Effects of different ligands on the bioaccumulation and subsequent depuration of dietary Cu and Zn in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract: The effects of different ligands on the bioavailability of dietary copper (Cu) and zinc (Zn) to fish have not been thoroughly investigated. We therefore exposed juvenile rainbow trout (*Oncorhynchus mykiss*; ~200 mg body weight) to control food or to food supplemented with different Cu (~400 $\mu g \cdot g^{-1}$ food) or Zn (~1000 $\mu g \cdot g^{-1}$ food) compounds. Tissue metal accumulation was compared among groups. Fish fed CuO showed no differences in tissue Cu concentrations relative to control fish, suggesting that Cu was not readily available for uptake in this form. In contrast, Cu in the form of $CuSO_4$, Cu-proteinate, or Cu-lysine was much more available for uptake, resulting in substantial increases in liver, gut tissue, and whole-body Cu concentrations during the loading phase and decreases during depuration, although liver and whole-body levels remained elevated after 2 weeks. We found no differences in tissue Cu accumulation among these three complexes. There were no effects on growth. For Zn, we found no differences among any of the treatments, including controls, in Zn accumulation or growth. Overall, there was homeostasis of whole-body and tissue-specific Zn concentrations despite the large differences in dietary Zn loads.

Résumé : Les effets de plusieurs ligands sur la biodisponibilité du le cuivre (Cu) et du le zinc (Zn) alimentaires chez les poissons ne sont pas entièrement connus. Nous avons donc exposé des jeunes truites arc-en-ciel (*Oncorhynchus mykiss*; ~200 mg de masse corporelle) à un régime alimentaire témoin ou à de la nourriture additionnée de divers composés de Cu (~400 $\mu g \cdot g^{-1}$ de nourriture) ou de Zn (~1000 $\mu g \cdot g^{-1}$ de nourriture). Nous avons ensuite comparé l'accumulation des métaux dans les tissus de chacun des groupes. Les poissons nourris de CuO ont des concentrations de Cu dans leurs tissus qui ne diffèrent pas de celles des poissons témoins, ce qui indique que le Cu n'est pas facilement assimilable sous cette forme. En revanche, le Cu sous forme de $CuSO_4$, de protéinate de Cu ou de complexe Cu-lysine est beaucoup plus assimilable; il en résulte des accroissements substantiels des concentrations de Cu dans le foie, les tissus du tube digestif et dans le corps entier durant la phase d'accumulation, ainsi que des déclins lors de l'élimination, bien que les concentrations dans le foie et le corps entier demeurent élevées après deux semaines. Il n'y a pas de différence dans les accumulations tissulaires entre ces trois complexes, ni d'effet sur la croissance. Dans le cas du Zn, il n'y a pas de différence entre les traitements expérimentaux, y compris les témoins, en ce qui a trait à l'accumulation de Zn ou à la croissance. Somme toute, il y a une homéostasie des concentrations de Zn à l'échelle du corps entier et des différents tissus, malgré les grandes différences dans les apports alimentaires de Zn.

[Traduit par la Rédaction]

Introduction

The transition metals copper (Cu) and zinc (Zn) are two of the three most abundant nutritive metals (Bury et al. 2003), and both are required in trace amounts by most organisms to maintain cellular functions. In humans, Cu has been linked to cellular respiration, free radical defense, synthesis of melanin, connective tissue biosynthesis, and cellular iron metabolism (for review, see Uauy et al. 1998). In fish, Cu deficiency can retard growth (Kamunde et al. 2002), and excess dietary Cu can also lead to reduced growth (e.g., Berntssen et al. 1999*b*; Kamunde et al. 2002), increased cellular apoptosis (e.g., Berntssen et al. 1999*a*), and death (e.g., Erickson et al. 1996). Consequently, Cu homeostasis tends to be tightly regulated in most animals (for review, see Clearwater et al. 2002; Bury et al. 2003).

Although fish can tolerate relatively high levels of Zn with no obvious adverse effects, it may become toxic at high

Received 15 December 2004. Accepted 27 October 2005. Published on the NRC Research Press Web site at http://cjfas.nrc.ca on 14 January 2006.
J18460

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 doi:10.1139/F05-230

levels as a result of interference with calcium homeostasis (Hogstrand and Wood 1996). Moreover, Zn deficiency has been shown to decrease growth and survival of various fish species; like Cu, therefore, Zn is thought to fall under tight homeostatic control (Hogstrand and Wood 1996). Previous research on mammals and birds has suggested that the main factor influencing the bioavailability, and therefore accumulation, of food-borne Cu and Zn may be diet composition; however, little is known about the subsequent depuration of Cu and Zn originally derived from different compounds (for review, see Clearwater et al. 2002; Bury et al. 2003).

