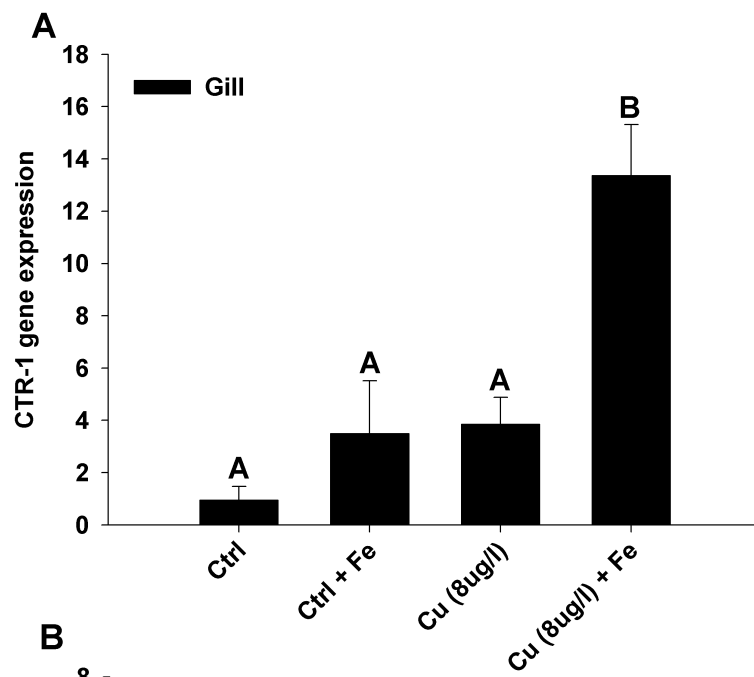
**Table 2:** Concentrations of water ions (µM), copper (µg/L), and iron (µg/L) for all experimental exposures. Values presented as mean ± SEM. \* indicates significant difference from respective control (p<0.05, n = 21).

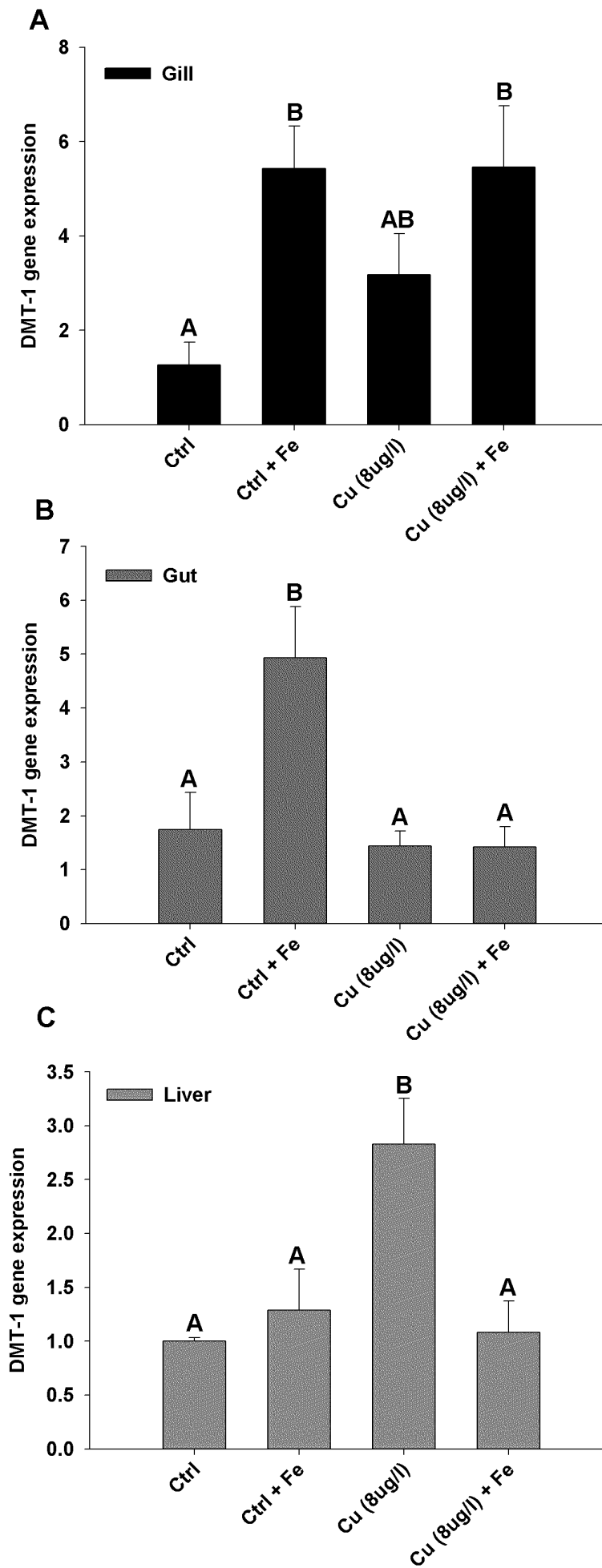
**Table 3:** Mean weight of fish before and after respective 21d treatment, including % change in weight. Values presented as mean ± SEM (n = 40-48 per treatment).

