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Role of metal speciation in the exposure medium on the toxicity, bioavailability and bio-reactivity of copper, silver, cadmium and zinc in the rainbow trout gut cell line (RTgutGC)

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ABSTRACT

The role of metal speciation on metal bioavailability, bio-reactivity and toxicity at the fish intestine is poorly understood. To investigate these processes, we used an in vitro model of the rainbow trout (*Oncorhynchus mykiss*) intestine, the RTgutGC cell line. Cells were exposed to two essential metals (copper and zinc) and two non-essential metals (cadmium and silver) in a medium of well-defined composition, which allowed the determination of metal speciation in solution. Concentrations resulting in a 50% cell viability reduction (EC_{50}) were measured using a viability assay based on two endpoints: metabolic activity and membrane integrity. Metal bioavailability and bio-reactivity was studied at non-toxic (300 nM all metals) and toxic (EC_{10}; Ag-0.6, Cu-0.9, Cd-3, and Zn-9 μM) concentrations. Bioavailability (i.e. intracellular metal accumulation) was determined by ICP-MS, while bio-reactivity (i.e. induction of a metal specific transcriptional response) was determined by measuring the mRNA levels of a known biomarker of metal exposure (i.e. metallothionein) and of copper and zinc transporters (i.e. ATP7A and ZnT1). Dominant metal species in the exposure medium were Zn^{2+}, $CuHPO_4$, $CdCl^+$, and $AgCl_2^-$ respectively for Zn, Cu, Cd, and Ag. The EC_{50s} showed the metal toxicity hierarchy: Ag > Cu > Cd > Zn. In RTgutGC cells, essential metal homeostasis was tightly regulated while non-essential metals accumulated more readily. Non-essential metals were also more bio-reactive inducing higher MT and ZnT1 mRNA levels. Taken together these findings indicate that metal toxicity in RTgutGC cannot solely be explained by extracellular metal speciation but requires the evaluation of metal bioavailability and bio-reactivity.

1. Introduction

The chemical composition of an aqueous environment determines the chemical form, or species, of metals in that environment. Metals can be present in water and other extracellular fluids in different species such as free metal ions (e.g. Ag^+, Cu^{2+}), complexed with inorganic ligands (e.g. Cl^-, OH^-, and CO_3^{2-}), organic ligands (e.g. amino acids, dissolved organic matter) and other solid or colloidal phases. However, not all metal species are equally toxic and bioavailable (Batley et al., 2004; Campbell and Hare, 2015). On the one hand, there is evidence that the free metal ion is highly bioavailable and toxic, especially in freshwater at the fish gill. This concept is at the base of two toxicity prediction models: the FIAM (Free Ion Activity Model) (Morel, 1983; Campbell and Tessier, 1996) and the BLM (Biotic Ligand Model) (Di Toro et al., 2001; Paquin et al., 2002). On the other hand, there is evidence that metal complexes, forming when salinity increases, can also be bioavailable and toxic in fish (Ferguson and Hogstrand, 1998; Grosell et al., 2007; Matson et al., 2016; Wang et al., 2016). For example, silver chloride complexes have negligible bioavailability in fresh water (below 1 ppt) (Bury and Hogstrand, 2002) where the gill uptake is dominant, but they become more toxic at higher salinity (above 5–10 ppt) (Ferguson and Hogstrand, 1998; Matson et al., 2016) in environments where fish drink for osmoregulatory purposes (Scott et al., 2006). These studies suggest that: i) the mechanism of metal uptake might differ depending on the route of uptake (i.e. branchial vs intestinal uptake) and ii) metal complexes can be bioavailable and toxic.

The intestine is the most important route of essential trace metal uptake in fish and appears to occur via a different mechanism than branchial metal uptake (Bury et al., 2003). For instance, there is evidence that copper can leak thorough the apical sodium channel at the

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gill whereas at the intestine it is likely transported by a high affinity copper transporter (i.e. copper transporter 1 – CTR1) (Handy et al., 2002; Minghetti et al., 2008). While there is evidence that factors such as competition between cations affect uptake of essential metals such as of zinc (Glover and Hogstrand, 2003) and iron (Kwong and Niyogi, 2009) at the intestinal epithelium, our knowledge of factors such as metal complexation with organic and inorganic compounds on intestinal metal bioavailability are poorly understood and require further studies (Wood, 2012; Zhao et al., 2016). This is in part due to the lack of an approriate model to study such processes but also to the difficulty to access the intestinal tissue (Minghetti et al., 2017).

To investigate the bioavailability and toxicity of inorganic metal complexes at the intestine, we have used an in vitro model of the intestinal epithelium from Rainbow trout (*Oncorhynchus mykiss*), the RTgutGC cell line (Minghetti et al., 2017). The RTgutGC cell line is a unique intestinal in vitro model that was shown to conserve several features of the polarized intestial epitethelium in vivo including apical expression of tight junction proteins and basolatral expression of the Na^+/K^+-ATPase and alkaline phosphatase enymes (Kawano et al., 2011; Minghetti et al., 2017). Therefore, this cell line represents a suitable model to study the interactions occurring at the cell/exposure medium interface.

