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Development of an *in Vitro* Digestion System to Study the Bioavailability and Bioreactivity of Zinc Sulfate and Zn-Bioplex in Fish Using the RTgutGC Cell Line

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ABSTRACT: Here, we report the development of a novel *in vitro* digestion system that mimics the digestive process in fish and uses the RTgutGC cell line, a model of the rainbow trout (*Oncorhynchus mykiss*) intestine, to study the bioavailability, bioreactivity, and toxicity of Zn added as an inorganic ($ZnSO_4$) and organic zinc compound (Zn-Bioplex). Our results showed that, before digestion, Zn-Bioplex was more bioavailable than $ZnSO_4$. However, after *in vitro* digestion, $ZnSO_4$ and Zn-Bioplex accumulated and were transported equally across the RTgutGC cell epithelium. However, Zn-Bioplex was shown to be less bioreactive than $ZnSO_4$, suggesting that peptide complexation might reduce the intracellular release of zinc as shown by a reduced induction of metallothionein and ZnT1 mRNA levels. This novel method shows promise as a standardized *in vitro* fish digestion model to study the interactions occurring at the lumen of the intestine and the bioavailability and toxicity of digested compounds in the fish gut.

KEYWORDS: zinc toxicity, Zinc bioavailability, Fish nutrition, in vitro fish digestion system, gene expression, in vitro alternative

1. INTRODUCTION

In vitro digestion models have been instrumental in improving our understanding of complex digestive processes in humans and other vertebrates. While such processes are well established for humans,[1] they are not in fish.[2] The development of a protocol that mimics fish digestion *in vitro* is important to evaluate the effect of mechanical and chemical transformations on nutrient or toxicant bioavailability. Moreover, *in vitro* digestion models can be used to screen the digestibility of novel ingredients in aquaculture at a lower cost than traditional *in vivo* studies and represent a more ethical approach, reducing the use of fish for research purposes.[3]

The anatomy of the teleost fish digestive system is diverse among different species.[4] For example, depending on the species and developmental stage, the first stage of fish digestion can occur in a true stomach, typical of carnivorous fish (e.g., trout), or in the absence of a stomach in some herbivore fish (e.g., carp). Different digestive processes therefore might occur in different fish species. For instance, in the acidic environment of the stomach, pepsin, an enzyme with high activity at a pH below 4, is responsible for protein digestion.[5] Moreover, it was shown that stomach digestion can inactivate some trypsin inhibitors present in protein-rich plant ingredients.[6] After the acidic digestion, the chyme enters the intestine, wherein bile and pancreatic secretions (i.e., zymogens and bicarbonate) further digest protein, carbohydrates, and fats at a slightly alkaline pH (~7.1−7.5 in salmonids).[7,8]

Previous studies have used a variety of approaches to study intestinal physiological processes. Surgical insertion of a catheter in live fish can guarantee dosage on gut exposure, maintaining neurological and endocrinological function in the digestive system. However, catheter surgery represents a technically demanding and invasive technique that can be

used only for short periods of time, ~3 h.[9] Dissection of the gut, such as the *ex vivo* gut sac model, can also be useful to study intestinal physiological processes such as osmoregulation.[10] However, isolating the gut removes the contribution of digestive juices produced by the pancreas and liver, and the gut sac is short-lived, as once removed it remains viable for roughly 4 h.[11] Several fish *in vitro* gastrointestinal models (i.e., just mimicking the gastrointestinal juices) have previously been developed and used to study ingredient digestibility; however, these procedures investigate only the mechanical and chemical breakdown of nutrients (e.g., protein hydrolysis) and do not include the digestibility or the bioavailability of the digested nutrients by the intestinal epithelium.[2]

In this study, we developed a static *in vitro* fish digestion model system that can be used in combination with an *in vitro* model of the fish intestinal epithelium, based on the rainbow trout (*Oncorhynchus mykiss*) gut cell line RTgutGC.[12,13] When cultured on transwells, RTgutGC cells polarize and express tight junctions mimicking the intact epithelial boundary between the intestinal lumen and the organismal blood circulation.[12] Moreover, these cells have been shown to express important enzymes for intestinal physiology such as sodium−potassium ATPase and the alkaline phosphatase[12,13] and genes involved in metal homeostasis,[45] immune and barrier function.[14] Therefore, the combination of the *in vitro*

digestion model with the RTgutGC cells allows the study of bioavailability and cellular effects (e.g., cytotoxicity, immune response, specific enzyme activities, etc.) of nutrients, food additives, or toxicants after the gastrointestinal digestive processes have occurred. Moreover, the use of transwells allows for the measurement of uptake and transport across the intestinal epithelium.

Increasing the bioavailability of nutrients, such as trace metals, in aquaculture feed can, on one hand, improve the nutritional quality of fish flesh while reducing the output of these elements in the environment.[15] For instance, it was shown that essential metal bioavailability can improve with peptide or amino acidic chelation.[16] Zinc chelated by organic molecules, such as methionine, glycine, or phytic acid, have been shown to increase Zn uptake, suggesting a Zn-amino acidic transport system.[17,18] Moreover, zinc chelated with partially hydrolyzed proteins such as the zinc Bioplex produced by Alltech[19] have also been shown to increase zinc bioavailability in poultry.[20] Zinc uptake and homeostasis has been studied extensively in fish; however, most studies focused on zinc salts.[21,22] Zinc is the most abundant trace metal within the organism, measuring in the micromolar range within the eukaryotic cell.[23,24] Zinc homeostasis is tightly regulated by specialized Zn transporters such as the ZiP and ZnT.[25] Roughly 9% of the eukaryotic proteome requires Zn for protein function as Zn is a cofactor for many essential enzymes and is vital for gene transcription.[26] Zinc is also an important pollutant in the aquatic environment as it can become toxic to aquatic organisms at high concentration.[19] Therefore, the bioavailability of zinc in fish diets is of critical importance for both fish health and to reduce the environmental impact of aquaculture.

