Effect of chloride concentration on the cytotoxicity, bioavailability, and bioreactivity of copper and silver in the rainbow trout gut cell line, RTgutGC

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Abstract

Chloride (Cl^-) influences the bioavailability and toxicity of metals in fish, but the mechanisms by which it influences these processes is poorly understood. Here, we investigated the effect of chloride on the cytotoxicity, bioavailability (i.e., accumulation) and bioreactivity (i.e., induction of mRNA levels of metal responsive genes) of copper (Cu) and silver (Ag) in the rainbow trout gut cell line (RTgutGC). Cells were exposed to metals in media with varying Cl^- concentrations (0, 1, 5 and 146 mM). Metal speciation in exposure medium was analyzed using Visual MINTEQ software. Cytotoxicity of $AgNO_3$ and $CuSO_4$ was measured based on two endpoints: metabolic activity and membrane integrity. Cells were exposed to 500 nM of $AgNO_3$ and $CuSO_4$ for 24 h in respective media to determine metal bioavailability and bioreactivity. Ag speciation changes from free ionic (Ag^+) to neutral (AgCl), to negatively charged chloride complexes ($AgCl_2^-$, $AgCl_3^-$) with increasing Cl^- concentration in exposure media whereas Cu speciation remains in two forms (Cu^{2+} and $CuHPO_4$) across all media. Chloride does not affect Ag bioavailability but decreases metal toxicity and bioreactivity. Cells exposed to Ag expressed significantly higher metallothionein mRNA levels in low Cl^- media (0, 1, and 5 mM) than in high Cl^- medium (146 mM). This suggests that chloride complexation reduces silver bioreactivity and toxicity. Conversely, Cu bioavailability and toxicity were higher in the high chloride medium (146 mM) than in the low Cl^- (0, 1, and 5 mM) media, supporting the hypothesis that Cu uptake may occur via a chloride dependent mechanism.

Clinical trials registration

This study did not require clinical trial registration.

Keywords Chloride · Metal speciation · Metal bioavailability · Metal toxicity · RTgutGC cells · In-vitro.

Introduction

Metals are present in the aquatic environment in different ionic phases such as free ions (Ag^+, Cu^{2+} etc.), inorganic (e.g. Cl^-, OH^-, and CO_3^{2-}) or organic (e.g., organic molecules and organic matters) complexes, and solid or colloidal phases (Zhao et al. 2016). Several studies in freshwater organisms have shown that free ionic species of metals are much more bioavailable than metal complexes (Di Toro et al. 2001; Paquin et al. 2002). However, it was shown that metal complexes may also become bioavailable and elicit toxicity in aquatic biota (Erickson et al. 1998; Fortin and Campbell 2000; Zhao et al. 2016). For instance, in fish, metals can become more bioavailable and toxic at higher salinity (e.g. brackish and marine) (Ferguson and Hogstrand 1998; Grosell et al. 2007; Matson et al. 2016) where fish drink for osmoregulatory purposes (Scott et al. 2006). This highlights the role of the intestine as an important route for the uptake of metal complexes. However, the bioavailability of metal complexes at the fish intestine is poorly understood and requires further studies. To study the bioavailability of dissolved metal complexes, we need to know which metal species are formed in the exposure medium. Considering the tendency of metal

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species to reach an equilibrium very rapidly, metal speciation in the water or exposure medium can be done by various equilibrium models such as MINEQL + (Schecher and McAvoy 1992) or Visual MINTEQ (Gustafsson 2013).

To further understand the toxicity and bioavailability of metal complexes in the fish intestine, we previously investigated how silver (Ag), copper (Cu), cadmium (Cd), and zinc (Zn) speciate in a synthetic medium that mimics the intestinal luminal buffer, and how the different metal species are related to intracellular effects (i.e., toxicity, bioavailability, and bioreactivity) in the rainbow trout (*Oncorhynchus mykiss*) gut cell line (RTgutGC) (Ibrahim et al. 2020). This in-vitro system was found to be an effective toxicological model, especially to study effects of extracellular metal speciation on intracellular effects (Ibrahim et al. 2020; Langan et al. 2017; Minghetti and Schirmer 2016). RTgutGC cells were isolated from the intestine of a healthy female rainbow trout fish (*Oncorhynchus mykiss*) (Kawano et al. 2011) and were shown to express tight junction proteins between adjacent cells and the Na$^+$/K$^+$-ATPase at the basolateral side of the cell (Minghetti et al. (2017)).

In this study, we chose an essential metal, Cu, and a non-essential metal, Ag to investigate the role of chloride concentration on their speciation in the exposure medium and the, toxicity, bioavailability, and bioreactivity of different metal species in RTgutGC cells. Chloride is an environmentally and physiologically relevant inorganic ligand that affects metal speciation in aqueous solutions. Chloride is the major anion in the aquatic environment and is also involved in key physiological process such as acid-base balance, ion osmoregulation, and cell volume regulation (Jentsch et al. 2002). Moreover, it was proposed that chloride may be required for copper uptake through a chloride dependent symporter mechanism at the fish intestine (Handy et al. 2000).