Furthermore, it is important to consider that absorptive epithelia are dynamic in nature and can regulate uptake and excretion of metals. The cell has evolved homeostatic processes, including metal transporters and metal chelating proteins, that regulate the intracellular concentration of essential metals (e.g. copper and zinc) (Minghetti et al., 2010, 2008; Zheng et al., 2008) while non-essential metals enter cells by hijacking essential metal transporters (Martinez-Finley et al., 2012). For instance, silver can be transported by copper transporters in mammalian cells, (Bertinato et al., 2010) a mechanism that seems to be conserved in fish as well (Bury et al., 1999; Grosell and Wood, 2002). In addition, there is evidence that cadmium can be transported by iron, zinc and calcium transporters in fish (McGeer et al., 2012). However, when metal concentration exceeds the transport and chelating capacity of the cell, metal can be present in non-complexed form and cellular toxicity can occur. Thus, in addition to the factors influencing the bioavailability of metals (i.e. cellular binding and accumulation) their bio-reactivity (i.e. capacity to induce a molecular response) should also be considered. Indeed, metals could be stored in the cell in a non-bioreactive form (Minghetti and Schirmer, 2016). Metallothionein (MT), is a multipurpose cysteine rich metal detoxification protein that is readily induced in fish and fish cell lines exposed to an excess of metals (Coyle et al., 2002; George and Olsson, 1994; Minghetti et al., 2011). MT is, therefore, an appropriate biomarker of metal bio-reactivity. Moreover, the zinc transporter 1 (ZnT1) and the copper ATPase (ATP7A) are metal transporter proteins which control cellular efflux of zinc and copper, respectively (Minghetti et al., 2010; Zheng et al., 2008). The quantification of mRNA levels of these genes is therefore a valid indicator of intracellular metal bio-reactivity and homeostasis.

In this study, we selected two essential metals (copper and zinc) and two non-essential metals (silver and cadmium) to investigate their toxicity, bioavailability and bio-reactivity in RTgutGC cells exposed in a synthetic medium: L-15/ex. L-15/ex is based on the commercially available Leibovitz's L-15 medium except it does not contain vitamins or amino acids to prevent metal complexation in the medium. This medium has a composition and osmolarity that is similar to that of the fish intestinal lumen in freshwater (Shehadeh and Gordon, 1969). The chemical speciation of these metals in the exposure medium was calculated using the chemical equilibrium model Visual MINTEQ (Gustafsson, 2013). The link between the metal species forming in solution and cellular responses could therefore be evaluated. Metal toxicity was determined using a multiple viability assay that allows simultaneous measurement of metabolic activity and membrane integrity (Schirmer et al., 1998), metal bioavailability by ICP-MS, and metal bioreactivity by measuring MTb, ZnT1 and ATP7A mRNA levels in RTgutGC cells.

2. Materials and methods

2.1. Cell culture

RTgutGC cell lines were cultured in Leibovitz's L-15 (L-15) medium (ATCC, VA, USA) supplemented with 5% of fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) and 1% of *Penicillin streptomycin* (Sigma-Aldrich, St. Louis, MO, USA). Cells were routinely cultured in 75 cm^2 flasks (Greiner Bio One, Monroe, NC, USA) at 19 °C in a normal atmosphere incubator. Once cells covered around 90% of the flask's growth area, they were sub-cultured and split into three new flasks. To passage or sub-culture a confluent flask, the L-15 medium was removed and cells were washed twice with 1 mL of Versene (Thermo Fisher Scientific, Waltham, MA, USA). After washing, cells were detached from the flask with 1 mL of 0.25% Trypsin (Thermo Fisher Scientific, Waltham, MA, USA). Viability of the cell suspension was evaluated using the Trypan Blue exclusion assay and simultaneously cells were counted on haemocytometer slides using Countess II™ Automated Cell Counter (Life Technologies Corporation, NYC, NY, USA). Only cell suspension with a viability over 90% were used for subsequent assays. For all experimental exposures RTgutGC cells were seeded in multi-well plates at 80,000 cells/cm^2 in L-15/FBS and incubated for 48 h before exposure to allow the formation of a confluent monolayer.

2.2. Exposure medium and preparation of exposure solutions

The synthetic exposure medium (L-15/ex) is based on the composition of the commercial medium Leibovitz's L-15. L-15/ex medium is identical to L-15 in terms of sugars and salt concentrations, but it does not contain amino acids and vitamins (Schirmer et al., 1997) (Table S1). Osmolality and pH were measured using the Vapro® Vapor Pressure Osmometer (Model5600, ELItech group, South Logan, UT, USA) and SI Analytics pH meter (SI Analytics, College Station, TX, USA), respectively. Stock solutions of $AgNO_3$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, and $CdCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) were prepared at 10 mM concentration in ultrapure water (16–18 mΩ, Barnstead GenPure Water, Thermo Fisher Scientific, Waltham, MA, USA). Exposure solutions were freshly prepared in the L-15/ex at metal concentrations ranging from 0.08 and 500 μM, vortexed for 20 s and immediately added to the washed cell monolayers. For metal accumulation and mRNA level analysis, two doses of exposure were chosen: non-toxic (300 nM, for each metal – no statistical difference from control unexposed cells, *t*-test, $p < 0.05$) and toxic concentrations, resulting in a 10% reduction in viability (Effect concentration 10% - EC10), as determined by the cell viability assays. Toxic concentrations were 600 nM, 900 nM, 3 μM and 9 μM for Ag, Cu, Cd, and Zn, respectively.