In this study, as a proof of principle, we compared bioavailability (i.e., intracellular accumulation), transport across the intestinal epithelium, toxicity and bioreactivity (i.e., induction of zinc-specific gene expression) of zinc sulfate, an inorganic zinc complex and zinc Bioplex (Zn-Bioplex, Alltech), and an organic zinc complex (i.e., zinc chelated by peptides[27]). This approach represents the first animal-free method to test nutrient bioavailability and toxicity simultaneously in fish.

2. METHODS

2.1. In Vitro Digestion Protocol. This section describes the in vitro digestion protocol, which is also summarized in Figure 1. All salts used for the preparation of the buffers used in the in vitro digestion protocol were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). A complete list of buffers' compositions can be found in Table 1. The digestion protocol was adapted from previous literature.[28,29] All of the buffers are prepared based on the cell culture medium Leibovitz's L-15 (Thermo Scientific, Waltham, MA, USA), but without amino acids and vitamins to prevent metal chelation.[35] This modified medium will be denoted as L-15/ex. Moreover, in the intestinal and luminal buffer (Table 1), phosphate was substituted with bicarbonate because of its physiological relevance and also because phosphate has a higher affinity for the free zinc ion (Zn^{2+}) than bicarbonate,[30] which can reduce zinc bioavailability. The pH values applied are within the range values measured in monogastric fish including trout.[7,8,31,32] All enzymes purchased from Sigma-Aldrich are of mammalian origin. Exposure stock solutions were prepared in ultrapure water (16−18 mΩ, Barnstead GenPure Water, Thermo Fisher Scientific, Waltham, MA, USA) using metal salts ($ZnSO_4 \cdot 6H_2O$; Sigma-Aldrich, St. Louis, MO, USA) or Zn-Bioplex (Alltech, Nicholasville, Ky, USA). Both the zinc compounds ($ZnSO_4$ and Zn-Bioplex) were processed through the digestion protocol. The
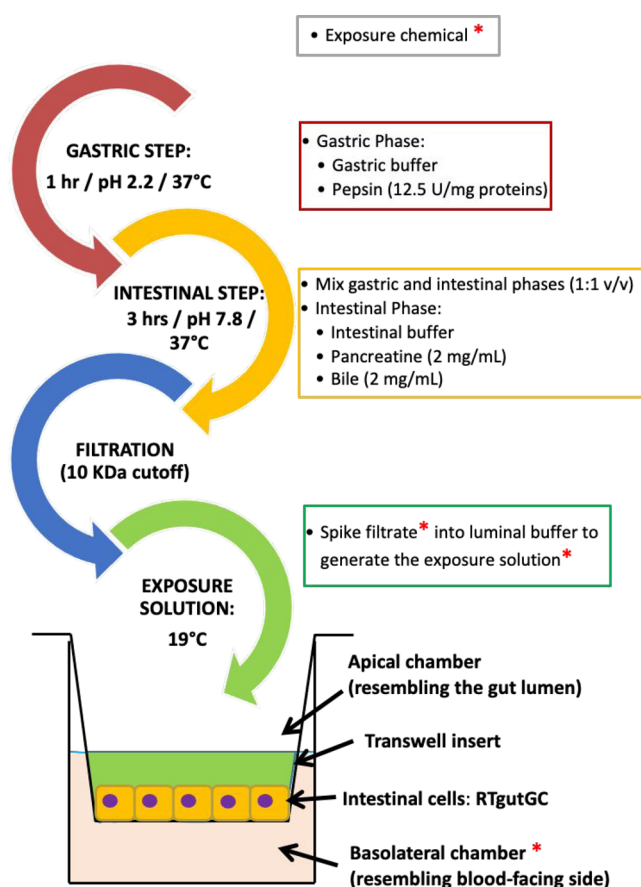
Figure 1. Schematic representation of the in vitro digestion system combined with a model of the fish intestine (i.e., polarized RTgutGC cells). Red asterisks denote a Zn measurement by ICP-OES, and values are reported in Figure 2A (exposure chemical) and Figure 4 (prefiltration, filtrate, and exposure).

Table 1. Buffer Compositions

component (mM)	basal buffer	gastric buffer	intestinal buffer	basolateral buffer	luminal buffer
Cl⁻	145.4	145.4	145.4	154.0	145.4
Ca^{2+}	1.3	1.3	1.3	1.3	1.3
Mg^{2+}	1.1	1.1	1.1	1.1	1.1
Na^+	142.5	142.5	143.5	157.0	142.5
K^+	3.0	3.0	3.0	3.8	3.0
PO_4	0.0	0.0	0.0	0.9	0.0
SO_4	1.1	1.1	1.1	1.1	1.1
HEPES	0.0	0.0	20.0	10.0	10.0
CO_3^{-2}	0.0	0.0	10.0	0.0	5.0
pyruvate	5.0	5.0	5.0	5.0	5.0
galactose	5.0	5.0	5.0	5.0	5.0
pH[a]	6.9	2.2	8.9	7.4	7.4
osmolality[a] (mOsm kg⁻¹)	252.3	278.0	301.7	344.3	335.3

[a]pH and Osmolality were measured by pH meter and Osmometer, respectively.