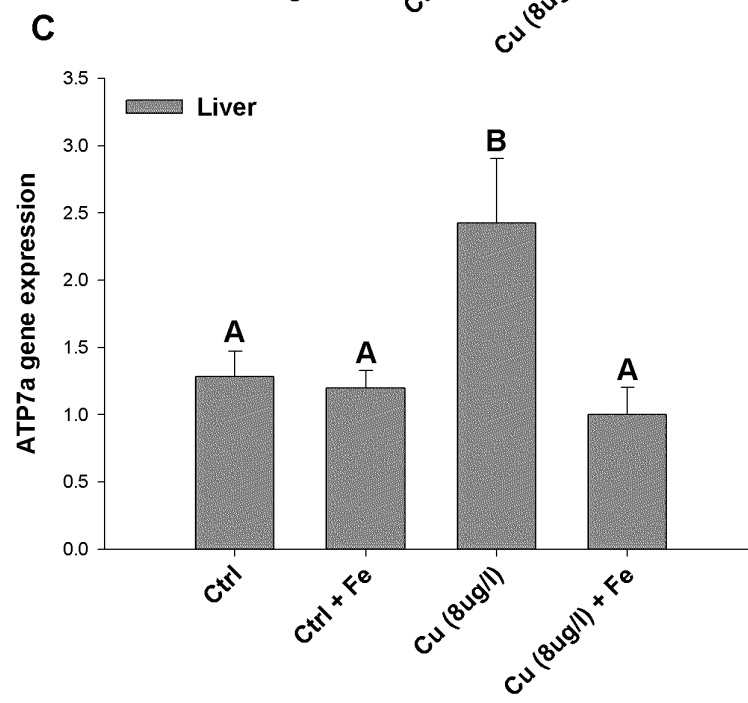
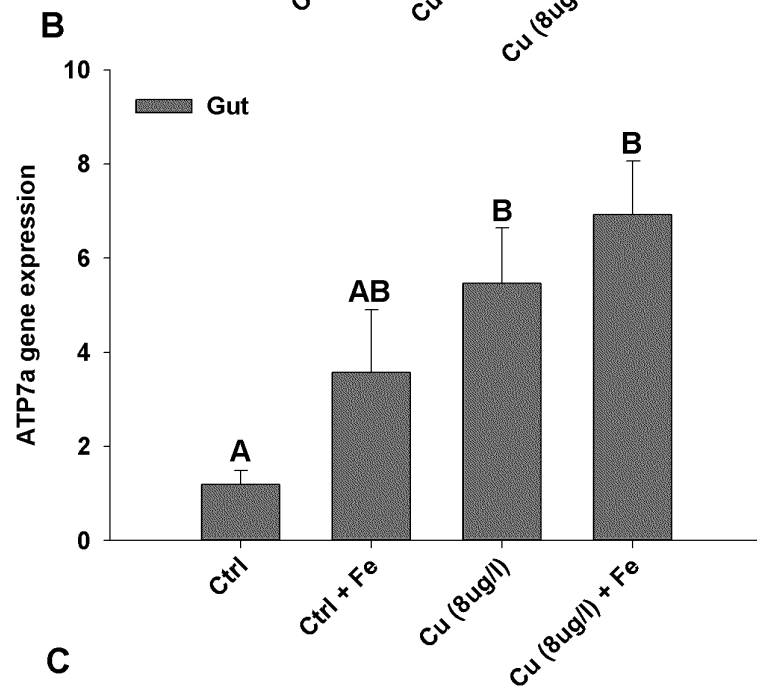
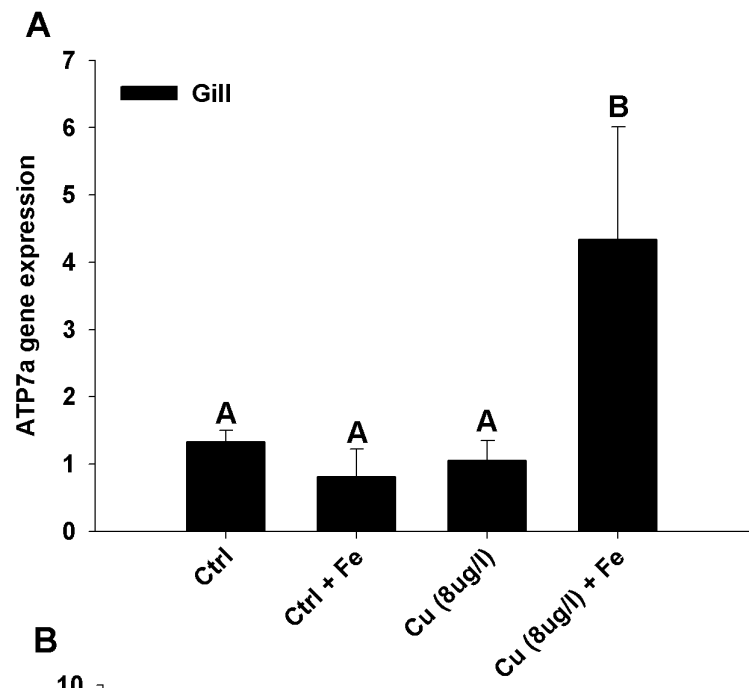


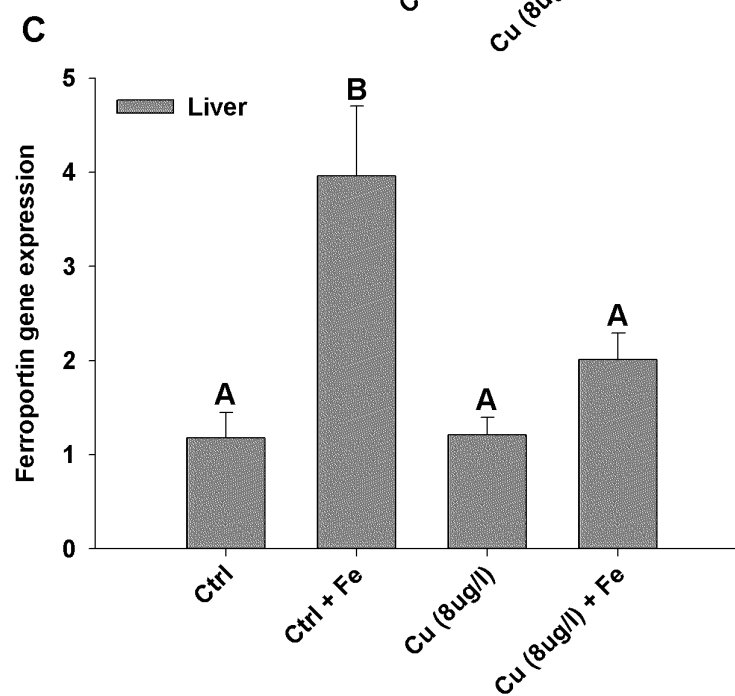
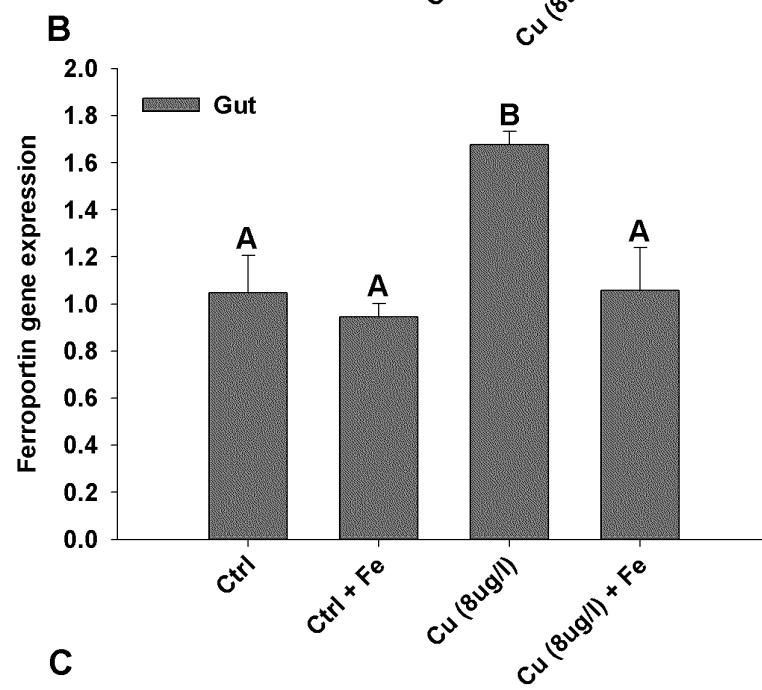
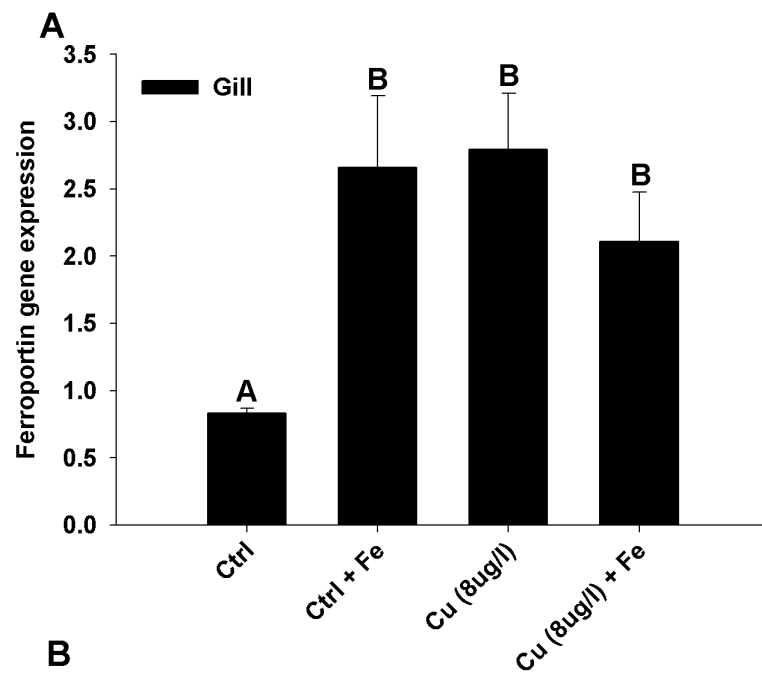


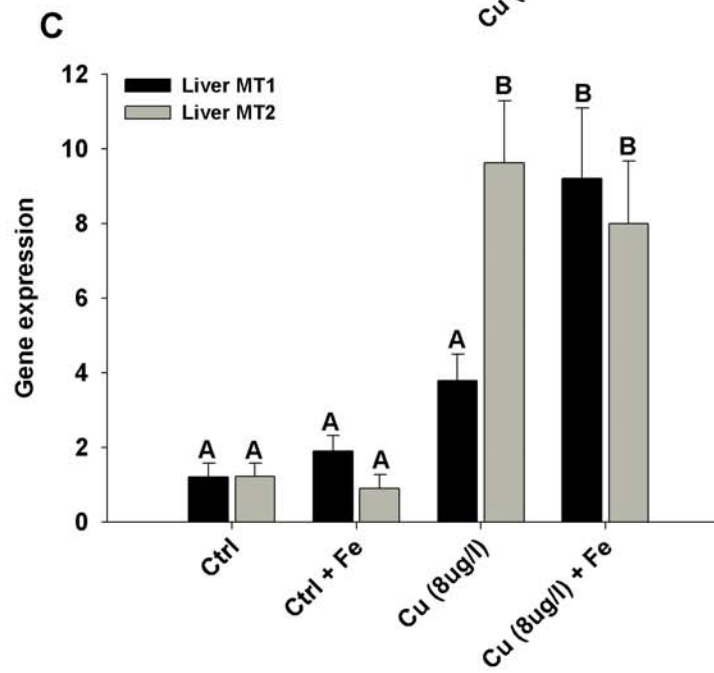