The same redox properties that make copper an important cofactor for several enzymes also make it extremely toxic when present unbound intracellularly. Thus, all organisms, including fish, have evolved mechanisms to maintain copper homeostasis using specific transporters and the metal chelators to scavenge its toxicity (Minghetti et al. 2010, 2008). In the intestine, Cu, can be transported into the cell by specific protein transporters such as copper transporter 1 (CTR 1), copper transporter 2 (CTR 2), and divalent metal transporter 1 (DMT 1) (Arredondo et al. 2003; Minghetti et al. 2008; Ohrvik and Thiele 2015). Bertinato et al. (2010) demonstrated the inhibition of Cu uptake by Ag and the role of human CTR1 in transporting both of these metal ions. Moreover, studies have suggested that Ag can be transported via CTR1 in fish as well as humans (Bury et al. 2003; Grosell and Wood 2002). For storage and detoxification of metals, two molecules, metallothionein

and glutathione, play a significant role. Metallothioneines (MTs) are a superfamily of proteins enriched in cysteine which has a very high binding affinity for metals. Specifically, metals bind with the thiol group of the cysteine to form a metal cluster in the MT (Coyle et al. 2002). Glutathione (GSH), a major non-protein reservoir composed of tripeptides, works as a chelating agent for different metal cations (Połeć-Pawlak et al. (2007)). The glutathione reductase (GR) has an important role in maintaining a reservoir of reduced GSH and in defense against oxidative stress (Srikanth et al. 2013). Another protein, ATP7A (a P-type ATPase) plays a pivotal role in maintaining Cu homeostasis by transporting copper from the intestine to the blood or plasma (Minghetti et al. 2010). To evaluate the bioreactivity of copper and silver, we quantified the mRNA levels of metallothionein b (MTb), copper transporter 1 (CTR1), divalent metal transporter 1 (DMT1), ATP7A, and glutathione reductase (GR).

The overall goal of our study was to understand the role of chloride concentration in the exposure medium on the speciation of copper and silver and determine the toxicity, bioavailability, and bioreactivity of different metal species in the rainbow trout gut cell line (RTgutGC). We hypothesized that along with free ionic metal species, metal-chloride complexes may also be bioavailable and toxic.

Materials and Methods

Cell culture

The rainbow trout gut cell line (RTgutGC) was routinely cultured using Leibovitz's L-15 (L-15) medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% of fetal bovine serum (FBS, Sigma-Aldrich, MO, USA) and 1% of *Penicillin streptomycin* (10000 U/ml penicillin, 10 mg/ml streptomycin, Sigma-Aldrich, St. Louis, MO, USA). Cell were grown in 75 cm^2 flasks (Greiner Bio One, Monroe, NC, USA) at 19 °C in a normal atmosphere incubator (B.O.D. Low Temperature Refrigerated Incubator, VWR, Radnor, PA, USA). Once cells reached approximately 90% confluency, they were sub-cultured or split into 3 flasks. To passage a confluent flask, culture medium (L-15/FBS) was removed and cells were washed twice with 1 mL of versene (Thermo Fisher Scientific, Waltham, MA, USA). Cells were then detached from the flask using 1 mL of 0.25% Trypsin (Thermo Fisher Scientific, Waltham, MA, USA) and counted using the Countess II^{TM} Automated Cell Counter (Life Technologies Corporation, NYC, NY, USA) which simultaneously counts and determines cell viability using the trypan blue exclusion assay. Only cell suspensions with more than 90% viability were seeded on multi-well plates (24 well plate for

cytotoxicity assays and 6 well plate for bioavailability and bioreactivity exposures) (Greiner Bio-One, Monroe, NC, USA) at 80,000 cells/cm^2 in L-15/FBS. Multi-well plates were not treated with any artificial chemicals for the attachment of cells. Cells were incubated for 48 h to allow the formation of the confluent monolayers. After 48 h, cells were exposed to AgNO$_3$ and CuSO$_4$ dissolved in different exposure media.

Preparation of exposure media along a chloride gradient

Synthetic exposure media with varying chloride concentration were prepared modifying the composition of L-15 (0–146 mM of chloride, L-15/ex$^{0 - 146\ Cl-}$). Sugar and salt concentrations of L-15/ex$^{0 - 146\ Cl-}$ media are identical to that of the commercial L-15 medium but they do not contain amino acids and vitamins (Schirmer et al. 1997) (Table S1). To maintain a similar ionic strength across all media, the chloride (Cl$^-$) concentration was adjusted using NO$_3^-$ (see Table S1 for composition). The osmolality and the pH of each medium were measured using the Vapro® Vapor Pressure Osmometer (Model 5600, ELItech group, South Logan, UT, USA) and SI Analytics pH meter (SI Analytics, College Station, TX, USA), respectively. The 10 mM stock solutions of AgNO$_3$ and CuSO$_4$.5H$_2$O (Sigma-Aldrich, St. Louis, MO, USA) were prepared in ultrapure water (16-18 mΩ, Barnstead GenPure Water, Thermo Fisher Scientific, Waltham, MA, USA). AgNO$_3$ concentrations ranging from 0.01 to 100 μM and CuSO$_4$ from 0.1 to 200 μM were used as exposure concentrations (Ibrahim et al. 2020; Minghetti and Schirmer 2016). Exposure solutions of AgNO$_3$ and CuSO$_4$ were prepared on the day of exposure by dissolving them in the respective media (L-15/ex$^{0\ Cl-}$, L-15/ex$^{1\ Cl-}$, L-15/ex$^{5\ Cl-}$, and L-15/ex$^{146\ Cl-}$). Exposure solutions were vortexed before exposing to cells. Stock concentrations and one exposure solution from each medium were measured by ICP-OES (iCAP 7400, Thermo Fisher Scientific, Waltham, MA, USA). The recovery rate of stock concentrations of both AgNO$_3$ and CuSO$_4$ were nearly 100% whereas it was between 85 and 90% for Ag exposure concentrations and nearly 95% for Cu solutions (see Table S5) supporting the accuracy of the test system. Therefore, all concentrations reported in this paper are nominal.

Ag and Cu speciation in each exposure medium

Metal speciation, including formation of solids, and ionic strengths of Ag and Cu in each exposure medium were determined by Visual MINTEQ (Gustafsson 2013), a chemical equilibrium software. Ag and Cu speciation were calculated for concentrations ranging from 0.1 to 100 μM in all L-15/ex exposure media.