intestinal buffer and gastric buffer were prepared from the basal buffer. For the gastric buffer, 69% HCl was added to the basal buffer to bring the pH to 2.2 after a 1 h incubation. To start the digestion process, $ZnSO_4$ and Zn-Bioplex stock solutions were spiked into separate sterile, plastic Falcon tubes (Thermo Scientific, Waltham, MA, USA) in gastric buffer to a concentration of 4 mM each, and pepsin was added to the gastric buffer at 12.5 U/mg of protein. The

concentration of 4 mM was chosen to account for dilution throughout digestion, toxicity concentration ranges, and the detection limit of ICP-OES. Considering that the enzymes used were of mammalian origin, and to allow optimal enzymatic activity, all *in vitro* digestion reactions were performed at 37 °C. The gastric phase was incubated at 37 °C for 1 h under agitation. During this time, the intestinal buffer was prepared by adding the porcine bile and pancreatin salts to the basal buffer at 4 mg/mL each and adding NaHCO₃ and HEPES into the solution at 10 mM and 20 mM, respectively. The mixture was stirred for 30 min at 37 °C on an orbital shaker (VWR; Avantor, Radnor, PA, USA), after which the pH was measured at 8.9. After incubation of the gastric phase, the intestinal phase was added at a ratio of 1:1 v/v to form the digesta (i.e., the complete digestive fluid mimicking the chyme mixing with pancreatic and hepatic juices), which brought the pH to 7.8 and the concentration of pancreatin and bile to 2 mg/mL. Then, the digesta was incubated at 37 °C for 3 h under agitation in the same manner as the gastric phase. To confirm pH stability, pH was measured after incubation. Subsequently, the digesta was transferred to an Amicon Ultra-4 Centrifugal Filter, 10 kDa cutoff (MilliporeSigma, Burlington, MA, USA) and centrifuged at 4000g for 30 min at 20 °C. After centrifugation, the filtrate was stored overnight at 4 °C. The exposure medium was prepared by spiking the filtrated digesta into the luminal buffer immediately before exposure to a nominal concentration specific for each experiment. Excess digesta, filtrate, and exposure medium were used for ICP-OES, pH, and osmolality measurements (Table 1; Figure 1).

2.2. Zn-Bioplex Characterization. Protein quantification was performed with both the Lowry assay (Thermo Scientific, Waltham, MA, USA) and the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Zinc concentration in Zn-Bioplex was measured by dispersing 1 mg compound in one mL of ultrapure water (16−18 mΩ, Barnstead GenPure Water, Thermo Fisher Scientific, Waltham, MA, USA). Stocks were vortexed and digested in 29% HNO₃ for 24 h. After digestion, the samples were diluted to 5% HNO₃ and samples were measured by ICP-OES (iCAP Qc, Thermo Fisher Scientific, Driesch, Germany). Zinc recovery in stocks, before digestion was within 10% of nominal concentration (Figure 4).

Zinc speciation and formation of solids in the luminal buffer was calculated using the chemical equilibrium model Visual MINTEQ.[33] The composition of the luminal buffer reported in Table 1 as well as 10 μM ZnSO₄, the dose used during the bioavailability and bioactivity cell exposures, were the parameters used in the Visual MINTEQ calculation of zinc speciation and of the medium ionic strength.

2.3. RTgutGC Cell Culture. RTgutGC cells were cultured in 75 cm² cell culture flasks (Greiner Bio One, Monroe, NC, USA) in the complete medium (L-15/FBS) at 19 °C in a normal atmosphere incubator. L-15/FBS is Leibovitz L-15 medium (Thermo Scientific, Waltham, MA, USA) supplemented with 5% Fetal Bovine Serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) and gentamicin (10 mg/L; Sigma-Aldrich, St. Louis, MO, USA). Once the cells reached a confluency of around 90%, they were washed twice with Versene (Thermo Scientific, Waltham, MA, USA) and detached from the flask using 0.25% trypsin (Thermo Scientific, Waltham, MA, USA). After trypsinization, cells were resuspended in L-15/FBS and then were counted using a Countess II Automated Cell Counter (Life Technologies Corporation, NYC, NY, USA), which measures cell number and viability simultaneously using the Trypan blue exclusion assay. As a quality control, only cell batches with >90% viability were used. The cell suspensions were then diluted with L-15/FBS to allow for a seeding density appropriate for each test. RTgutGC cells were seeded at 72 000 cells per cm² on flat bottom wells, then incubated at 19 °C for 48 h to allow formation of a confluent cell monolayer. Polarized RTgutGC epithelium was prepared as previously described.[12] Briefly, cells were seeded at 62 500 cells per cm² on six-well transwells and incubated for 21 days at 19 °C to allow for cell polarization and tight junction formation (previously demonstrated in Minghetti et al.[12]). When ready for exposure, the L-15/FBS was aspirated from both the apical and basolateral side and washed twice with the luminal and basolateral buffer, respectively.

2.4. Cell Viability Assay. Cell viability was evaluated in RTgutGC cell monolayers and on RTgutGC polarized cells. Before metal exposure, cells were washed twice with the luminal buffer to remove any traces of L-15/FBS from the well. The ZnSO₄ and Zn-Bioplex filtrate were spiked individually into the luminal buffer (Table 1) at 500, 100, 50, 25, 10, 5, and 1 μM in the luminal buffer. The exposure solutions were applied in triplicate, and cells were incubated at 19 °C for 24 h before viability was measured. All exposure solutions were made just before exposure to minimize the effects of precipitation and allow for an accurate exposure concentration. The zinc concentration in the exposure solutions was measured by ICP-OES. Effective concentration 50 (EC_{50}) is based on measured values. Cell viability was measured using a two-end-point cytotoxicity assay based on two fluorescent dyes: Alamar Blue (Resazurin; Invitrogen, Eugene, OR, USA) was used for metabolic activity, and CFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester; Invitrogen, Eugene, OR, USA) was used for membrane integrity, in accordance with the methods of Schirmer et al.[34] Alamar Blue and CFDA-AM excitation/emission were read using the Cytation 5 plate reader (BioTek, Winooski, VT, USA) at 530/595 and 485/530 nm wavelength, respectively.