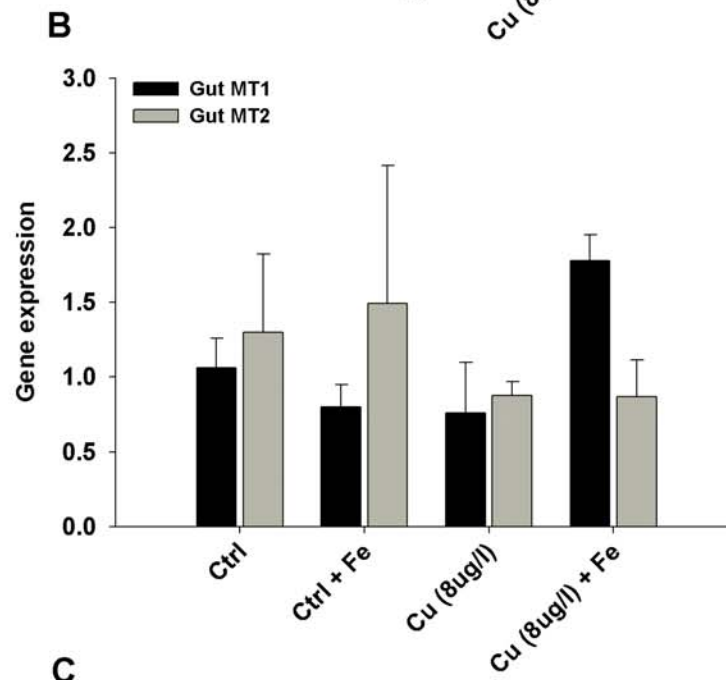
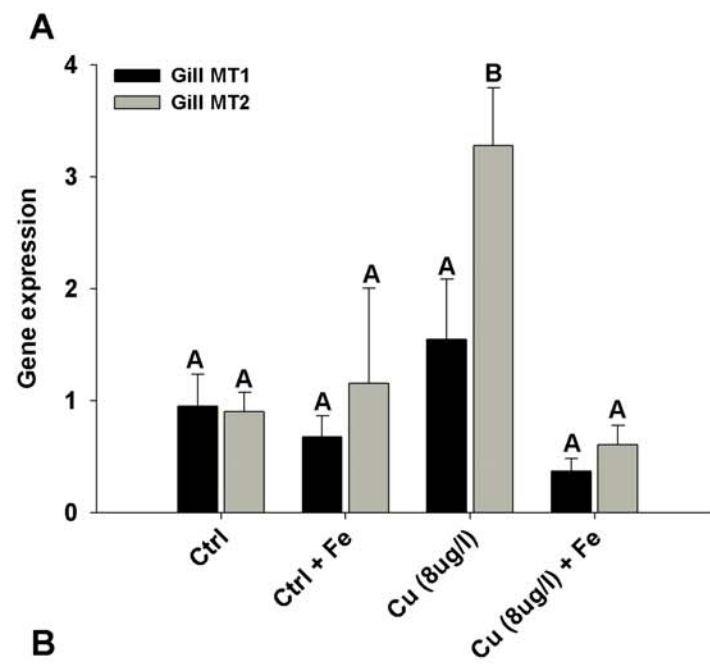


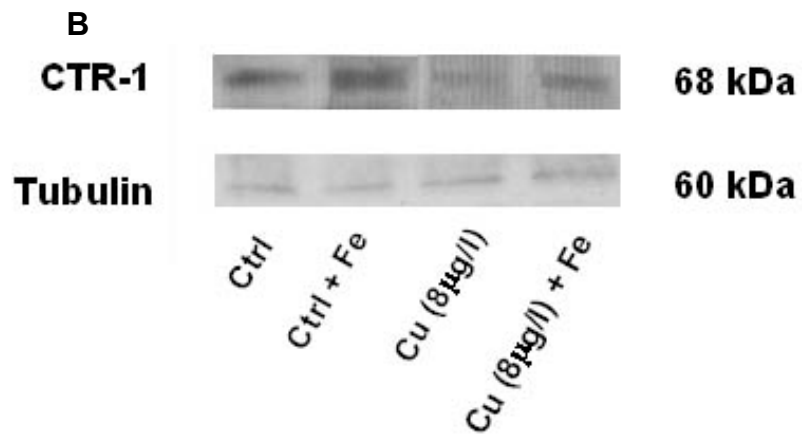
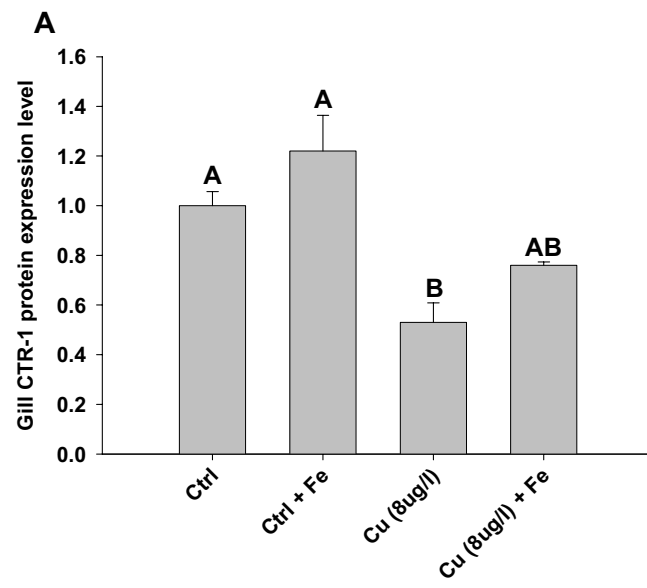




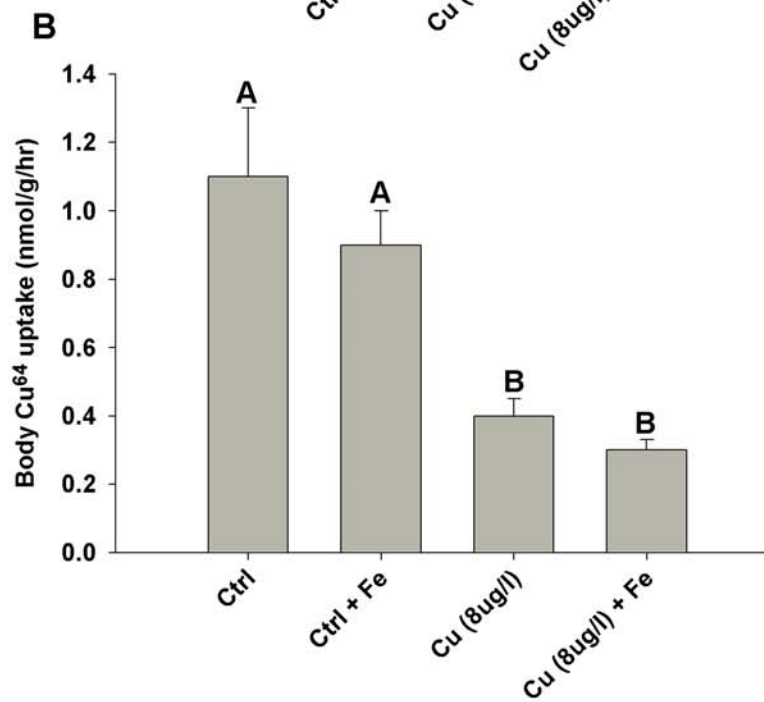
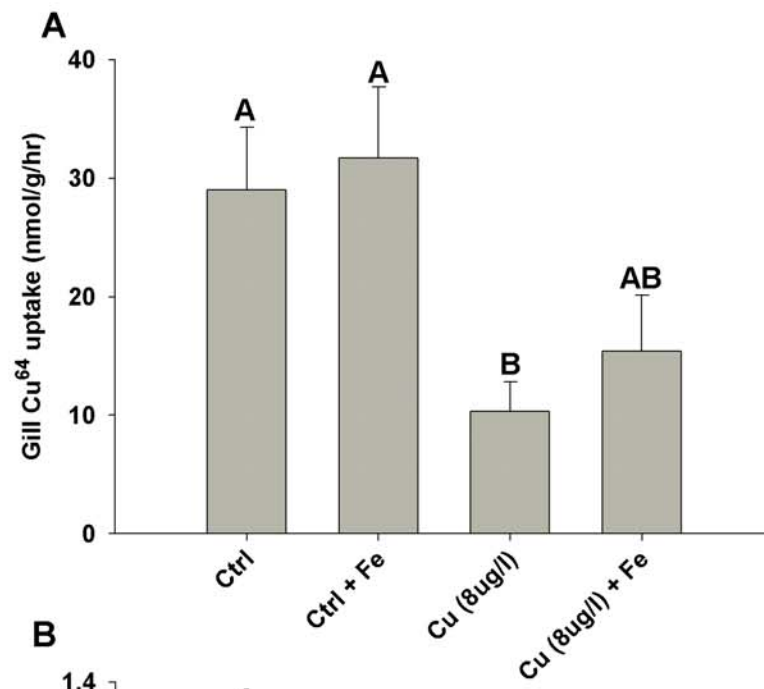












Gene	Primer	Acession #	Amplicon size (BP)