2.3. Chemical speciation and ionic strength

Chemical speciation, including formation of solids, and ionic strength of each metal in L-15/ex (See Section 2.2 and Table S1) were determined by Visual MINTEQ (Gustafsson, 2013), a chemical equilibrium software. Metal speciation was calculated for each metal at concentrations ranging from 0.1 to 500 μM in L-15/ex.

2.4. Cell viability assays

Cell viability assays were performed at least 5–7 times independently with cells of different passages using an assay that simultaneously measures two endpoints: metabolic activity and cell membrane integrity. Metabolic activity was measured by alamarBlue® (Invitrogen, Eugene, OR, USA), and cell membrane integrity by CFDA-AM (5-carboxylfluorescein diacetate acetoxymethyl ester) (Invitrogen, Eugene, OR, USA) (Minghetti and Schirmer, 2016; Schirmer et al.,

1997). alamarBlue® (AB) is a chemical dye known as resazurin that enters into cells as a non-fluorescent molecular substrate and is converted into a fluorescent product (resorufin) by mitochondrial, microsomal and cytoplasmic oxidoreductases (Schreer et al., 2005). A decrease in AB-fluorescence demonstrates a decline in the cellular metabolic activity (O'Brien et al., 2000). 5-carboxylfluorescein diacetate acetoxymethyl ester (CFDA-AM) is an esterase substrate that can enter into living cells as a non-fluorescent form and can be converted into a fluorescent product by different non-specific cytoplasmic esterases. It can be used as an indicator of cell membrane integrity which is necessary to retain its florescent product (Schirmer et al., 1997). Cell viability assays were performed by seeding 152,000 cells/mL (80,000 cells/cm^2) in L-15/FBS on 24 well plates (Greiner Bio-One, Monroe, NC, USA) and incubated for 48 h. After 48 h, each well was washed twice with phosphate buffer saline (PBS; (Thermo Fisher Scientific, Waltham, MA, USA) and cells were exposed to 1 mL of metal solution dissolved in L-15/ex at concentrations ranging from 0.08 to 500 μM. The concentration range varied depending on the metal toxicity. Exposed cells were then incubated in 19 °C incubator for 24 h. Exposure solutions were then removed and each well was washed with PBS. After washing, cells were exposed to a solution of AB (5% *v*/v) and CFDA-AM (4 μM) both dissolved in PBS. Exactly after 30 mins, fluorescence was measured using the Cytation 5 Plate Reader (BioTek, Winooski, VT, USA) at respective excitation/emission wavelengths of 530/595 nm for AB, 485/530 nm for CFDA-AM.

2.5. Quantification of metal accumulation

For the metal accumulation experiment, cells were seeded into six well plates (Greiner Bio-One, Monroe, NC, USA) at 80,000 cells/cm^2 (768,000 cells/well in 3 mL of L-15/FBS) for 48 h. After 48 h of incubation, each cell monolayer was washed twice with PBS, and then cells were exposed to non-toxic concentrations as determined by alamarBlue viability assay (300 nM of each metal) and toxic concentrations corresponding to the EC10 (Ag-600 nM, Cu-900 nM, Cd-3 μM, and Zn-9 μM) of metal dissolved in L-15/ex for 24 h. After 24 h of exposure, media were removed, and then to ensure thorough removal of loosely bound metal, each well was washed twice with a solution of 0.5 mM cysteine (Sigma-Alrdrich, St. Louis, MO, USA) dissolved in PBS. Next cells were lysed by applying 1 mL of 50 mM NaOH (Sigma-Alrdich, St. Louis, MO, USA) for 2 h at room temperature. Cell lysates were then collected in 1.5 mL Eppendorf tubes and 100 μL from each tube was taken for protein quantification 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-Alrdrich, St. Louis, MO, USA) and 0.2 mL H_20_2 (Sigma-Aldrich, St. Louis, MO, USA). Finally, samples were diluted 10 times with ultra-pure water (16–18 $M\Omega cm^{-1}$ Barnstead GenPure Water, Thermo Fisher Scientific, Waltham, MA, USA). 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. Metal accumulation data were presented as nanomoles metal per milligram of protein determined from the same samples to take into account cell growth (Minghetti and Schirmer, 2016).

2.6. Determination of MTb, ZnT1 and ATP7A mRNA levels by quantitative PCR

To quantify MTb, ZnT1 and ATP7A mRNA levels in RTgutGC, cells were seeded into 12 well plates at 80,000 cells/cm^2 in complete medium (L-15/FBS). Cells were exposed exactly as in the metal accumulation experiment. After 24 h of exposure, 600 μL TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was applied to each well. The total RNA was extracted following the manufacturer instructions and on average, 1.6 μg of RNA was obtained from each well. The purified 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 Cytation 5 Plate Reader (Bio-Tek, Winooski, VT, USA) and by electrophoresis using 0.5 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. Normalized genes copy numbers are reported in Fig. S1. Normalization of copy number of the target genes MTb, ZnT1 and ATP7A was conducted based on the geometric mean of reference genes (Elongation factor 1a and Ubiquitin). The PCR efficiency of all genes was above 93%. Primer sequences are reported in Table S3. Detailed procedures on RNA extraction, DNAase treatment, cDNA synthesis, and qPCR measurement of mRNA levels of target genes have been described previously (Minghetti et al., 2014).