2.5. Quantification of Intracellular Zinc. Quantification of zinc bioavailability (i.e., intracellular zinc) was performed in polarized RTgutGC cells (i.e., cells cultured on transwells). The exposure medium was prepared in the same manner as for the cytotoxicity assay. Cells were exposed to a low (2.5 μM) and a high (5 μM) concentration of ZnSO₄ and Zn-Bioplex for 3, 24, and 72 h. The time frame used was determined considering the tolerance of RTgutGC cells to the exposure medium without amino acids or FBS (e.g., L-15/ex)[35] and the time necessary to evacuate a meal *in vivo* in rainbow trout, which was shown to range from ∼14 to 53 h.[36] In addition, cells were also exposed to the luminal buffer without zinc compound added. Exposure medium zinc concentrations were measured by ICP-OES. After 3, 24, and 72 h, samples were taken from both the apical and basolateral side for ICP-OES measurement. Each RTgutGC epithelium was washed with luminal buffer supplemented with 0.5 mM cysteine to remove any loosely bound zinc. Cells were lysed using 1 mL of 50 mM NaOH solution, and the plates were incubated at room temperature and mixed at 200 rpm for 2 h. After mixing, the cell lysates were carefully transferred into a 1.5 mL Eppendorf tube, and a 100 μL aliquot was taken for total protein quantification using the Lowry assay (Thermo Scientific, Waltham, MA, USA). The remaining 900 μL was desiccated using a concentrator (concentrator plus, Eppendorf, Hauppauge, USA). The desiccated samples were then digested for 16 h using 0.8 mL of 68% HNO₃ and 0.2 mL of 30% H₂O₂ at room temperature to allow for complete dissolution of all the metals. The whole solution was then diluted to have a final concentration of 5% HNO₃ in solution. The samples were stored in 4 °C refrigeration until the ICP-OES analysis.

2.6. RNA Extraction, cDNA Synthesis, and Quantitative PCR (qPCR). Polarized cells were cultured on transwells in the same manner as for the zinc bioavailability method. After 24 h of exposure, 900 μL of TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was applied to the RTgutGC cell epithelium, and the RNA was extracted following the manufacturer's instructions. The purified RNA was treated with the TURBO DNase kit (Thermo Fisher Scientific, Waltham, MA, USA) to remove any trace of DNA. Quality and quantity of RNA was determined spectrophotometrically using a Cytation 5 plate reader and by electrophoresis using 1 μg of RNA in 1% agarose gel. Complementary DNA (cDNA) synthesis was performed from 1 μg of total RNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) by following the manufacturer's instructions. Quantitative PCR (qPCR) 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 (mRNA) levels were measured using the absolute quantification method and are reported as a fold change of the treated groups from their respective controls. Controls were cell monolayers exposed to the luminal buffer. Gene absolute copy numbers are
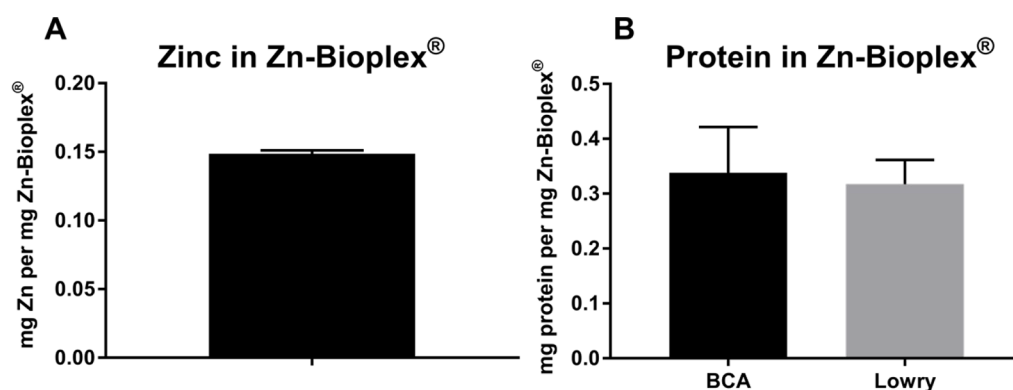
Figure 2. Analytical characterization of the Zn-Bioplex. Values represent mean ± standard deviation ($n = 3$). (A) Total zinc measured by ICP-OES; (B) protein content measured with the BCA and the modified Lowry assay. Statistical analysis showed that the protein quantification did not differ between the BCA and Lowry assay ($p < 0.05$, t test, $n = 6$, six independent measurements with three technical replicates in each measurement).

reported in Figure S6. Normalization of target genes Zinc Transporter 1 (ZnT1), Metallothionein b (MT), and Glutathione reductase (GR) was based on the geometric mean of the reference genes Ubiquitin (Ubi) and Elongation Factor 1 alpha (EF1α). The PCR efficiency of all genes was above 90%. Primer sequences are reported in Table S4. Detailed procedures on RNA extraction, DNase treatment, cDNA synthesis, and qPCR measurement of mRNA levels of target genes have been described previously.[37]

2.7. Statistical Analysis. Statistical analysis was performed using GraphPad Prism Version 8 (GraphPad Software Inc., San Diego, CA). Viability data were calculated as the percentage of the mean fluorescent units of exposed cells by their respective control. The effect concentration 50 (EC_{50}) was calculated by a nonlinear regression sigmoidal dose−response curve fitting module using the Hill-slope equation. EC_{50}'s are presented as the mean plus or minus the standard deviation ($n \geq 3$). All data were assessed for normality using the D'Agostino and Pearson normality test, and where necessary, data were transformed using a base-10 logarithm to improve normality. Analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to determine the statistical significance among the different experimental treatments, and a Dunnett's test was performed when comparing to a control. Values of $p < 0.05$ were considered statistically significant.