Research on rats has shown that complexation to amino acids may make Cu more available for uptake, whereas the presence of competing ligands (e.g., Zn) or the complexation to nonsoluble dietary constituents such as fiber can make it less available (for review, see Du et al. 1996). In calves, $CuSO_4$ was highly available for uptake, whereas CuO was a very poor source of dietary Cu (Xin et al. 1991). Similarly in heifers, $CuCO_3$ was considered to be less bioavailable than $CuSO_4$ (Ward et al. 1996). In chicks, a Cu-lysine complex was found to be relatively more bioavailable than $CuSO_4$ (Guo et al. 2001), and in rats, Cu-lysine and a Cu-proteinate complex were both more available than $CuSO_4$ (Du et al. 1996).

While Cu supplements have been well studied in livestock and laboratory mammals, research on fish remains relatively sparse. It is generally assumed that metals may be biologically incorporated into prey organisms of natural fish diets in forms that are more easily absorbed by fish (e.g., bound to proteins or other organic molecules), whereas most laboratory-prepared diets are supplemented with metals in an inorganic form (e.g., $CuSO_4$; Clearwater et al. 2002). Woodward et al. (1995) concluded that for both fish and invertebrates, ionic metals are not absorbed as efficiently and may not be as toxic as metals bound to proteins. Commercial fish feed is often supplemented with Cu, but to our knowledge, the relative bioavailability of different Cu supplements to fish has received little attention. Clearwater et al. (2002) compared a number of studies that investigated the bioavailability and toxicity of dietary Cu to fish. However, the majority of studies in that review used commercial fish feed supplemented only with $CuSO_4$; thus, the relative availability of Cu when bound to other complexes remains unclear.

In the absence of excess Zn in the natural aquatic environment, dietary Zn is the dominant route of exposure for teleosts, and Zn homeostasis is tightly controlled (for review, see Bury et al. 2003). However, the bioavailability of dietary Zn is considered to be quite low (Maage et al. 2001) and is reported to be influenced by the protein source in the food, the diet type, and chemical form in which Zn occurs in the diet (Apines et al. 2001).

Zn as $ZnSO_4$ was found to be only approximately one-third as available as Zn-methionine to juvenile disk abalone (*Haliotis discus hannai*; Tan and Mai 2001) and channel catfish (*Ictalurus punctatus*; Paripatananont and Lovell 1995), although Li and Robinson (1996) concluded that the bioavailabilities of $ZnSO_4$ and Zn-methionine were similar in the latter species when incorporated into a typical practical diet. Bioavailabilities of these two compounds were also similar for rainbow trout (*Oncorhynchus mykiss*; Apines et al. 2001). In a review of the subject, Clearwater et al. (2002) concluded that the relative bioavailability of different Zn compounds to fish appears to vary from species to species.

From the standpoint of animal husbandry and aquaculture, it is of interest to determine which type(s) of Cu and Zn compounds to use to promote optimum growth and development while at the same time keeping costs at a minimum. If the use of certain compounds reduces the amount of feed required, this can lead to reductions in aquatic pollution associated with aquaculture facilities (Tan and Mai 2001). Similarly, from the standpoint of environmental toxicology (Clearwater et al. 2002), it is of interest to determine how the form of Cu or Zn in a contaminated diet affects its bioavailability, and therefore its contribution to body metal burdens, as well as its subsequent depuration.

The purpose of this study was to evaluate the effects of different ligands on the relative bioavailability of dietary Cu and Zn to juvenile salmonids. We exposed rainbow trout fry to control food or to one of eight different kinds of food, each supplemented with a different Cu or Zn compound (CuO, $CuSO_4$, Cu-proteinate, Cu-lysine, ZnO, $ZnSO_4$, Zn-proteinate, or Zn-methionine). Cu and Zn concentrations were then measured in various tissues over several weeks of dietary loading and over several weeks of depuration. In light of a potential complication with the first Cu-feeding trial (see Discussion), we conducted a second experiment to verify the results of the first.

Methods

Experiment 1: Cu and Zn (short-term loading and depuration)

Fish acclimation

Juvenile rainbow trout (mean weight ~200 mg) were obtained from a local trout farm and were allowed to acclimate to laboratory conditions for 1 week. During this time, fish were equally distributed among nine circular polyethylene tanks, with a total of 160 fish per tank. Each tank was supplied with a continuous flow of dechlorinated municipal (Hamilton, Ontario, Canada) tap water (composition: 0.6 $mmol \cdot L^{-1}$ Na^+, 0.7 $mmol \cdot L^{-1}$ Cl^-, 1.0 $mmol \cdot L^{-1}$ Ca^{2+}; hardness = 140 $mg \cdot L^{-1}$ as $CaCO_3$, alkalinity = 95 $mg \cdot L^{-1}$ as $CaCO_3$, and dissolved organic C = 3.0 $mg \cdot L^{-1}$). Natural background levels of Cu and Zn in the water were approximately 3 $\mu g \cdot L^{-1}$ and 20 $\mu g \cdot L^{-1}$, respectively. All tanks were individually aerated with airstones. Water temperature averaged 12.5 °C during the study. A 12 h light cycle was established, and all treatment groups were fed an excess of trout starter feed (Corey Feed Mills Ltd., Fredericton, New Brunswick, Canada; [Cu] = 4.6 $\mu g \cdot g^{-1}$; [Zn] = 91.3 $\mu g \cdot g^{-1}$) for 1 week using 12 h automated feeders. Ration was not monitored during this acclimation period. Tanks were siphoned daily, prior to the start of daily feeding. All mortalities were recorded.