Cell viability assays

Cell viability assays were performed in each exposure medium 3-6 times using cells of different passages each time. The cell viability assay is based on two endpoints: cell metabolic activity and cell membrane integrity. The metabolic activity was measured by alamarBlue® (AB; Invitrogen, Eugene, OR, USA), and the cell membrane integrity by CFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester) (Invitrogen, Eugene, OR, USA) (Schirmer et al. 1997). For both dyes, a decline in fluorescence means a decrease in cell viability.

Prior to exposure, each well of the plate was washed twice with phosphate buffer saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA). Cells were then exposed to 1 mL of exposure solutions (i.e., AgNO$_3$ and CuSO$_4$ dissolved in respective media) or to the respective control media and incubated for 24 h at 19 °C. After 24 h of exposure, exposure solutions were aspirated and washed twice with PBS. After washing, cells were incubated with AB (5% v/v) and CFDA-AM (4 μM) dye solutions in PBS for 30 min. The Cytation 5 Plate Reader (BioTek, Winooski, VT, USA) was used to measure fluorescence at excitation/emission wavelengths of 530/595 nm for AB and 485/530 nm for CFDA-AM.

Quantification of metal bioavailability

To investigate the effects of chloride concentration on Ag and Cu bioavailability cells were seeded in quadruplicate (control and exposure) in 6 well plates at 80,000 cells/cm^2 (768,000 cells/well in 2 mL of L-15/FBS) for 48 h and incubated at 19 °C. In this study, the term bioavailability means intracellular accumulation. After 48 h of incubation, confluent cell monolayers were washed twice with PBS. A single nominal concentration, 500 nM of AgNO$_3$ and CuSO$_4$ was selected for exposure. This concentration was selected keeping in consideration the detection and toxicity of intracellular metal. Exposure media of both metals were made by spiking the appropriate volume in the respective media from 10 mM stock. After washing, cells were exposed to 2 mL of exposure solutions for 24 h. After 24 h of exposure, each well was washed once with 1 mL of PBS and twice with a solution of 0.5 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA) dissolved in PBS to ensure thorough removal of loosely bound metal. Next cells were lysed by applying 1 mL of 50 mM NaOH (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at room temperature on an orbital shaker. Cell lysates were then transferred to 1.5 mL Eppendorf tubes and 100 μL from each well was transferred into another tube for the quantification of total proteins using the Lowry Assay (Thermo Fisher Scientific, Waltham, MA, USA). For metal quantification, cell lysates were

desiccated using a concentrator (concentrator plus, Eppendorf, Hauppauge, USA) and digested overnight by adding 0.8 mL of 69% HNO_3 (Sigma-Aldrich, St. Louis, MO, USA) and 0.2 mL H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA). Finally, samples were diluted 10 times with ultra-pure water to dilute the nitric acid concentration to 5%. Diluted samples were kept at 4 °C until measurement. Samples were measured using inductively coupled plasma mass spectrometry (ICP-MS; iCAP Qc, Thermo Fisher Scientific, Dreieich, Germnay). Standard reference material (NIST SRM 1643f; National Institute of Standards and Technology, MD, USA) was analyzed with each set of samples for quality control. Ag and Cu bioavailability data were normalized by total protein concentrations in each well and presented as pmol metal/mg of protein (Ibrahim et al. 2020).

Determination of mRNA levels of metal responsive genes by quantitative PCR

In this study, the term bioreactivity indicates the induction of a metal responsive gene expression (Ibrahim et al. 2020). To quantify mRNA levels of metal responsive genes (MTb, GR, CTR1, ATP7A, and DMT1) in RTgutGC cells, cells were seeded and exposed in an identical manner as for metal bioavailability. After 24 h of exposure, the exposure solution was aspirated and 900 μL of TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was applied to the cell monolayer. Cells were lysed by pipetting the TRIzol up and down several times. Total RNA was extracted following the manufacturer instructions and an average of 1.93 μg of RNA was collected from each well. To remove any trace of DNA from samples, total RNA was treated with the TURBO DNase kit (Thermo Fisher Scientific, Waltham, MA, USA). Quality and quantity of the isolated RNA were determined spectrophotometrically using the Cytation 5 Plate Reader (Bio-Tek, Winooski, VT, USA) and by electrophoresis using 1 μg of total RNA in a 1% agarose gel.

Complementary DNA synthesis was performed from 0.5 μg of total RNA using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) by following the manufacturer instructions. Quantitative PCR was performed in triplicate using the SYBR Premix Ex Taq II (Clontech, Mountain View, CA, USA) and the CFX Connect Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Messenger RNA levels were measured using the absolute quantification method and are reported as fold change of the treated groups from the controls. Normalization of copy number of the target genes MTb, GR, CTR1, ATP7A, and DMT1 was conducted based on the geometric mean of reference genes (Elongation factor 1a and Ubiquitin) (Minghetti and Schirmer 2016). The PCR efficiency of all genes was above 93%. Primer sequences are reported in Table S6.

Data analysis

All statistical analyses were done using GraphPad Prism Version 7.0 (GraphPad Software Inc., San Diego, CA). The cell viability data are shown as percentages by dividing the fluorescence units of each exposed sample by the fluorescence unit of respective controls. The effective concentrations leading to 50% reduction in viability (EC50) were determined by the nonlinear regression sigmoidal dose–response curve fitting module using the Hill slope equation. The effective concentration 30 and 50 values (EC30 and EC50) are presented as mean and standard deviation, SD ($N = 3$–6). All data were assessed for normality with the D'Agostino & Pearson normality test and where necessary, data were transformed to improve normality. Analysis of variance (ANOVA) followed by Tukey's post hoc test were performed to determine the statistical significance among different experimental groups.