3. RESULTS

3.1. Zn-Bioplex Characterization. Zn-Bioplex total zinc concentration was consistent with the information provided by Alltech. Total zinc concentration was 0.148 ± 0.002 mg of Zn per mg of Zn-Bioplex (Figure 2). Total protein was measured at 0.310 ± 0.099 and 0.322 ± 0.039 mg of protein per mg of Zn-Bioplex using the BCA and Lowry assay, respectively. There was no statistical difference between the two methods (t test, $p > 0.05$).

3.2. Zinc Recovery after in Vitro Digestion Protocol. The *in vitro* digestion process resulted in a postfiltration recovery of 48.8 ± 10.04% for ZnSO$_4$ and 43.93 ± 7.07% for Zn-Bioplex (Figure 3A), which was not statistically different (t test; $p > 0.05$). Moreover, the zinc filtration recovery was not dose dependent (one-way ANOVA; $p > 0.05$; Figure S1). This loss of Zn through the digestion process was taken into consideration in the toxicity, bioavailability, and bioreactivity experiments (i.e., reported exposure concentrations were measured). Protein concentration of the filtrate was equal between ZnSO$_4$, Zn-Bioplex, and Zn-free control (one-way ANOVA; $p > 0.05$, Figure 3B).

3.3. Zinc Sulfate and Zn-Bioplex Cytotoxicity. Metabolic activity EC_{50} values for ZnSO$_4$ and Zn-Bioplex
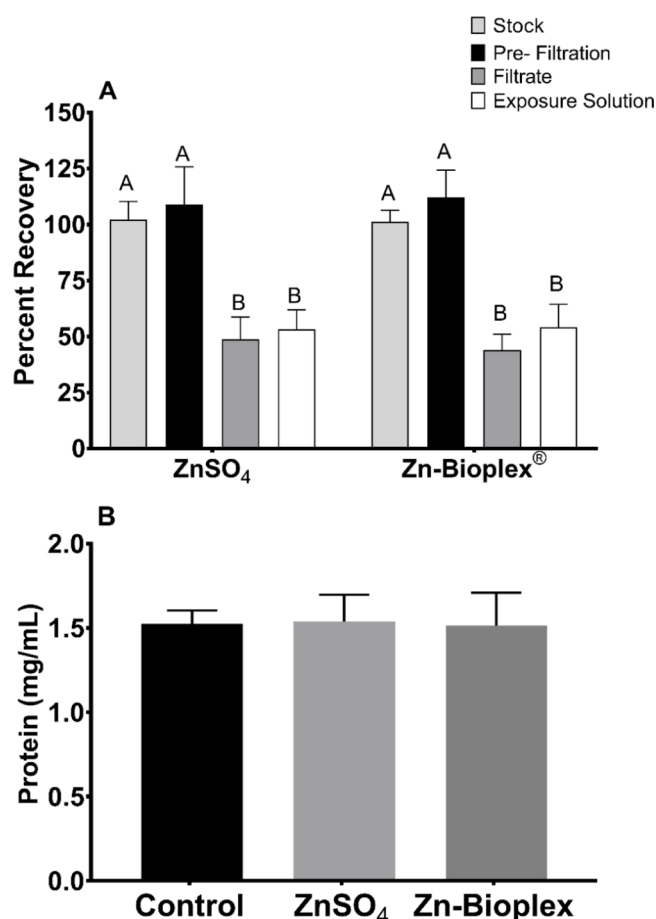
Figure 3. Total zinc recovery (A) and protein content in the filtrate (B). (A) Total zinc recovery in primary stock solution (Stock), after digestion (Pre-Filtration), after filtration, and in the exposure solution. The percentage recovery is calculated by dividing the ICP-OES measured value in the collected fractions (see Figure 1) by the nominal concentration. (B) Protein concentration in the filtrate after *in vitro* digestion and filtration of control medium alone or supplemented with 10 μM of ZnSO$_4$ or Zn-Bioplex. Values are mean ± standard deviation, $n \geq 3$. Columns bearing different lettering are significantly different ($p < 0.05$; two-way ANOVA, Tukey post hoc).

were 19.64 ± 6.68 and 23.93 ± 4.58 μM, respectively. For membrane integrity, the EC_{50} values were 25.04 ± 3.96 and