Diet preparation

Diets consisted of trout starter feed alone (controls) or trout starter feed supplemented with one of eight different Cu or Zn compounds: copper sulfate ($CuSO_4 \cdot 5H2O$; Fisher

Scientific, Toronto, Ontario, Canada), copper oxide (CuO; Aldrich Chemical Co., Milwaukee, Wisconsin, USA), copper proteinate (Cu-proteinate; Bioplex® copper proteinate, Alltech Inc., Lexington, Kentucky, USA), copper lysine (Cu-lysine; Cuplex 100, Zinpro Corporation, Eden Prairie, Minnesota, USA), zinc sulfate ($ZnSO_4 \cdot 7H_2O$; Fisher Scientific), zinc oxide (ZnO; Aldrich Chemical Co.), zinc proteinate (Zn-proteinate, Bioplex® zinc proteinate, Alltech Inc.), or zinc methionine (ZnMeth; Zinpro 180, Zinpro Corporation) to elevate metal concentrations. All diets were processed as follows: 110 mL of deionized water (Sybron Barnstead, Boston, Massachusetts, USA) with predissolved metal complex (or with no metal supplement in the control group) were added to 250 g of starter feed and mixed thoroughly in a commercial pasta maker. Food was then extruded in small pellets, dried at 60 °C for 1 h, and allowed to cool to room temperature prior to being crushed into fine powder by hand. Diets were stored at 4 °C until use. In total, 250 g of each of the metal diets and 750 g of the control diet were prepared. The target Cu concentration of each Cu-supplemented diet was 400 μg $Cu \cdot g^{-1}$ food; actual measured concentrations of Cu (in μg $Cu \cdot g^{-1}$ food ± standard error of the mean, SEM) in the experimental diets were as follows: control: 4.6 (±0.04, $n = 3$), CuO: 292.8 (±2.0, $n = 3$), $CuSO_4$: 323.4 (±1.6, $n = 3$), Cu-proteinate: 312.7 (±7.2, $n = 3$), Cu-lysine: 359.1 (±4.9, $n = 3$). The target Zn concentration of each Zn-supplemented diet was 1000 μg $Zn \cdot g^{-1}$ food; actual measured Zn concentrations (μg $Zn \cdot g^{-1}$ food) were as follows: control: 91.3 (±2.2, $n = 3$), ZnO: 771.9 (±4.4, $n = 3$), $ZnSO_4$: 1035.8 (±16.5, $n = 3$), Zn-proteinate: 653.3 (±15.3, $n = 3$), ZnMeth: 988.7 (±1.8, $n = 2$).

Sampling and feeding

Sampling occurred every third day. Eighteen fish were randomly selected from all treatments on day 0; 12 fish were sampled from each treatment on days 3, 6, and 9; and approximately 10 fish were sampled from each treatment on all remaining sampling days through to the end of the experiment. Fish were starved for a minimum of 24 h prior to being sacrificed by an overdose of tricaine methanesulfonate (Syndel Laboratories Ltd., Vancouver, British Columbia) at 1 $g \cdot L^{-1}$. To increase the analytical accuracy for these very small fish, two to four individuals were pooled together, and carcass weights, tissue weights, and all subsequent analyses were recorded per pooled group ($N = 3$–4 pools per treatment group).

After sampling on day 0, all groups were switched from the control diet to their respective metal-supplemented diets. Diets were fed for 2 weeks at 8% body weight per day using an automatic feeder that delivered the daily ration to each tank in 12 equal amounts administered hourly. At the end of the metal-loading phase, Cu (or Zn) depuration was initiated by switching all metal groups back to the control diet. Depuration continued for 2 weeks at the same sampling schedule and the same ration as during the loading phase. As no food was found remaining in the tanks several hours after feeding, we assumed that all food was consumed.