2.7. Data analysis

Statistical analysis was performed using GraphPad Prism Version 7.0 (GraphPad Software Inc., San Diego, CA). Viability data are shown as percentages by dividing the fluorescence units of each exposed sample by the fluorescence unit of controls (L-15/ex). The concentrations leading to 50% and 10% reduction in viability (effective concentration, EC 50 and EC 10) were determined by the nonlinear regression sigmoidal dose–response curve fitting module using the Hill slope equation. EC values are presented as mean and standard error of the mean (N = 5–7). 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. When comparing to a control group, Dunnett's test was applied after the ANOVA ($p < 0.05$).

3. Results

3.1. Chemical speciation in exposure medium (L-15/ex)

Metal species distribution, predicted based on Visual MINTEQ analysis, varied significantly among all the metals dissolved in L-15/ex (Fig. 1). Silver speciation was dominated by negatively charged species: $AgCl_2^-$ (~73%) and $AgCl_3^{2-}$ (~22%). The free silver ion (Ag^+) was 0.025% of total silver. The dominant species of copper were Cu^{2+} (~47%) and $CuHPO_4$ (~43%). Zinc formed three major species: Zn^{2+} (~75%), $ZnHPO_4$ (~11%), and $ZnCl^+$ (~11%). For Cd, the dominant species were $CdCl^+$ (~61%), $CdCl_2$ (~22%), and Cd^{2+} (~13%). Varying the metal concentrations between 0.1 and 500 μM resulted in a negligible variation in metal species distribution for silver, cadmium and zinc in L-15/ex medium. For copper, increasing the metal concentration above 50 μM results in a reduction of Cu^{2+} of about 1% and in the increase in $CuHPO_4$ (aq.) of about 1%.

The formation of metal precipitates is reported in Figs. 1, 2 and Table S2. Precipitation analysis conducted by Visual MINTEQ software showed no precipitation of Cd in the L-15/ex at the exposure concentrations up to 200 μM. Precipitation started (1% of total metal) at concentrations of 3.1 μM for $CuSO_4$, 4.7 μM for $AgNO_3$ and 5.6 μM for $ZnSO_4$, respectively. Fifty percent of the Cu, Ag and Zn precipitated at 6 μM, 9 μM and 11 μM, respectively.

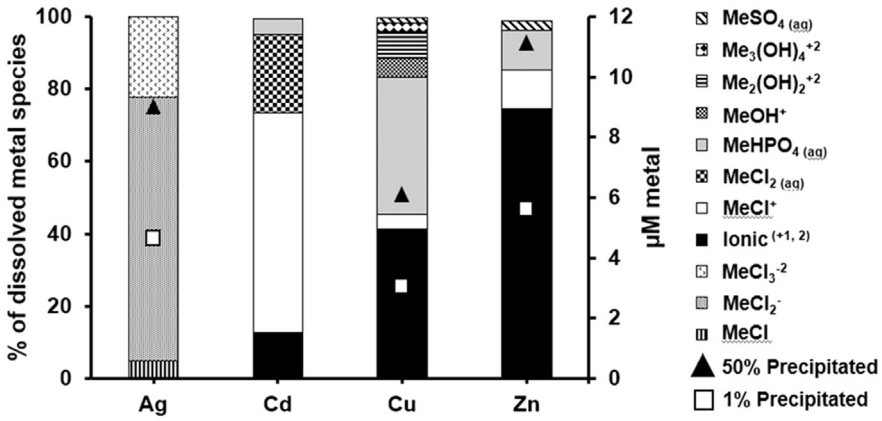

Fig. 1. Metal (Me) species distribution in the exposure medium (L-15/ex), calculated using VISUAL MINTEQ 3.1 assuming an initial concentration of 1 µM. At initial concentrations of ionic metal between 0.1 and 500 µM differences in dissolved silver species distribution were negligible. Left axis denotes total percentage all metal in the media, while the right Y axis denotes at what concentration the metal is during precipitation A complete table of metal precipitate is reported in Table S2.

3.2. Cell viability after 24-hour exposure to metals

Cell viability analysis and EC_{50} calculations showed that toxicity was Ag > Cu > Cd > Zn. This order of toxicity was the same for both alamarBlue® (metabolic activity indicator) and CFDA-AM (indicator of the cell membrane integrity) (Table 1; Fig. 2). Moreover, the analysis of the dose response curves highlighted that while Ag and Cu affected cellular metabolic activity more than cell membrane integrity, for Cd and Zn there were no significant differences between the cell metabolic activity and the cell membrane integrity curve (Fig. 2).

Table 1
EC_{50} values (in µM) calculated exposing RTgutGC cells to $AgNO_3$, $CuSO_4$, $ZnSO_4$, and $CdCl_2$ for 24 h.