Results

Metal speciation in exposure media

Varying the concentration of $AgNO_3$ and $CuSO_4$ from 0.01 μM to 100 μM contributed a negligible change in Ag and Cu speciation determined by Visual MINTEQ. Data presented in Fig. 1 were calculated using 1 μM of $AgNO_3$ and $CuSO_4$. Silver species distribution varied significantly among the 4 media. Ag speciation changes from free ionic form to complexed forms as the chloride concentration increases. Free ionic Ag^+ (~93%) is the dominant species in the chloride free medium (L-15/ex$^{0\ Cl-}$) whereas $AgCl_2^-$ (~77%) and $AgCl_3^{-2}$ (~17%) are the two dominant species in the highest chloride-containing medium (L-15/ex$^{146\ Cl-}$). Ag^+ (~44%) and $AgCl_{(aq)}$ (~48%) are the two major species formed in L-15/ex$^{1\ Cl-}$ whereas in L-15/ex$^{5\ Cl-}$, the dominant species is $AgCl_{(aq)}$ (~61%) followed by $AgCl_2^-$ (~26%) (Fig. 1). Copper speciation remained stable across the chloride concentration gradients, mainly complexing to hydrogen phosphate, $CuHPO_4$ (~42%) or remained in ionic form, Cu^{2+} (~43%) (Fig. 1). The formation of metal precipitates in each medium is reported in Figs. 1, 2 and Table S2–4. Precipitation analyses showed that copper precipitates as $Cu_3(PO4)_2$ and that 1% of total Cu started forming in L-15/ex$^{0\ Cl-}$, L-15/ex$^{1\ Cl-}$ and L-15/ex$^{5\ Cl}$ at 3.1 μM of $CuSO_4$ and fifty % percent occurred in these media at 7.4 μM of $CuSO_4$. In L-15/ex$^{146\ Cl-}$ medium, 1% and 50% precipitation of total Cu occurred at 3 μM and 7.1 μM of $CuSO_4$, respectively (Fig. 1 and Table S2–4).
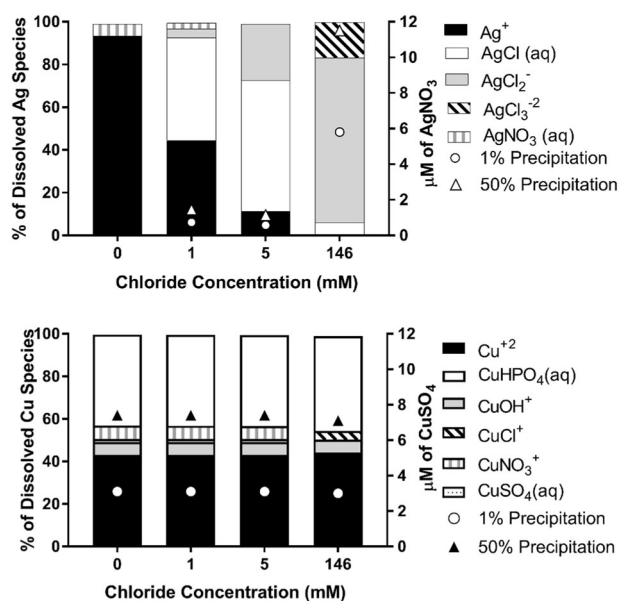
Fig. 1 Ag and Cu species distribution in different chloride (Cl−) containing media (L-15/ex$^{0 Cl−}$ L-15/ex$^{1 Cl−}$, L-15/ex$^{5 Cl−}$, and L-15/ex$^{146 Cl−}$) calculated using VISUAL MINTEQ 3.1. Species contributing less than 1% were excluded on the graphs. Exposure media are represented on the X-axis by chloride concentration in the media. Left Y-axis denotes total percentage of metal (Ag or Cu) in the media while right Y-axis represents the concentrations at which metal precipitates. Precipitations were calculated (silver as cerargyrite (AgCl) and copper as Cu$_3$(PO4)$_2$.3H$_2$O) using the software Visual MINTEQ 3.1. A complete table of metal precipitation is given in Table S 2–4

Silver precipitated as cerargyrite (AgCl). There was no precipitation of Ag (up to 500 μM) in the chloride free medium L-15/ex$^{0 Cl−}$. Precipitation started (1% of total) in L-15/ex$^{1 Cl−}$, L-15/ex$^{5 Cl−}$, and L-15/ex$^{146 Cl−}$ at 0.73 μM, 0.57 μM, and 5.8 μM of AgNO$_3$ respectively. Fifty % of precipitation occurred in these three media (same order) at 1.45 μM, 1.14 μM, and 11.5 μM of AgNO$_3$, respectively (Fig. 1 and Table S2–4).

Cell viability after 24 h exposure to metals in media of different chloride concentration

When metabolic activity was used as an indicator of cell viability, EC50 calculations showed significantly lower toxicity (EC50: 1.41 μM) of Ag in the highest chloride-containing medium (L-15/ex$^{146 Cl−}$) in comparison to the other 3 media (L-15/ex$^{0 Cl−}$, L-15/ex$^{1 Cl−}$, and L-15/ex$^{5 Cl}$−) (Fig. 2 and Table 1). The same trend was found when membrane integrity was used as the indicator of cell viability. There was no variation in the toxicity among L-15/ex$^{0 Cl−}$, L-15/ex$^{1 Cl−}$, and L-15/ex$^{5 Cl−}$ for both cell metabolic activity and cell membrane integrity (Fig. 2 and Table 1).

Conversely, copper toxicity increased with the increase of chloride concentration in the media for both cell

metabolic activity and cell membrane integrity. In terms of metabolic activity, cells exposed to Cu in L-15/ex$^{0 Cl−}$ (EC50: 6.93 μM) had significantly higher viability when compared with cells in L-15/ex$^{1 Cl−}$ (EC50: 4.73 μM), L-15/ex$^{5 Cl−}$ (EC50: 4.67 μM) and L-15/ex$^{146 Cl−}$ (EC50: 3.01 μM) (Fig. 2 and Table 1). A similar trend was found when cell membrane integrity was used as an indicator of cell viability having highest EC50 value 11.26 μM in L-15/ex$^{0 Cl−}$ and lowest 6.15 μM in L-15/ex$^{146 Cl−}$ (Fig. 2 and Table 1).