30.30 ± 2.95 μM for $ZnSO_4$ and Zn-Bioplex, respectively. Although a slight trend for a higher toxicity of $ZnSO_4$ could be observed, the statistical analysis did not show a significant difference between $ZnSO_4$ and Zn-Bioplex (t test, $p < 0.05$; Figure 4; Figure S2). Moreover, RTgutGC cells exposed to 5
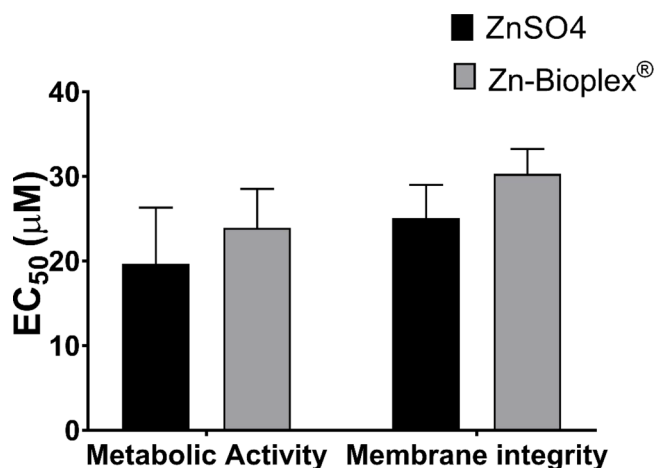
Figure 4. Mean EC_{50} values for $ZnSO_4$ and Zn-Bioplex in RTgutGC. All cells were exposed to digested $ZnSO_4$ and Zn-Bioplex. EC_{50} values were determined using the nonlinear regression sigmoidal dose–response curve fitting module using the Hill slope equation (see Figure S2). Values are mean ± standard deviation. Metabolic activity and membrane integrity were measured using the Alamar Blue and the CFDA-AM assay.[34] No difference between treatments was found ($p < 0.05$, t test, $n = 4$).

μM (measured postdigestion) of $ZnSO_4$ or Zn-Bioplex, used for bioavailability and bioreactivity studies, did not show any toxicity (Figure S3).

3.4. Zinc Sulfate and Zn-Bioplex Bioavailability and Transport. Exposure to $ZnSO_4$ or Zn-Bioplex did not significantly change intracellular zinc concentrations which remained similar to that of control cells (cell kept in luminal buffer without added zinc) for all tested exposure treatments (i.e., ~2.5 and 5 μM of $ZnSO_4$ and Zn-Bioplex) and time points (i.e., 3, 24, and 72 h; one-way ANOVA, $p > 0.05$; Figure 5). However, transport across the RTgutGC epithelium was affected by the dose and time of exposure but not by the zinc compound (two-way ANOVA, $p < 0.05$). The Zn concentration in the basolateral compartment increased over time in cells exposed to both zinc compounds but not in cells exposed to the control condition (i.e., luminal medium without added zinc; two-way ANOVA, $p < 0.05$) indicating that RTgutGC cells actively maintained zinc homeostasis by excreting excess zinc in the basolateral compartment.

Zinc diffused similarly across cell-free transwells independently from the compound type (i.e., ZnSO4 or Zn-Bioplex), reaching equilibrium between the apical and basolateral chamber in 72 h (Figure S4).

Exposures of RTgutGC cells to the undigested compounds ($ZnSO_4$ or Zn-Bioplex), directly dissolved into the luminal buffer, showed that zinc in the Zn-Bioplex was more bioavailable than $ZnSO_4$ and that more zinc was transported through the RTgutGC epithelial layer than for undigested $ZnSO_4$ (Figure 6).

3.5. Messenger RNA levels of MT, ZnT1, and GR. The gene expression data are represented in Figure 7 and Figure S6. Metallothionein mRNA levels were higher in cells exposed to
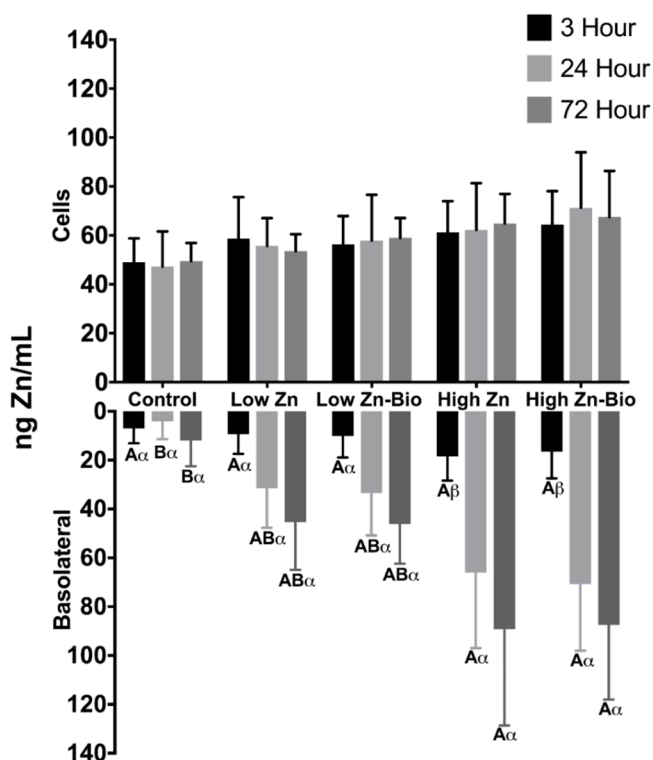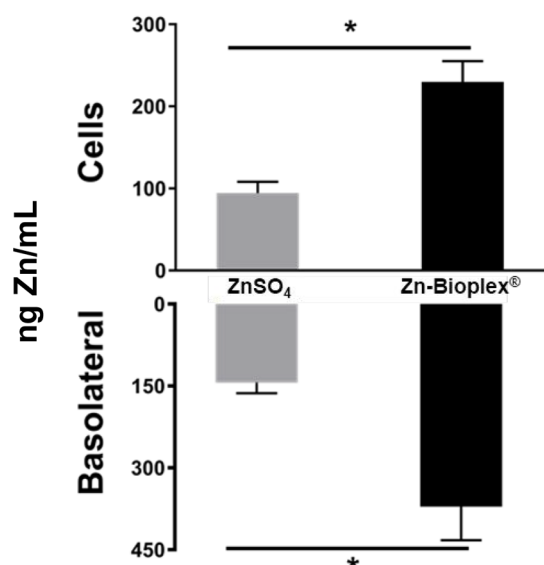