Metals	Metabolic activity	Cell membrane integrity
Ag	1.69 ± 0.22 [a]	2.15 ± 0.07 [A]
Cu	3.63 ± 0.21 [a]	7.84 ± 0.63 [B]
Cd	24.13 ± 2.72 [b]	39.22 ± 6.71 [C]
Zn	98.38 ± 9.68 [c]	126.2 ± 24.89 [D]

Values are means ± SEM from at least 5 independent experiments (N = 5–7). EC_{50} concentrations were determined using non-linear regression sigmoidal dose-response curve following the Hill slope equation on GraphPad Prism software (GraphPad Prism Ver. 7.0, San Diego, CA, USA). Values bearing different letters indicate statistical significance among different groups (One-way ANOVA, Tukey test, P < 0.05).

3.3. Metal bioavailability in RTgutGC cells after 24-hour of exposure to non-toxic and toxic concentrations of metals

Metal accumulation in RTgutGC cells exposed to non-toxic and toxic concentrations of metal is reported in Table 2. RTgutGC cells exposed to an identical non-toxic concentration of metal (300 nM) show some difference in metal bioavailability. While cells exposed to copper accumulate about 3-fold more copper than control cells, cells exposed to zinc did not show any increase in intracellular zinc concentration compared to controls. RTgutGC cells accumulated the same concentration of the non-essential metal cadmium and silver.

When cells were exposed to toxic metal concentrations inducing a 10% reduction in viability (i.e. Ag 600 nM; Cu 900 nM; Cd 3 µM; Zn 9 µM), differences in metal homeostasis could be detected. RTgutGC cells appear to be able to regulate intracellular concentration of the essential metal copper and zinc more efficiently in comparison to the non-essential metal silver and cadmium. Cells exposed to toxic concentration of copper (3-fold higher than the non-toxic concentration) did not show any variation in intracellular copper concentration. Similarly, cells exposed to toxic zinc concentration (30-fold higher than the non-toxic concentration) showed a 1.2-fold increase in intracellular

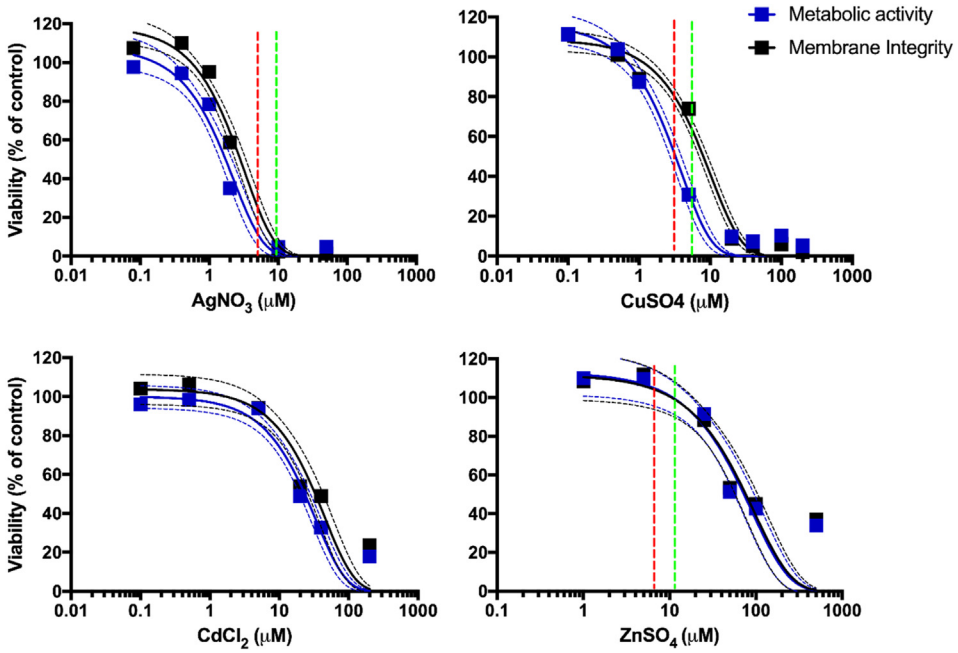

Fig. 2. Toxicity of $AgNO_3$, $CuSO_4$, $CdCl_2$, and $ZnSO_4$ dissolved in RTgutGC cells. Cell viability assay endpoints: metabolic activity (Alamar blue) and membrane integrity (CFDA-AM) were taken after 24 h of exposure. Values are shown as averages and dashed lines indicate confidence intervals (95%) of at least 5 independent experiments. EC_{50} 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. For Ag and Cu the metabolic activity curve was more sensitive than the cell membrane integrity curve. No differences among curves for Cd and Zn. All graphs and statistical analysis 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. Cadmium did not precipitate at any exposure concentration. Precipitation was determined using Visual MINTEQ and is reported in Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Intracellular metal content in RTgutGC cells expressed in nmol per mg of protein.