To evaluate if EC30 values are more statistically sensitive than EC50 values, we calculated and compared EC30 values found among 4 different exposure media. Overall, EC50 values were more statistically sensitive among different groups than EC30 values. However, one difference that can be noted from EC30 comparison is the significantly higher Ag toxicity (cell membrane integrity) found in L-15/ex$^{0 Cl−}$ (EC30: 0.93 μM) and L-15/ex$^{1 Cl−}$ (EC30: 1 μM) in comparison to the L-15/ex$^{146 Cl−}$ (EC50: 1.67 μM) (Table 1).

Bioavailability of Ag and Cu in RTgutGC cells exposed for 24 h in media of different chloride concentrations

Copper and silver bioavailability in RTgutGC cells exposed in medium of different chloride concentrations is shown in Table 2. The average silver concentrations, in pmol/mg of proteins, were 285.1, 249.1, and 202.6 respectively for L-15/ex$^{0 Cl−}$, L-15/ex$^{1 Cl−}$, and L-15/ex$^{5 Cl−}$. Ag bioavailability was not significantly different among different exposure media, although there is a decreasing trend of Ag bioavailability as the chloride concentration increases from 0 mM Cl− to 5 mM Cl− in exposure media. However, in the medium with the highest chloride concentration, L-15/ex$^{146 Cl−}$, bioavailability of Ag (291.1 pmol/mg of proteins) went back to the level of Ag bioavailability in L-15/ex$^{0 Cl}$.

The highest bioavailability of Cu (309.4 pmol/mg of proteins) was found in the medium containing the highest amount of chloride, L-15/ex$^{146 Cl−}$, and it was significantly higher than the chloride-free medium, L-15/ex$^{0 Cl−}$. In L-15/ex$^{1 Cl−}$, and L-15/ex$^{5 Cl−}$, bioavailability of Cu was not significantly different from L-15/ex$^{146 Cl−}$ and L-15/ex$^{0 Cl−}$. As Cu is an essential element, thus it was found in cells exposed to control medium (Table 2) and excluding the exposure in L-15/ex$^{1 Cl−}$, it accumulated significantly in the copper exposed cells.

Metal bioreactivity in RTgutGC cells exposed to 500 nM of AgNO$_3$ or CuSO$_4$ for 24 h

The mRNA levels of 5 metal responsive genes, MTb, GR, CTR1, ATP7A, and DMT1, are shown in Fig. 3.
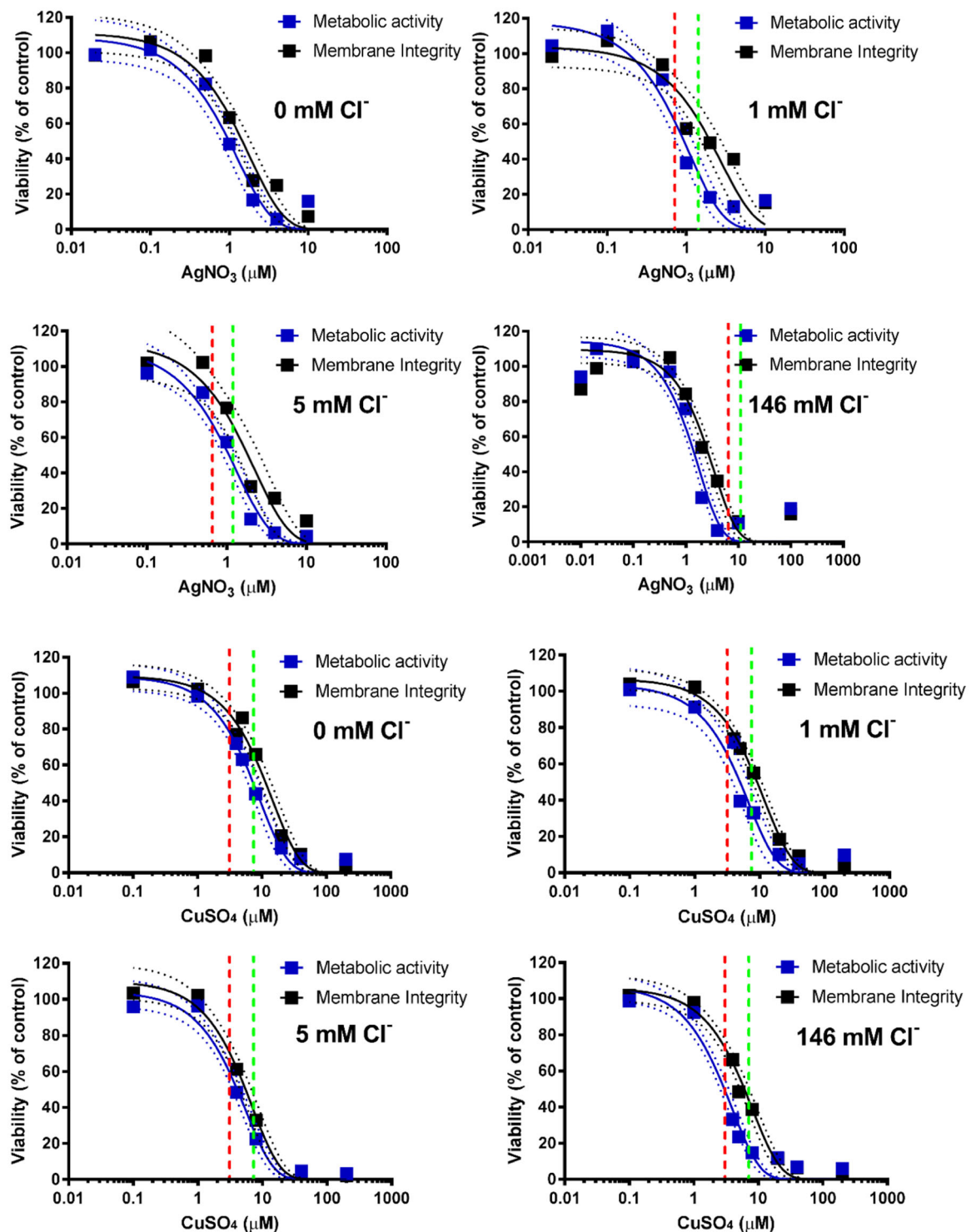
Fig. 2 Toxicity of $AgNO_3$ (top) and $CuSO_4$ (bottom) in RTgutGC cells exposed in 4 exposure media (L-15/ex[0 Cl−], L-15/ex[1 Cl−], L-15/ex[5 Cl−], and L-15/ex[146 Cl−]). Cell viability assay endpoints: metabolic activity (alamar blue) and membrane integrity (CFDA-AM) were collected after 24 h of exposure. Values shown are averages and dashed lines indicate confidence intervals (95%) of at least 3 experiments. EC50 concentrations were determined using non-linear regression sigmoidal dose-response curve following the Hill slope equation. Differences among curve fits within the same graph were tested using the F-test. All graphs and statistical analyses were performed using GraphPad Prism Software Ver. 7.0 (CA, USA). The red and green vertical dashed lines indicate the concentration at which 1% and 50% of the metal will be precipitated, respectively