Figure 5. Metal bioavailability and transport. Zinc concentration in the cells (top graph) and the basolateral compartment (bottom graph). X axis represents treatments (Zn = $ZnSO_4$ and Zn-Bio = Zn-Bioplex; low = 2.5 μM and high = 5 μM; measured values are shown in Table S1). Bar values are mean ± standard deviation. Control is digested medium without $ZnSO_4$ or Zn-Bioplex. Bars bearing different lettering are statistically different between treatments. Bars bearing a different Greek letter show a difference between time points within the same treatment. Treatment and time effects were analyzed by using two-way ANOVA and time ($p < 0.05$, Tukey post hoc, $n = 3$; three independent experiments, see Figure S5 for individual data sets). There was no difference among treatments or time points in intracellular zinc.

$ZnSO_4$ or Zn-Bioplex than respective controls. Moreover, MT expression in cells exposed to $ZnSO_4$ was greater than in cells exposed to Zn-Bioplex (one-way ANOVA; $p < 0.05$; Tukey post hoc). Similarly, ZnT1 mRNA levels were greater in cells exposed to $ZnSO_4$, in comparison to cells exposed to Zn-Bioplex (one-way ANOVA; $p < 0.05$; Tukey post hoc). Glutathione reductase mRNA levels did not differ among any of the conditions tested (one-way ANOVA; $p < 0.05$).

4. DISCUSSION

This paper reports the development of a static *in vitro* fish digestion protocol that can be used in combination with an *in vitro* model of the fish intestinal epithelium based on the RTgutGC cell line.[12] This combined approach allowed us to study the role of the digestion processes on the bioavailability of an inorganic $ZnSO_4$ and an organic Zn-Bioplex zinc supplement. To the best of our knowledge, this study is the first to employ an animal-free *in vitro* system utilizing commercially available digestive enzymes and adapted buffers and a fish intestinal cell line to study bioavailability, toxicity, and transport across the intestinal epithelium of inorganic and organic zinc compounds.

Figure 6. Undigested $ZnSO_4$ and Zn-Bioplex bioavailability and transport. Measurements were taken after 24 h of exposure to 10 μM of $ZnSO_4$ and Zn-Bioplex. Values are the mean ± standard deviation (n = 3). Statistical significance was calculated between treatments within the cell layer and basolateral chamber. $ZnSO_4$ was found to be less bioavailable and transported less efficiently than Zn-Bioplex (t test, $p < 0.05$).
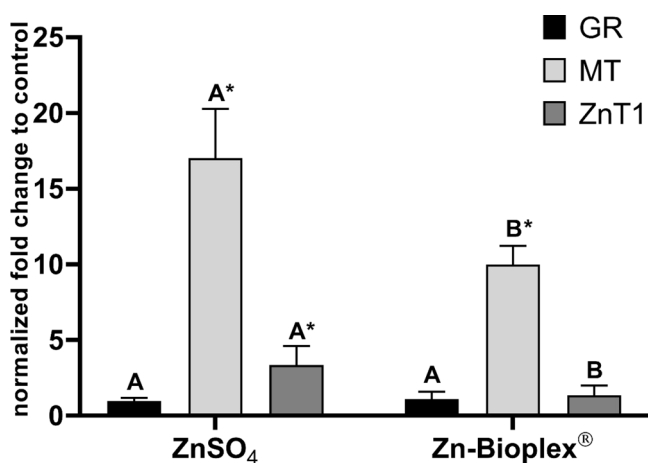
Figure 7. Normalized mRNA levels of MT, GR, and ZnT1. Values are mean ± SD ($n \geq 3$). Target gene normalized expression is reported as the ratio of the expression in cells exposed to a control medium (reported in Figure S6). Bars bearing different letters are statistically different between treatments (one-way ANOVA, Tukey post hoc; $p < 0.05$). Differences from the respective control are represented with an asterisk (one-way ANOVA, Dunnett post hoc; $p < 0.05$).

While it is debatable if the use of commercially available mammalian digestive enzymes is appropriate to study the role of digestion on nutrient bioavailability in fish,[2] for instance, the use of mammalian enzymes demands temperatures that are not relevant to fish (i.e., 37 °C), our choice was driven by the need of an animal-free method as a more ethical, repeatable, and less expensive method for prescreening of nutrients developed for aquaculture. Moreover, there is evidence that the use of digestive extracts from fish represent a more physiologically relevant approach.[3,38] However, this approach brings also some limitations, including experimental variability due to different enzymatic compositions of different fish extracts, difficulty to

characterize the exact composition of the extracts, and use of fish which ultimately will increases the cost of the overall experiment, reduces the throughput, and represents a less ethical approach.

The *in vitro* fish digestion protocol shown here mimics the gastrointestinal digestive processes in fish and allows tight control of the composition of the luminal content. Moreover, the use of RTgutGC cells allows the measurement of zinc bioavailability and toxicity threshold. However, one other important limitation of this protocol is the need to remove digestive enzymes by filtration from the digesta (i.e., the compound digested in the gastric and intestinal steps). The removal of the digestive enzymes is necessary because it was shown that unfiltered digesta can disrupt the RTgutGC cells epithelium integrity and viability (data not shown). This filtration step is incompatible with uptake or bioavailability studies of particulate matter and/or molecular complexes bigger than 10 kDa. In this study, after the filtration process, about 50% of the zinc was lost, likely due to complexation with peptide complexes bigger than 10 kDa. The loss of zinc was not compound specific (i.e., $ZnSO_4$ and Zn-Bioplex showed the same loss of zinc), indicating that the complexation occurs with components of the gastric or intestinal phases and was not affected by chelation of zinc by peptides (Figure 3). In addition, it was established that Bioplex chelates are ranging between 0.5 and 5 kDa in size.[39] This effect can be explained by the high affinity of Zn^{2+} for proteins, especially cysteine rich proteins.[24] Thus, the proteins in the pepsin, porcine bile and pancreatin mix added in the gastric and intestinal phases could bind free Zn^{2+} in the medium, preventing the Zn from passing freely through the 10 kDa filter. Measuring zinc after each key step of the *in vitro* digestion protocol (Figure 1) allowed us to evaluate this effect and to account for it by adjusting the nominal concentration to the actual measured zinc concentration in the filtrate.