	Cu	Zn	Cd	Ag
Control	0.418 ± 0.01	4.228 ± 0.227	BDL	BDL
300 nM	1.243 ± 0.144*[b]	4.054 ± 0.091[a]	0.491 ± 0.035[c]	0.407 ± 0.117[c]
EC10	1.245 ± 0.115*[b]	5.016 ± 0.616*[a]	1.826 ± 0.313[b]	1.052 ± 0.198[b]

Metal determination by ICP-MS in RTgutGC cells exposed to non-toxic concentrations (300 nM of each metal) and toxic concentrations (EC10: Ag-600 nM, Cu-900 nM, Cd-3 μM, and Zn-9 μM). Cellular-associated metal was normalized to protein content to take into account differences in cell numbers. Data are presented as mean ± SD, $n = 3–4$. Statistical difference from respective control, i.e. untreated cells, is indicated by an asterisk (ANOVA, Dunnet test $p < 0.05$). Cadmium and silver were below the detection limit (BDL). Differences in metal content among the same treatment is indicated by different letters (One-way ANOVA, Tukey test, $P < 0.05$).

zinc. On the other hand, cells exposed to toxic concentration of cadmium (10-fold higher than the non-toxic concentration) showed a 3.7-fold increase in intracellular cadmium and cells exposed to toxic concentration of silver (2-fold higher than the non-toxic concentration) showed a 2.6-fold increase intracellular silver.

3.4. MTb, ZnT1 and ATP7A mRNA levels in RTgutGC after 24-hour of exposure to non-toxic and toxic concentrations of metals

MTb, ZnT1 and ATP7A mRNA levels in RTgutGC cells exposed to non-toxic and toxic concentrations of metal is reported in Fig. 3. At non-toxic concentrations of metal exposure (300 nM), only cells exposed to Cd showed higher MTb mRNA levels than controls. Moreover, ZnT1 mRNA levels were increased, compare to the respective mRNA levels in control cells, by zinc only and ATP7A by silver only. ZnT1 mRNA induction could explain the reduction in zinc intracellular concentration compared to the control cells.

In cells exposed to metal concentrations inducing a 10% reduction in viability (Ag-600 nM, Cu-900 nM, Cd-3 μM and Zn-9 μM), MTb mRNA levels were significantly higher than controls in cells exposed to Ag, Cd and Zn. Cells exposed to Cu did not induce significantly MTb compared to respective controls. ZnT1 mRNA levels were induced by the non-essential metals, silver and cadmium but not by the essential metals, copper and zinc. ATP7A mRNA levels were not changed by any of the metals at toxic doses of exposure.

4. Discussion

In this study, we show that in RTgutGC intestinal cells, the toxicity and bioavailability of two essential metals (copper and zinc) and two non-essential metals (cadmium and silver) cannot be explained solely by metal complexation in the exposure medium and that factors like metal bio-reactivity and metal homeostasis play a role in determining the toxicity of metals at the cellular level.

The intestinal luminal composition can vary significantly from that of the surrounding water (Shehadeh and Gordon, 1969; Genz et al., 2011) affecting metal uptake and toxicity at the intestinal epithelium. The complexation and precipitation of metals in L-15/ex could explain, at least partially, the difference in toxicity measured in RTgutGC in vitro with the toxicity of these metals in rainbow trout in vivo. Metal speciation in L-15/ex was performed using a chemical equilibrium model which takes into consideration only the interaction between metals and the medium components and did not take into consideration the possible complexation of metals with organic compounds (glycoproteins, proteins, amino acids, etc.) and inorganic compounds (i.e. HCO_3^-) which could be excreted by RTgutGC cells. L-15/ex composition was based on the commercial medium Leibovitz's L-15 and did not contain bicarbonate or glycoprotein present in mucus which is physiologically relevant in the fish intestine (Glover and Hogstrand, 2003). However, substitution of bicarbonate with phosphate would not have changed the speciation of silver, cadmium and zinc but would have changed the speciation of copper which would have complexed with bicarbonate (data not shown). Future in vitro studies should evaluate mucus and bicarbonate secretion by RTgutGC cells. Moreover, it should be noted that this study investigates the toxicity and bioavailability of inorganic metal complexes not of organic metal complexes. Inorganic complexes are relevant for intestinal exposures to waterborne metal in brackish and seawater conditions where fish drink for osmoregulatory purposes (Scott et al., 2006). Conversely, organic metal complexes are relevant for intestinal diet borne metal exposures. Dietary metal exposures do not exert acute toxicity (Wang, 2013) although chronic toxicity has been reported for copper, zinc and cadmium (Clearwater et al., 2002; Tan et al., 2010). Exposure to dietary silver (3.1 μg/g) did not induce any adverse effect in rainbow trout (*Oncorhynchus mykiss*) (Galvez et al., 2001). Dietary metal toxicity is likely reduced by protein complexation as shown previously in vitro for silver (Minghetti and Schirmer, 2016) but also due to the role of the liver in metal detoxification after intestinal uptake (Kamunde and MacPhail, 2011).