Table 1 EC50 and EC30 concentrations (µM) of Ag and Cu using alamarBlue and CFDA-AM dyes as indicators of cellular metabolic activity and cell membrane integrity respectively. EC concentrations were determined using non-linear regression sigmoidal dose-response curve following the Hill slope equation on GraphPad Prism software (CA, USA). Data are presented as mean ± SD, $n = 3$-6. Superscript letters on numerical values indicate statistical significance among different groups within the same endpoint (e.g. metabolic activity) (One-way ANOVA, Tukey test, $P < 0.05$)

	Ag EC50 in µM		Cu EC50 in µM	
Exposure media	Metabolic activity	Cell membrane integrity	Metabolic activity	Cell membrane integrity
L-15/ex$^{0\ Cl-}$	1.05 ± 0.22^A	1.37 ± 0.05^A	6.93 ± 0.71^A	11.26 ± 1.14^A
L-15/ex$^{1\ Cl-}$	0.89 ± 0.12^A	2.03 ± 0.76^A	4.73 ± 0.98^B	$8.56 \pm 1.57^{A,B}$
L-15/ex$^{5\ Cl-}$	1.09 ± 0.07^A	2.05 ± 0.78^A	$4.67 \pm 0.25^{B,C}$	6.59 ± 1.41^B
L-15/ex$^{146\ Cl-}$	1.41 ± 0.16^B	2.64 ± 0.83^A	3.01 ± 0.46^D	6.15 ± 2.18^B
	Ag EC30 in µM		Cu EC30 in µM	
Exposure media	Metabolic activity	Cell membrane integrity	Metabolic activity	Cell membrane integrity
L-15/ex$^{0\ Cl-}$	0.63 ± 0.11^A	0.93 ± 0.09^A	4.07 ± 0.49^A	7.22 ± 1.31^A
L-15/ex$^{1\ Cl-}$	0.63 ± 0.16^A	1.00 ± 0.18^A	$2.83 \pm 0.66^{B,C}$	$5.04 \pm 0.56^{A,B}$
L-15/ex$^{5\ Cl-}$	$0.77 \pm 0.07^{A,B}$	$1.20 \pm 0.18^{A,B}$	2.97 ± 0.25^B	4.36 ± 0.96^B
L-15/ex$^{146\ Cl-}$	1.07 ± 0.16^B	1.67 ± 0.39^B	1.95 ± 0.45^C	3.70 ± 1.11^B

Table 2 Quantification of Ag and Cu by ICP-MS in RTgutGC cells exposed to a fixed concentration (500 nM of each metal). Metal units are presented as pmol of metal/mg of proteins. Data are presented as mean ± SD, $n = 3$ or 4. Superscript letters on numerical values indicate statistical significance among different groups within the same treatment (e.g. control, 500 nM of AgNO$_3$) (One-way ANOVA, Tukey test, $P < 0.05$). Asterisks indicate significant increase of Cu concentrations in exposed groups from respective control groups (t-test, $P < 0.05$)

	Ag (pmol/mg of proteins)		Cu (pmol/mg of proteins)	
Exposure media	Control	500 nM AgNO$_3$	Control	500 nM CuSO$_4$
L-15/ex$^{0\ Cl-}$	BDL	285.1 ± 44.1^A	194.8 ± 31.84^a	$245.1 \pm 18.09*^A$
L-15/ex$^{1\ Cl-}$	BDL	249.1 ± 31.09^A	197.1 ± 38.71^a	$254.2 \pm 33^{A,B}$
L-15/ex$^{5\ Cl-}$	BDL	202.6 ± 36.79^A	150.8 ± 23.66^a	$255.6 \pm 8.21*^{A,B}$
L-15/ex$^{146\ Cl-}$	BDL	291.1 ± 75.6^A	154.5 ± 30.3^a	$309.4 \pm 46.68*^B$

The mRNA levels of MTb were significantly higher than control in RTgutGC cells exposed to 500 nM AgNO$_3$ dissolved in L-15/ex$^{0\ Cl-}$, L-15/ex$^{1\ Cl-}$, and L-15/ex$^{5\ Cl-}$ but not in L-15/ex$^{146\ Cl-}$. The mRNA levels of other genes (GR, CTR 1, ATP 7A, and DMT 1) were not significantly different than controls in RTgutGC cells exposed to AgNO$_3$. In cells exposed to 500 nM of CuSO$_4$ in L-15/ex$^{1\ Cl-}$, mRNA levels of GR were significantly higher than respective control cells. Although not significant, mRNA levels of CTR1 and ATP7A showed an increasing trend in RTgutGC cells exposed to CuSO$_4$ dissolved in L-15/ex$^{0\ Cl-}$. The direct comparison of normalized copy number of control and respective exposed groups are shown in Fig. S1.