The following tissues were sampled for Cu (or Zn) concentration and growth rate: liver, gut (i.e., the entire gastrointestinal tract with the intestinal and stomach contents removed), and remaining carcass. Livers were only sampled on days 15–30 (i.e., on the final day of the loading phase and during the depuration phase). At earlier times, the liver was too small to accurately remove. Tissues were digested in 1 $mol \cdot L^{-1}$ nitric acid overnight at 55–60 °C. Samples were allowed to cool to room temperature prior to being vortexed and centrifuged. Duplicate samples were then diluted in 1% nitric acid for analysis on a graphite furnace atomic absorption spectrophotometer (Varian AA-1275 with GTA-95 graphite tube atomizer, Mulgrave, Australia) using Fisher-certified standards. Accuracy and precision of the analyses were controlled by three separate internal controls consisting of 0.5 g of three different diets digested along with the unknown samples.

Experiment 2: Cu (long-term uptake and depuration)

In this experiment, which was conducted to corroborate results from Experiment 1, we evaluated only Cu uptake and depuration. The source of fish, water chemistry, experimental design, feeding method, and basic setup were similar to those of Experiment 1, with the following exceptions. Fish were larger at the onset of the experiment (~1.2 g), and they were allowed to acclimate to laboratory conditions for 2 weeks, during which they were fed a 4% ration of control food. Tanks were exposed to natural daylight cycles for June, July, and August, and tank water was maintained around 15.9 °C through the use of a recirculating chiller system supplemented with municipal water.

The same four Cu complexes were used for the diets, in addition to a control. Target [Cu] in the diets was again 400 μg $\cdot g^{-1}$, and actual measured concentrations (in μg $Cu \cdot g^{-1}$ food ± SEM) were as follows: control: 23.4 (±0.4, $n = 3$), CuO: 411.9 (±9.1, $n = 3$), $CuSO_4$: 401.4 (±5.6, $n = 3$), Cu-proteinate: 403.6 (±0.2, $n = 3$), and Cu-lysine 379.8 (±5.9, $n = 3$). In this experiment, the Cu-loading phase lasted for 4 weeks at a 4% ration, followed by a 2-week depuration period during which fish were fed control food at a 4% ration. Sampling occurred twice weekly, and tissues were collected and processed in the same manner as described in Experiment 1, except that in Experiment 2, livers were collected and analyzed for all sampling days. Also, because fish used in this experiment were larger, there was no need to pool samples.

Calculations

In both experiments, whole-body Cu (or Zn) concentrations were calculated by summing all tissue measurements on a weight-proportional basis. Specific growth rates (% per day) were calculated as the slope of a regression of natural logarithm of body mass on time (days) using data from all sampled fish at all times (typically $N \sim 130$) in each treatment. Mean apparent relative bioavailability (%) of Cu or Zn was calculated as $100 \times [(M_e - M_s)/M_t]$, where M_e and M_s are the absolute amount of metal (μg) per fish at the end and at the start of a given time period, respectively, and M_t is the total amount of metal (μg) fed during that same time.

Statistical analyses

Data are presented as means ± 1 SEM (N), where N is the number of pooled samples or individual samples, as appropriate. Within each experiment, tissue [Cu] (or [Zn]) and body mass on any given sampling day were compared among groups

with an analysis of variance (ANOVA) or a Kruskal–Wallis test, depending on whether the data met the conditions of a Bartlett's test for equal variance. Note that different treatments were run in different tanks, and ANOVAs were only performed within discrete sampling days. Thus, the individual samples were independent, but we cannot eliminate the possibility that the responses were influenced by tank effects, because treatment tanks were not replicated. ANOVA results (*F* values, *P* values) for each tissue type are given as ranges spanning all sampling days. Overall specific growth rates were similarly compared by ANOVA. Differences among means were tested with a Tukey's honestly significant difference test or a nonparametric comparison of means test, as appropriate. All statistics were conducted with Statistix for Windows (Analytical Software, Tallahassee, Florida), and the alpha value was set at 0.05.

Results

Within each experiment, we found few significant differences in body mass among fish on any given sampling day (data not shown), and within each experiment, there were no significant differences in overall growth rates among treatments, indicating a lack of stimulatory or inhibitory effect of this level of dietary Cu or Zn supplementation (Table 1).

Table 1. Mean whole-body specific growth rates (%·day^{-1} ± 1 standard error of the mean) of fish fed diets supplemented with different Cu or Zn complexes.

Treatment	Experiment 1	Experiment 2
Control	6.7±0.1	3.6±0.2
CuO	6.4±0.3	4.2±0.4
$CuSO_4$	6.6±0.2	3.1±0.4
Cu-proteinate	6.2±0.3	3.1±0.3
Cu-lysine	6.1±0.3	3.4±0.2
ZnO	6.6±0.3	—
$ZnSO_4$	6.3±0.4	—
Zn-proteinate	6.4±0.3	—
Zn-methionine	6.4±0.3	—

Note: Specific growth rate was calculated as the slope of a regression of natural logarithm of body mass on time (days) for all fish at all sample times in a treatment (N = 130). There were no significant differences ($P \geq 0.05$) among treatments within experiments.