ATP7a	F: 5'-GGCTCGACTTCTCGCAGCT-3' R: 5'-ATTCCGCATTTTCACTGCCT-3'	NM_001042720	50
CTR-1	F: 5'-GAATCAGGTGAACGTGCGCT-3' R: 5'-CCATCAGATCCTGGTACGGG-3'	AY077715	51
DMT-1	F: 5'-CAATCGACACTACCCACGG-3' R: 5'-TCCACCATAAGCCACAGGATG-3'	AF529267	51
Ferroportin	F: 5'-ACATTCTGGCACCGATGCTT-3' R: 5'-AGTGTGAGCCAAATGCCATG-3'	NM_131629	51
MT-1	F: 5'-CGTCTAACAAAGGCTAAAGAGGGA-3' R: 5'-GCAGCAGTACAAATCAGTGCATC-3'	AY514790	51
MT-2	F: 5'-GGAGGAGGGTCAGAGGAACC-3' R: 5'-AGCAACTGAAGCTCCATCCG-3'	NM_194273	51
EF1- $\alpha$	F: 5'-GTGCTGTGCTGATTGTTGCT-3' R: 5'-TGTATGCGCTGACTTCCTTG-3'	NM131263	201

Ion	Ctrl	Ctrl + Fe diet	Cu (8μg/L)	Cu (8μg/L) + Fe diet
Na <sup>+</sup>	65.3 ± 3.6	53.4 ± 3.8	49.0 ± 3.0	51.6 ± 3.2
Mg <sup>2+</sup>	20.5 ± 3.6	12.5 ± 0.9	12.4 ± 1.4	12.8 ± 1.9
Fe <sup>2+</sup>	2.5 ± 0.7	2.2 ± 0.6	2.1 ± 0.8	2.2 ± 0.7
Ca <sup>2+</sup>	49.2 ± 3.8	42.2 ± 2.9	38.0 ± 2.8	42.2 ± 2.8
Cu <sup>2+</sup>	1.8 ± 0.2	1.2 ± .0.2	8.0 ± 0.4*	8.7 ± 0.3*

<b>Treatment</b>	<b>Before (g)</b>	<b>After (g)</b>	<b>% Change</b>
Ctrl	0.35 ± 0.01	0.50 ± 0.05	28.5
Ctrl + Fe Diet	0.43 ± 0.04	0.55 ± 0.13	21.6
Cu (8μg/L)	0.45 ± 0.11	0.38 ± 0.02	-17.7
Cu (8μg/L) + Fe diet	0.50 ± 0.06	0.45 ± 0.03	-10.1