Discussion

In this study, we show that chloride concentration in the exposure medium has opposing effects on copper and silver bioavailability and toxicity in a model of the fish intestinal epithelium based on the cell line, RTgutGC. Although silver and copper have been shown to enter mammalian (Bertinato et al. 2010) and possibly fish (Bury et al. 1999) cells using the same transporter mechanism (i.e., CTR1), their oxidation state in aqueous solution is different, copper is divalent and silver is monovalent. Interestingly, to allow copper transport into cells, Cu^{2+} needs to be reduced to Cu^{1+} by specific membrane reductases (Ohgami et al. 2006) or it is reduced directly at CTR1 amino domain (Shenberger et al. 2018). Thus, CTR1 transports specifically monovalent elements which explains its affinity and ability to transport Ag$^+$ (Bertinato et al. 2010). On the other hand, in the exposure solution, copper and silver react differently with chloride. While the monovalent metal silver possesses a high affinity for the chloride anion, the divalent metal copper does not. Indeed, in the exposure media used in this study, copper was preferentially bound to phosphate or remained as free ion (Cu^{2+}) as its affinity for chloride is relatively low (Fig. 1).
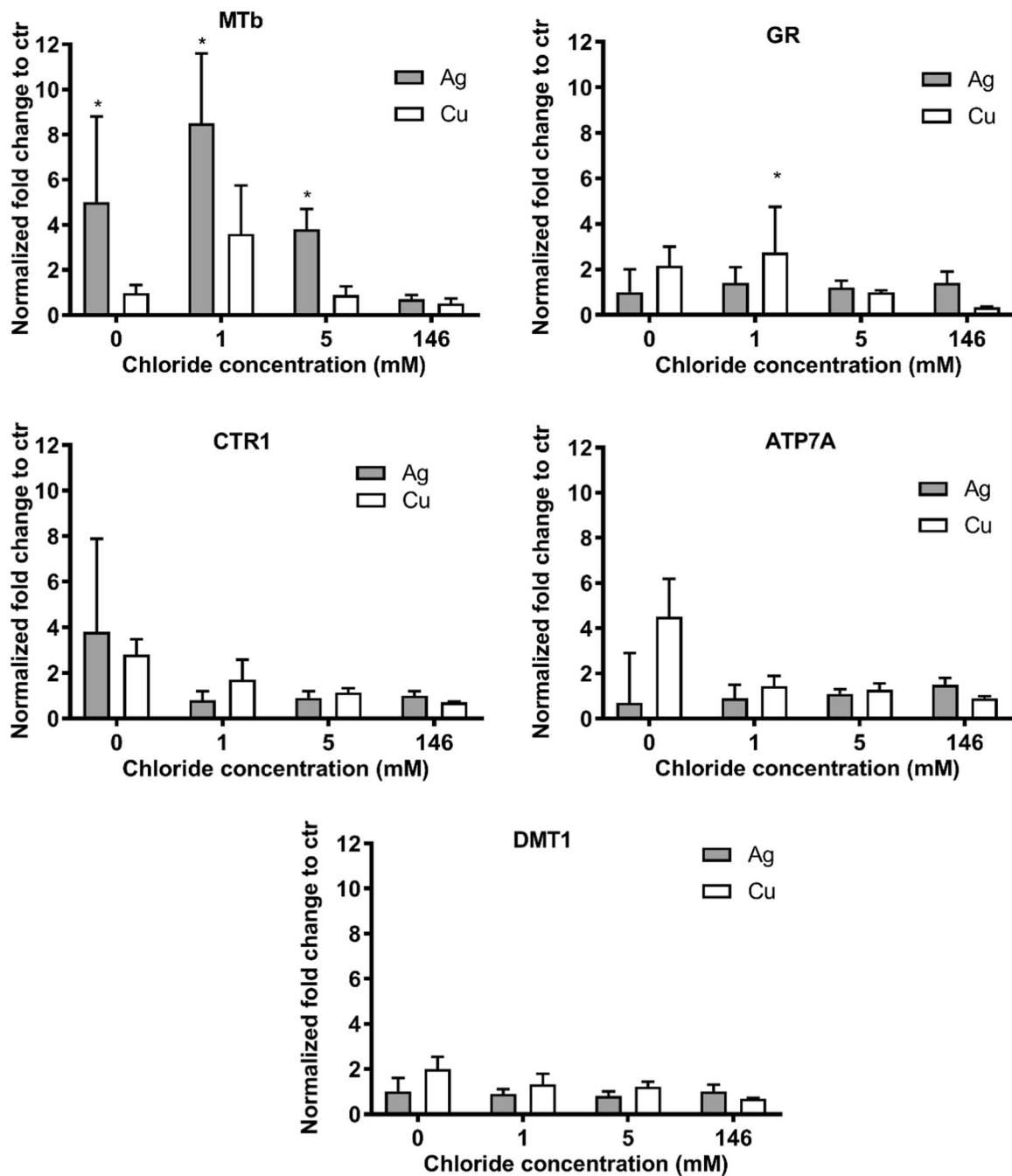
Fig. 3 Metallothionein b (MTb), Glutathione reductase (GR), copper transporter 1 (CTR 1), copper ATPase (ATP 7A), a divalent metal transporter 1 (DMT 1) mRNA levels (normalized fold change) in RTgutGC cells exposed to 500 nM of AgNO$_3$ and CuSO$_4$. All values are expressed as ratio of the expression in those cells exposed to control media. Data are presented as mean ± SD, $n = 3$–4. All values are expressed as ratio of the expression in those cells exposed to control medium. Statistical difference from respective control, i.e., untreated cells, is indicated by an asterisk (Mann-Whitney t-test, $p < 0.05$)

Although the behaviors of silver and copper in aqueous solutions are well known (Blanchard and Grosell 2006; Bury and Hogstrand 2002; Wood et al. 2004), the implications of the interactions occurring in the extracellular environment on metal uptake are less well understood.