In Experiment 1, [Cu] was highest in the liver, intermediate in the gut tissue, and lowest in the whole body, a pattern that was maintained in all groups throughout the experiment (Fig. 1). Fish in the CuO group did not differ from control fish at any time in any of the sampled tissues. In contrast, fish supplemented with $CuSO_4$, Cu-proteinate, or Cu-lysine typically had higher tissue [Cu] than controls and CuO-fed fish throughout most of the experiment (whole-body: df = 4, *F* range = 3.27–67.13, *P* range = 0.000–0.041, Fig. 1*a*; liver: df = 4, *F* range = 3.30–11.05, *P* range = 0.0004–0.066, Fig. 1*b*; gut: df = 4, *F* range = 0.47–36.74, *P* range = 0.0000–0.744, Fig. 1*c*). During the Cu-loading phase (days 0–15), Cu-proteinate, $CuSO_4$, and Cu-lysine groups showed substantial increases in tissue [Cu], which in turn decreased during the depuration phase (days 16–30). While there were some significant differences among groups at specific times (Figs. 1*a*–1*c*), overall the patterns were very similar in the Cu-proteinate, Cu-lysine, and $CuSO_4$ treatments during both loading and depuration. In contrast, the control and CuO groups showed no remarkable increases; their tissue [Cu] remained relatively stable throughout the experiment. At the end of the loading period (day 15), whole-body and liver [Cu] were approximately doubled in the Cu-proteinate, Cu-lysine, and $CuSO_4$ groups relative to the controls, whereas gut tissue [Cu] was approximately tripled. At the end of the depuration period (days 21–30), whole-body [Cu] (Fig. 1*a*) and liver [Cu] (Fig. 1*b*) remained elevated in these treatments relative to controls and CuO, whereas gut [Cu] did not differ among the five groups (Fig. 1*c*).

In Experiment 2, whole-body [Cu] quickly attained the bimodal pattern during the loading phase that characterized whole-body [Cu] in Experiment 1 (i.e., control and CuO fish had consistently lower values than the other three groups) (Fig. 2*a*); this pattern persisted through the depuration phase (whole-body: df = 4, *F* range = 1.25–65.07, *P* range = 0.0000–0.299). The same pattern was mirrored in liver [Cu], although it took an extra week for the two peaks to differentiate during the loading phase (Fig. 2*b*; liver: df = 4, *F* range = 1.16–35.5, *P* range = 0.0000–0.337). Here as well, liver [Cu] in the $CuSO_4$, Cu-proteinate, and Cu-lysine treatments did not return to control levels by the end of the depuration period. Gut [Cu] also showed significant increases in the $CuSO_4$, Cu-proteinate, and Cu-lysine treatments relative to control and CuO fish during the loading phase (Fig. 2*c*), but in this compartment, Cu levels returned to control levels by the end of the depuration period (gut: df = 4, *F* range = 0.86–8.62, *P* range = 0.0000–0.49). Overall, in terms of the relative magnitude and time course of the response, the results were very similar to those of Experiment 1.

Although the values differed between Experiments 1 and 2, the same trends in relative Cu bioavailability were seen during the loading phase (i.e., bioavailability was highest in the control group, lowest in the CuO group, and similar in the other three treatments). During depuration in Experiment 1, Cu bioavailability increased in all treatments, becoming greatest in the control group and lowest in the Cu-lysine treatment. In Experiment 2, bioavailability remained unchanged in the control group, increased in the CuO and Cu-lysine treatments, and became negative in the $CuSO_4$ and Cu-proteinate treatments (Table 2).

In contrast with [Cu], [Zn] was similar in the three sampled tissue compartments and was at most twofold higher in the liver at the end of the loading phase in all treatments (Fig. 3). Also in contrast with Cu, where we observed consistent significant differences between the CuO complex and control treatments versus all other Cu compounds (cf. Figs. 1, 2), no such striking differences were evident among the different Zn compounds. Surprisingly, dietary Zn supplementation in a variety of forms had negligible effects on tissue or whole-body Zn burdens. Moreover, in the whole body (Fig. 3*a*; df = 4, *F* range = 0.28–8.51, *P* range: 0.0009–0.888) and gut (Fig. 3*c*; df = 4, *F* range = 0.89–10.94; *P* range = 0.0002–0.495), no differences were apparent between the loading and the depuration phases in any of the treatments, although the liver showed a trend towards de-

Fig. 1. Mean (+1 standard error of the mean) (*a*) whole-body [Cu], (*b*) liver [Cu], and (*c*) gut [Cu] of juvenile rainbow trout (*Oncorhynchus mykiss*) in Experiment 1. Fish were fed Cu-supplemented diets during the loading phase and control diet during the depuration phase at a ration of 8% body weight·day^{-1}. For each sampling day, different letters above bars indicate significant differences ($P < 0.05$) among groups. Comparisons were not made between sampling days.