In fish, waterborne metal toxicity (LC_{50}) varies widely depending on the water chemistry, however, the order of toxicity is usually Ag > Cd > Cu > Zn (McDonald and Wood, 1993; Naddy et al., 2015; Hogstrand and Wood, 1998). In RTgutGC, the order of metal toxicity (EC_{50}) was Ag > Cu > Cd > Zn (Fig. 2; Table 1). Therefore, with the exclusion of cadmium that is normally more toxic than copper in vivo, the order of toxicity (LC_{50} vs EC_{50}) of the other metals in RTgutGC cell is comparable to that of rainbow trout in vivo (McDonald and Wood, 1993). The toxicity of cadmium in RTgutGC exposed in L-15/ex is 428- fold lower than that measured in rainbow trout exposed in soft water (Naddy et al., 2015) and could be explained by chloride complexation. Similarly, chloride complexation could explain the reduced toxicity of silver (between 4- and 40-fold lower) in RTgutGC cells in comparison to that in rainbow trout measured in fresh water conditions (Hogstrand and Wood, 1998). Moreover, it should be noted that silver toxicity in RTgutGC in this study is identical to that measured in a previous study (Minghetti and Schirmer, 2016) supporting the reliability of this in vitro cell culture system. Copper toxicity is only about 2-fold lower in RTgutGC than that measured in rainbow trout (Naddy et al., 2015). About 40% of total copper is present in L-15/ex as free metal ion (Cu^{2+}) and is over 99% dissolved at the concentration inducing a 50% reduction in metabolic activity. Thus, copper speciation in L-15/ex could explain the closer discrepancy between in vitro and in vivo toxicity of copper. Zinc was about 20- fold less toxic in RTgutGC exposed in L-15/ex in comparison to rainbow trout exposed in soft water (Naddy et al., 2015) but it was comparable to that of killifish exposed in brackish water (Bielmyer et al., 2012). Furthermore, the difference between in vitro and in vivo toxicity could also be explained by the formation of zinc phosphate solids in L-15/ex, that could reduce zinc bioavailability (Fig. 2; Table S2). Indeed, when exposing RTgutGC to zinc in a phosphate free medium, which inhibited the formation of zinc solids, zinc toxicity was 40% higher than in our study (Prabhu et al., 2018).

The lower toxicity of cadmium in comparison to that of copper in RTgutGC cells was surprising, especially because cadmium has been shown to induce mitochondria dysfunction in fish (Adiele et al., 2012). This would suggest a bigger reduction in the metabolic activity assay used in this study. Moreover, in a previous in vitro study it was shown

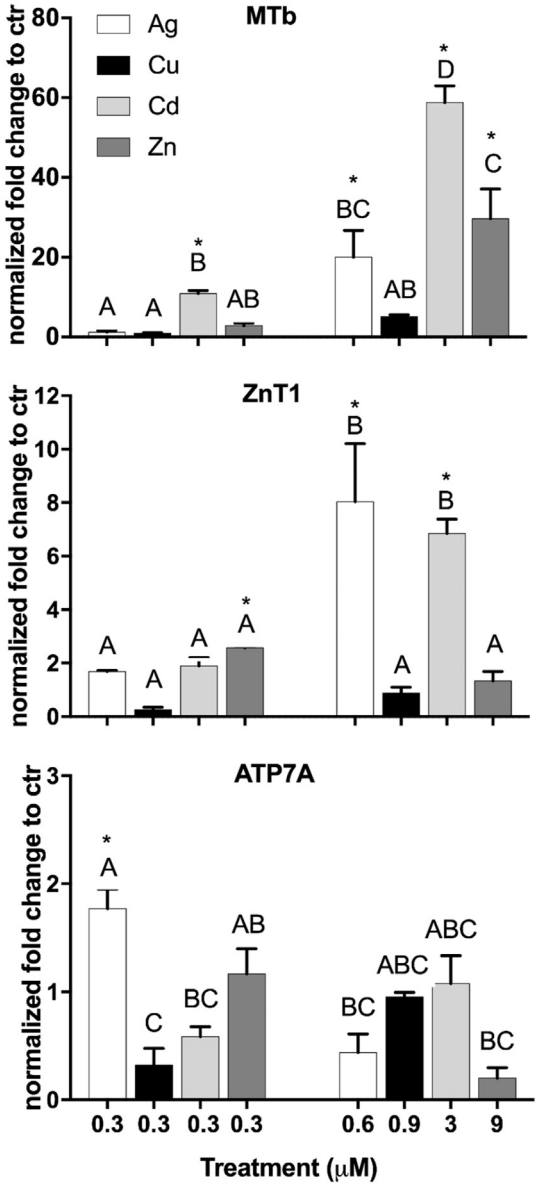

Fig. 3. Metallothionein b (MTb), Zinc Transporter 1 (ZnT1) and copper ATPase (ATP7A) mRNA levels (normalized fold change) in RTgutGC cells exposed to non-toxic concentrations (300 nM for all metal) and toxic concentrations (EC10: Ag-600 nM, Cu-900 nM, Cd-3 μM, and Zn-9 μM). All values are expressed as ratio of the expression in those cells exposed to control media (see Fig. S1). Data are presented as mean ± SD, $n = 3–6$. Values bearing different letters indicate statistical significance among different groups (One-way ANOVA, Tukey test, $P < 0.05$). 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 (ANOVA, Dunnet test $p < 0.05$).