In RTgutGC cells, reduction of chloride concentration in the exposure medium resulted in a reduction of copper bioavailability whereas silver bioavailability was not affected by the chloride concentration. We hypothesize that the mechanistic role of chloride on the uptake mechanism of copper and silver is different. Previous studies showed that Cu can be transported via a chloride-dependent Cu/anion symporter mechanism at the intestine of African catfish (Handy et al. 2000). Moreover, it was proposed

that a chloride-dependent anionic transport mechanism is involved in the uptake of copper at the apical surface of human intestinal (Zimnicka et al., (2011)) and red blood cells (Alda and Garay 1990). These studies support the hypothesis that in RTgutGC cells, chloride is necessary for copper uptake. Furthermore, the increase in copper bioavailability explains the increase in copper toxicity that we see in RTgutGC exposed in the medium with the highest chloride concentration (Table 1). For silver, the increase in chloride concentration in the exposure solution results in the progressive formation of neutral and negatively charged silver chloride species. Our data show that at the intestinal epithelium the free ion (i.e., Ag^+) possesses equal bioavailability to the negative AgCl complexes (i.e., $AgCl_2^-$ and $AgCl_3^{2-}$). This is in contrast to studies focusing on silver uptake at the gill epithelium where the bioavailability of negative AgCl complexes is negligible (Bury and Hogstrand 2002). Environmental concentration of chloride has a protective effect against Ag toxicity in rainbow trout (Bury and Hogstrand 2002) in low osmolarity medium, such as fresh water, where fish do not drink. However, in brackish or seawater, where fish drink for osmoregulatory purposes and the intestinal route becomes important for metal uptake, chloride does not protect against metal toxicity (Bielmyer et al. 2008; Ferguson and Hogstrand 1998; Matson et al. 2016). While the exact mechanism of uptake of silver chloride complexes at the fish intestinal epithelium is not clear, these in vivo data (Bielmyer et al. 2008; Ferguson and Hogstrand 1998; Matson et al. 2016) and additional in vitro data in RTgutGC cells (Minghetti et al. 2019; Minghetti and Schirmer 2016) suggest that silver chloride complexes are bioavailable at the fish intestinal epithelium. One possible explanation of the discrepancy between the uptake of copper and silver at the gill and intestinal epithelium could be that while at the gill copper and silver seem to enter mainly via the sodium channel (Bury and Wood 1999; Grosell and Wood 2002), at the intestine, copper seems to enter via a sodium independent mechanism, likely CTR1 (Burke and Handy 2005). Thus, at the fish gill, it is possible that only silver ions (Ag^+) can compete with sodium ions (Na^+), whereas negatively charged AgCl ions do not bind to the biotic ligand (i.e. Na channel). At the intestinal epithelium, Ag^+ is likely entering via CTR1 (Bertinato et al. 2010; Burke and Handy 2005). The mechanism of uptake of AgCl negatively charged complexes is not clear and requires further studies. On the other hand, while negative silver chloride complexes are as bioavailable as free silver ions, they are less toxic. The reduced toxicity of the silver chloride complexes could be explained by the gene expression data. Indeed, silver chloride complexes are less bioreactive as they do not induce an increase in MTb mRNA

levels. Conversely, cells exposed at lower chloride concentrations, where the silver free ion is present, show increased MTb mRNA levels. Metallothionein forms complexes with metals, including silver, and decrease the metal toxicity (Coyle et al. 2002). Overall, this data set suggests that silver chloride species might enter the cell in a less bioreactive form. Importantly, the uptake of copper via CTR1 was shown to involve endocytosis (Clifford et al. 2016). Thus, considering that Ag is also transported via CTR1 (Bertinato et al. 2010), we could speculate that endocytosis might internalize both silver complexes and silver ions, but the free silver ion could be transported into the cytoplasm more efficiently than silver chloride.

Conversely, the exposure to non-toxic doses of copper did not result in any induction of MTb or copper transporters. It was previously shown that copper homeostasis is mainly regulated at the post-translational level (i.e., ATP7A protein trafficking) and not at the transcriptional level (Minghetti and Schirmer 2019). However, copper-induced a moderate induction of GR only in cells exposed in $L-15/ex^{1Cl}$ indicative of oxidative stress (Minghetti et al. 2008).

Conclusion

In this study, we show that RTgutGC cells can be used to simultaneously evaluate the toxicity, bioavailability, and bioreactivity of Ag and Cu across a gradient of chloride concentrations. We show that chloride assumes a different role in mediating the bioavailability and toxicity of copper and silver. This study adds to our understanding of the mechanism of uptake and toxicity of metal and metal complexes at the intestine which is important to improve computer-based metal toxicity prediction models for environmental risk assessment. Future studies should investigate uptake mechanisms of metal complexes, including complexations with various anions (e.g., amino acids, carbonate), which are currently poorly understood.

Author contributions Both authors contributed to conceptualization, data curation, formal analyses, investigation, methodology, software, validation, and visualization. MM supervised this work and provided necessary resources to MI to conduct this research. MI wrote the first draft which was further edited and reviewed by MM. Both authors approved the final paper.

Compliance with Ethical Standards

Conflict of Interest The authors declare no competing interests.

Ethics Statement This study did not use any vertebrate animal. All data was acquired using the cell line RTgutGC. RTgutGC cells were kindly gifted to MM by Professor Kristin Schirmer (Eawag, CH).

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