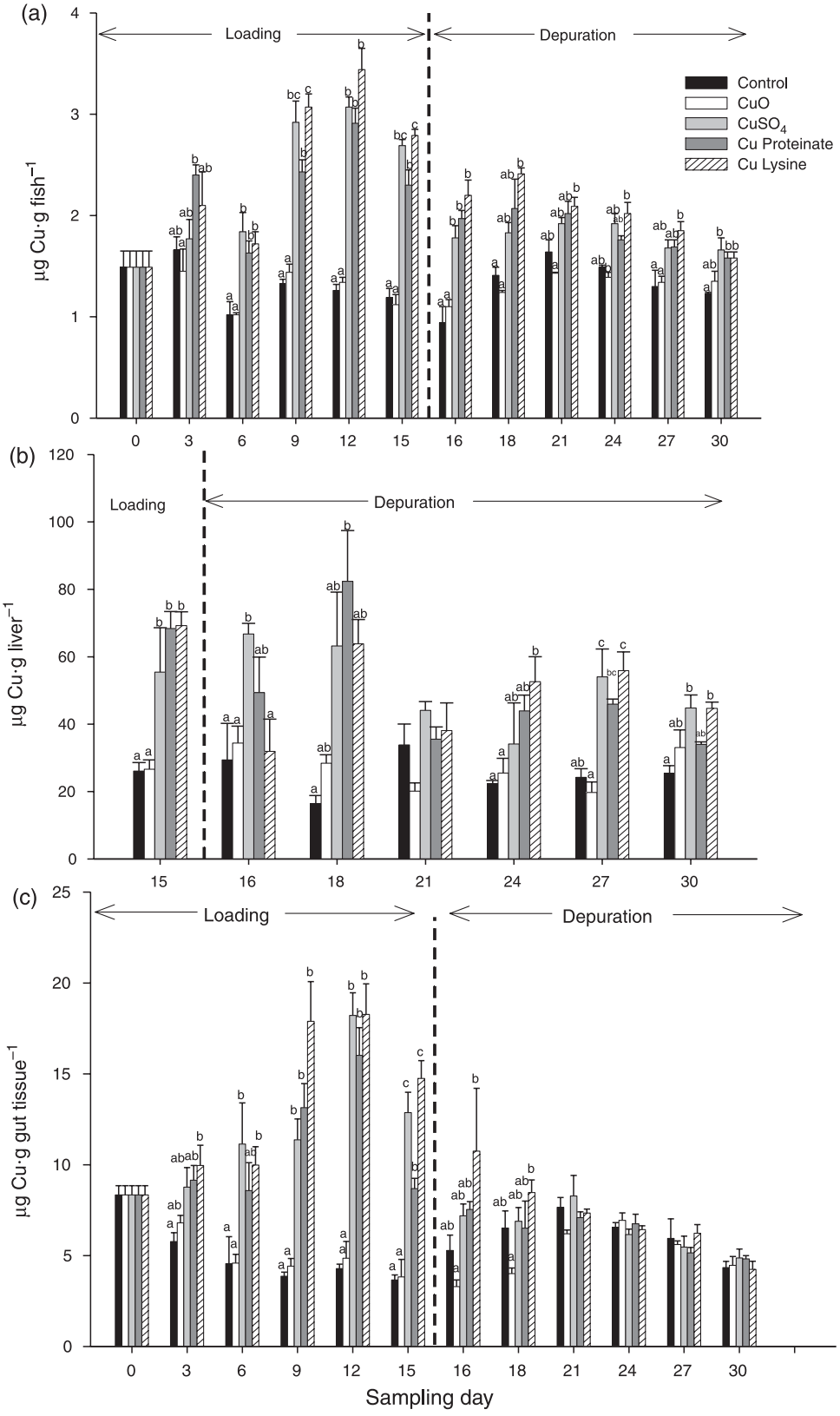

Fig. 2. Mean (+1 standard error of the mean) (*a*) whole-body [Cu], (*b*) liver [Cu], and (*c*) gut [Cu] of juvenile rainbow trout (*Oncorhynchus mykiss*) in Experiment 2. Fish were fed Cu-supplemented diets during the loading phase and control diets during the depuration phase at a ration of 4% body weight·day^{-1}. For each sampling day, different letters above bars indicate significant differences ($P < 0.05$) among groups. Comparisons were not made between sampling days.