Previous studies have shown that methionine chelated Zn has higher bioavailability than $ZnSO_4$ in RTgutGC cells[17] and that amino acidic chelation does increase bioavailability of zinc in rainbow trout[40] but not in Atlantic salmon (*Salmo salar*)[18] *in vivo*. Moreover, inorganic (i.e., $ZnSO_4$) and organic (i.e., Zn-Bioplex) zinc were shown to equally improve the quality of semen in rainbow trout[41] and to be equally bioavailable in rainbow trout and common carp (*Cyprinus carpio*).[42,43] Similarly, exposures to the digested compounds showed that $ZnSO_4$ or Zn-Bioplex are equally bioavailable in RTgutGC. However, direct exposure to undigested $ZnSO_4$ or Zn-Bioplex showed that the latter was about twice as bioavailable. We could not explain this effect, but a possible explanation is that *in vitro* digestion could displace zinc from the Bioplex peptides which thus reduces the Bioplex efficacy in increasing bioavailability. Alternately, $ZnSO_4$ is completely dissolved in the luminal buffer (Figure S7), whereas the Zn-Bioplex compound appeared as a suspension and tended to precipitate out of solution, which might affect cellular uptake (Oldham personal observation). Addressing this question will require further studies. It should be noted that zinc speciation calculations are only possible in the luminal buffer before *in vitro* digestion. The complex nature of the gastric and intestinal phases would make Visual MINTEQ calculations impossible.

The RTgutGC epithelium is an effective barrier between the apical and basolateral chambers.[12] For instance, while zinc concentration within RTgutGC cells did not change after exposure to $ZnSO_4$ or Zn-Bioplex, excretion of zinc in the

basolateral chamber increased in a time- and dose-dependent manner, showing the ability to maintain intracellular zinc homeostasis. The maintenance of zinc intracellular concentration as well as the dose- and time-dependent increase of zinc excretion in the basolateral chamber demonstrates a tight control of the essential metal zinc through the epithelial layer. This effect is consistent with previous results obtained in the trout intestinal epithelium *in vivo*[44] and supports the sustainability of the RTgutGC intestinal model to study metal homeostasis in fish.[45]

One remarkable difference between $ZnSO_4$ and Zn-Bioplex exposure is that although the cells accumulated identical amounts of total zinc intracellularly, $ZnSO_4$ induced a higher metallothionein and ZnT1 gene expression than Zn-Bioplex. This effect suggests that zinc enters the cells complexed differently after exposure to $ZnSO_4$ and Zn-Bioplex, and the former is more bioreactive (i.e., induces a higher transcriptional response). Thus, these data show that $ZnSO_4$ induces a higher concentration of intracellular labile zinc (i.e., Zn^{2+}) than Zn-Bioplex, which in turns triggers an upregulation of MT and ZnT1 via the metal transcription factor 1 (MTF1).[46] Moreover, this effect could explain the slightly higher toxicity of $ZnSO_4$ and suggests that Zn-Bioplex provides a slower release of zinc intracellularly. This lower bioreactivity (i.e., lower gene expression) may prove to be beneficial for fish health and growth rate as Zn-Bioplex provides an equal zinc supply to the organism but a reduced intracellular metal detoxification response which is energetically costly and can inhibit fish growth.[47,48]

We have developed a method simulating the fish gastro-intestinal digestion and nutrient bioavailability in rainbow trout (*Oncorhynchus mykiss*) intestines *in vitro*. A similar model has not been reported before. This model has potential to be used for the testing of the toxicity, bioavailability, and bioreactivity of several more ingredients and/or compounds. Our data showed that, following *in vitro* digestion, zinc organic complexes such as Zn-Bioplex accumulated in the RTgutGC cells and were transported across the intestinal epithelium in a similar manner to inorganic salts (i.e., $ZnSO_4$). However, Zn-Bioplex was less bioreactive, suggesting that it may be a superior supplement for fish health. Further studies will be necessary to evaluate the impact of Zn-Bioplex on fish growth and general health *in vivo*. Lastly, this *in vitro* model allows the study of several conditions in high throughput, using small volumes, making a less expensive and mechanistically valid model. This approach shows that a standardized *in vitro* model can be used to fine-tune feed formulations, lowering the cost of feed development for the aquaculture industry. Moreover, this method does not require the use of fish, thus representing a more ethical approach to study nutrient and toxicant bioavailability and toxicity.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.2c00307.

Zinc concentration in the exposure solutions; primer sequences for qPCR; zinc recovery analysis; dose–response curves of $ZnSO_4$ and Zn-Bioplex; RTgutGC cells' viability during exposure to 5 μM of $ZnSO_4$ or Zn-Bioplex; zinc transport across the empty (cell-free) transwell; zinc bioavailability and transport shown in three independent experiments; qPCR shown as absolute copy numbers; zinc speciation in the luminal buffer (PDF)

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Notes

The authors declare no competing financial interest.

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