that cadmium induced higher toxicity (including mitochondria dysfunction) than copper in gill and liver trout cell lines (RTgill-W1 and RTH-149) although at longer exposure times (i.e. 120 h) and using exposures in presence of amino acids and proteins (Krumschnabel et al., 2010). The presence of proteins and amino acids in the exposure solution result in metal complexation and possibly in different uptake pathways for cadmium and copper which might also affect their toxic responses as it was previously shown for silver (Minghetti and Schirmer, 2016). Moreover, in our study, the reduced toxicity of cadmium in comparison with copper could be explained by the higher induction of metallothionein per μM of intracellular metal (Table 2, Fig. 3). Cadmium has the highest affinity for MT among silver, copper and zinc resulting in higher displacement of zinc from zinc-metallothionein complexes (Waalkes et al., 1984) which is linked to MT induction (Günther et al., 2012). The higher MTb mRNA levels in RTgutGC exposed to cadmium compared to silver, copper and zinc supports these studies. Moreover, the role of MT induction on the tolerance to cadmium have been shown previously in turbot (*Scophthalmus maximus*) (George et al., 1996) and marine grunt, (*Terapon jarbua*) (Dang and Wang, 2009). Cadmium was also shown to induce higher MT mRNA levels than copper and zinc in a fish fibroblast cell line (SAF1) (Minghetti et al., 2011). Therefore, MTb induction can further explain the lower toxicity of cadmium in comparison to copper in RTgutGC.

Silver chloride complexes have been shown to be non-bioavailable in rainbow trout exposed in freshwater where the gill uptake is dominant (Hogstrand et al., 1996; Bury and Hogstrand, 2002). Whereas, in brackish and seawater where intestinal metal uptake is more important, silver chloride complexes appear to be more bioavailable and toxic in rainbow trout and killifish (Ferguson and Hogstrand, 1998; Matson et al., 2016). Our data shows that in intestinal RTgutGC cells silver chloride complexes, which are over 99.9% of total silver species in L-15/ex, are bioavailable and toxic (Figs. 2, 3; Minghetti et al., 2019) supporting the role of the intestine as an important route of silver uptake in brackish and seawater. There is also evidence that copper (Grosell et al., 2007) and cadmium (Wang et al., 2016) toxicity increases significantly from brackish to seawater conditions suggesting that intestinal uptake might be an important route of entry for metals in high salinity environments. Conversely, salinity have been shown to have a protective role on zinc toxicity (Bielmyer et al., 2012). Taken together, these studies suggest that the mechanisms of metal uptake might differ at the gill and at the intestine and that the intestine is an important route of metal uptake in brackish and seawater.

Metal bioavailability and homeostasis in RTgutGC cells appears to be strongly associated with whether the metal is essential or non-essential, wherein non-essential metals accumulate at higher concentrations with lower extracellular concentrations (Table 2). This is consistent with previous studies showing that cadmium and silver have a long biological half-life in fish (Chowdhury et al., 2003, 2004; Hogstrand et al., 2003; Galvez et al., 2002). Silver and cadmium accumulation could be explained by the way these metals are handled at the cellular lever. For instance, there is evidence that luminal cadmium is transported in mammals by zinc transporters (i.e. ZIP and ZnT transporters) (Girijashanker et al., 2008; Zalups and Ahmad, 2003) and in mammals and fish by the divalent metal transporter 1 (DMT1) (Elisma and Jumarie, 2001; Kwong et al., 2010) and that silver is transported by copper transporters (Behra et al., 2013). These transporters have been shown to be expressed in RTgutGC cells (Minghetti and Schirmer, 2019). Thus, we could speculate that higher accumulation of silver and cadmium in comparison to copper and zinc could be in part due to their ability to enter the cell via the copper and zinc importers (e.g. ZIPs, DMT1, CTR1) (Martinez-Finley et al., 2012; Bertinato et al., 2010; Kwong et al., 2010) but then their excretion is not as efficient as shown by a lower affinity for zinc and copper exporters (e.g. ZnTs and ATP7A) (Hoch et al., 2012; Monty et al., 2005). Moreover, our data suggest that RTgutGC maintain a tight regulation of zinc homeostasis via the regulation of ZnT1 mRNA levels (Table 2 and Fig. 3), a mechanism shown previously in zebrafish (*Danio rerio*) (Zheng et al., 2008). Furthermore, copper homeostasis is tightly regulated by all organisms (Kim et al., 2008) including fish (Minghetti et al., 2010, 2008). However, in our study in RTgutGC cells, copper exposure did not affect ATP7A mRNA levels suggesting that copper homeostasis is mainly regulated at the post-translational level (Minghetti and Schirmer, 2019; Lutsenko et al., 2007).

5. Conclusions

In this study, we used an in vitro model of the fish intestine based of

RTgutGC cells in order to study the link between extracellular metal speciation and intracellular effects (i.e. toxicity, bioavailability and bioreactivity). In freshwater, where the main route of metal uptake in fish is the gill, the free ion was shown to be the most bioavailable and toxic species (Paquin et al., 2002). In brackish and seawater where the intestine plays an important role in metal uptake, other metal species might be bioavailable and toxic as shown in this study. Moreover, in RTgutGC cells, metal toxicity cannot be explained solely by the extracellular metal speciation. Factors like metal homeostasis and intracellular sequestration (e.g. MT complexation) also play a major role in determining metal toxicity. In summary, the RTgutGC cell system proved to be an effective model to study the bioavailability, bio-reactivity and toxicity of different metal complexes. Moreover, this model allows a more high-throughput and more ethical approach than whole fish studies. Given that the mechanisms of metal complexes uptake are poorly understood, this model may well prove to be a robust tool in addressing these processes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2020.108816.

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