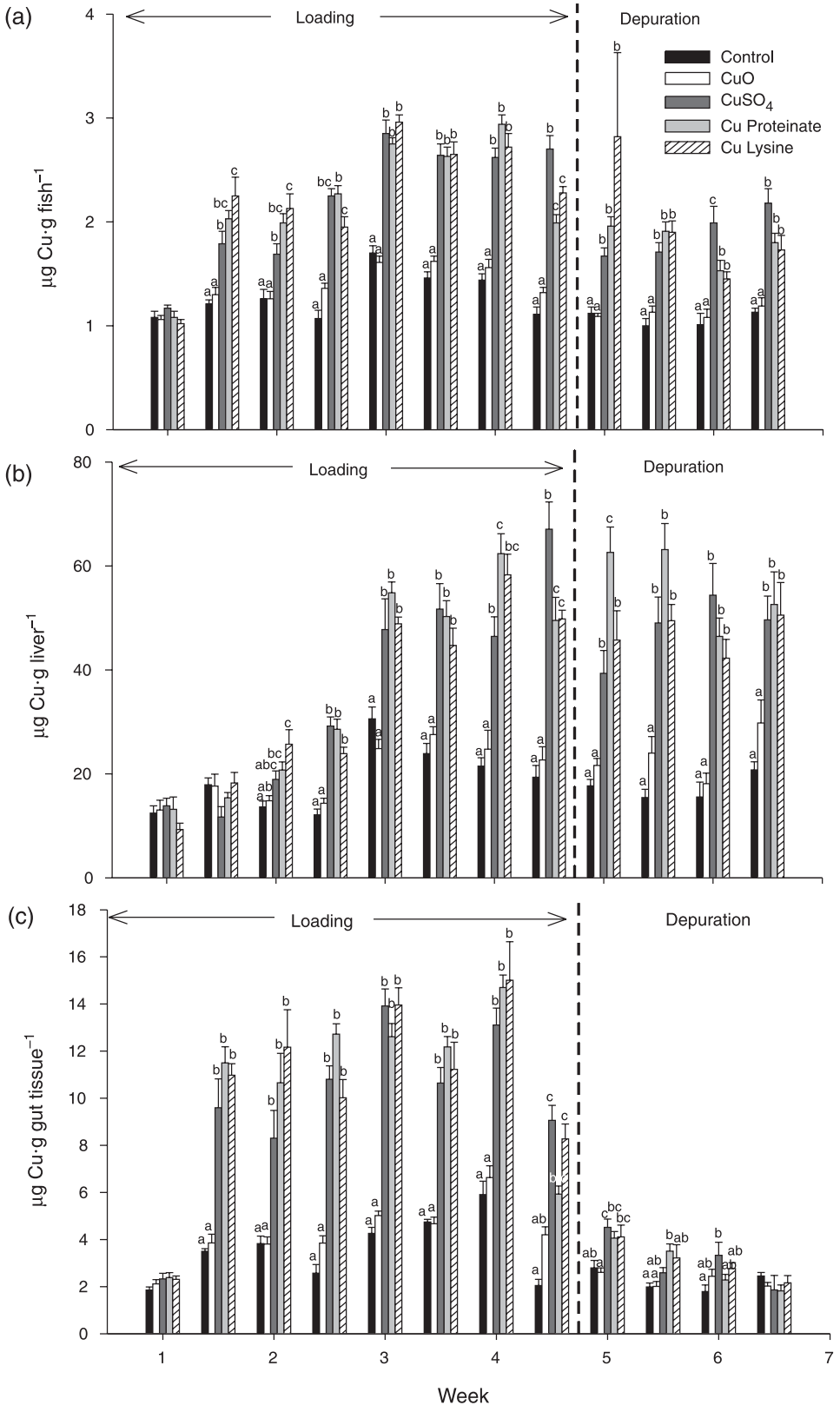

Table 2. Relative bioavailability (%) of Cu and Zn.

Group	Cu	Group	Zn
Experiment 1			
Loading			
Control	14.42	Control	14.48
CuO	0.30	ZnO	2.28
$CuSO_4$	0.85	$ZnSO_4$	1.55
Cu-proteinate	0.87	Zn-proteinate	2.55
Cu-lysine	0.89	Zn-methionine	1.63
Depuration			
Control	29.68	Control	29.16
CuO	26.75	ZnO	24.75
$CuSO_4$	24.37	$ZnSO_4$	28.14
Cu-proteinate	20.41	Zn-proteinate	26.49
Cu-lysine	9.71	Zn-methionine	26.60
Experiment 2			
Loading			
Control	3.42		
CuO	0.36		
$CuSO_4$	0.75		
Cu-proteinate	0.52		
Cu-lysine	0.61		
Depuration			
Control	4.23		
CuO	4.77		
$CuSO_4$	–2.62		
Cu-proteinate	–0.83		
Cu-lysine	4.28		

creased [Zn] during the depuration phase (Fig. 3*b*; df = 4, *F* range = 0.37–2.71, *P* range = 0.077–0.82).

During the loading phase, the relative bioavailability of Zn in the control group was similar to that of Cu in the same experiment (~15%), whereas the other groups showed much lower bioavailability values (Table 2). As for Cu, these values increased and were generally similar among all groups during the depuration phase.

Discussion

Both experiments clearly demonstrated that the form in which Cu was complexed in the diet affected how much Cu was accumulated by juvenile rainbow trout. In both experiments and in all measured body compartments (i.e., liver, gut, or whole body), CuO-supplemented fish showed results similar to those of control fish, suggesting that Cu was not readily available for uptake in this form. In Experiment 1, the measured [Cu] in the CuO food (292 $\mu g \cdot g^{-1}$) was somewhat lower than that in the other three experimental treatments (313–359 $\mu g \cdot g^{-1}$). Although this could potentially have contributed to the observed differences, it seems unlikely that it could explain the extreme discrepancy between this group and the other Cu-supplemented groups. Nevertheless, principally for this reason, the experiment was repeated. The concentration of Cu in the CuO treatment of Experiment 2 was much closer to the target 400 $\mu g \cdot g^{-1}$ and was in fact the highest [Cu] of all treatments, yet the patterns observed were very similar to those of Experiment 1. Thus, it is clear that Cu was not readily available for uptake by fish as CuO, an insoluble form. These results are similar to those reported by Xin et al. (1991), who exposed very young calves to food supplemented with either $CuSO_4$ or CuO and concluded that CuO was a very poor source of Cu for these animals.

In contrast, tissue [Cu] did not differ consistently among groups treated with $CuSO_4$, Cu-proteinate, or Cu-lysine, suggesting that these three compounds were more or less equally available for uptake by juvenile trout. This contrasts with earlier work on poultry (Guo et al. 2001) and rats (Du et al. 1996), where Cu-lysine and (or) Cu-proteinate complexes were found to be taken up more readily than $CuSO_4$. Similarly, Cu-proteinate was more bioavailable to heifers than $CuSO_4$ was, at least in the presence of molybdenum (Mo), although in the absence of Mo the bioavailabilities of the two complexes were similar (Ward et al. 1996). In ewes, Eckert et al. (1999) reported that liver [Cu] tended to decrease with increasing dietary Cu-proteinate but increased with increasing dietary $CuSO_4$. At the same time, ewes fed Cu-proteinate had higher intestinal [Cu] than ewes fed $CuSO_4$. Thus, although $CuSO_4$ is inorganic, it seems that at least in some animals, including juvenile rainbow trout, its relative bioavailability is similar to that of Cu-proteinate and Cu-lysine, both of which are organic forms.

Differences in the relative bioavailability of Cu in the two experiments suggest that relative bioavailability changes with dietary [Cu], size of fish, and the amount of Cu already present in the fish at a given sampling time. Overall, values from Experiment 1 were similar to the percent bioavailability (retention) values observed in a recent study that used similar-sized fish, similar dietary [Cu] (control: 6 $\mu g \cdot g^{-1}$; high Cu: nominally 500 $\mu g \cdot g^{-1}$ supplemented as $CuSO_4$), and a 4% daily food ration for 28 days (Kjoss et al. 2005). Fish in that study also had comparable tissue [Cu]. Experiment 2, however, yielded very different results, again probably because of differences in fish size, ration, and duration of the experiment.

In this study, we did not routinely measure water [Cu] during the experiments. However, a similar study on dietary Cu uptake indicated that regular siphoning to remove uneaten food and faeces prevented any significant leaching of Cu from the diet into the water (Kjoss et al. 2005); therefore, because the tanks in our study were siphoned regularly, we are confident that any metal leaching from the experimental diets into the water was minimal and hence did not affect our results. Nevertheless, the natural background level of Cu in the exposure water for all groups was appreciable (3 $\mu g \cdot L^{-1}$), and the ability of trout to take up substantial amounts of Cu from this concentration of Cu in the water is now well established (Grosell and Wood 2002; Kamunde et al. 2002; Pyle et al. 2003). Undoubtedly, some of the Cu accumulated by the fish was acquired from the water rather than from the food, and probably a greater proportion came from the water in the non-Cu-loaded treatments (control, CuO), based on the findings of Kamunde et al. (2002) that dietary Cu loading will down-regulate branchial Cu uptake in juvenile rainbow trout. For this reason, dietary bioavailability values must be considered apparent rather than absolute values.

Whole-body [Cu] showed similar trends between the two experiments, although in Experiment 2, the discrepancy between the control–CuO peak and the $CuSO_4$ – Cu-proteinate –

Fig. 3. Mean (+1 standard error of the mean) (*a*) whole-body [Zn], (*b*) liver [Zn], and (*c*) gut [Zn] of juvenile rainbow trout (*Oncorhynchus mykiss*) in Experiment 1. Fish were fed Zn-supplemented diets during the loading phase and control diet during the depuration phase at a ration of 8% body weight·day^{-1}. For each sampling day, different letters above bars indicate significant differences ($P < 0.05$) among groups. Comparisons were not made between sampling days.

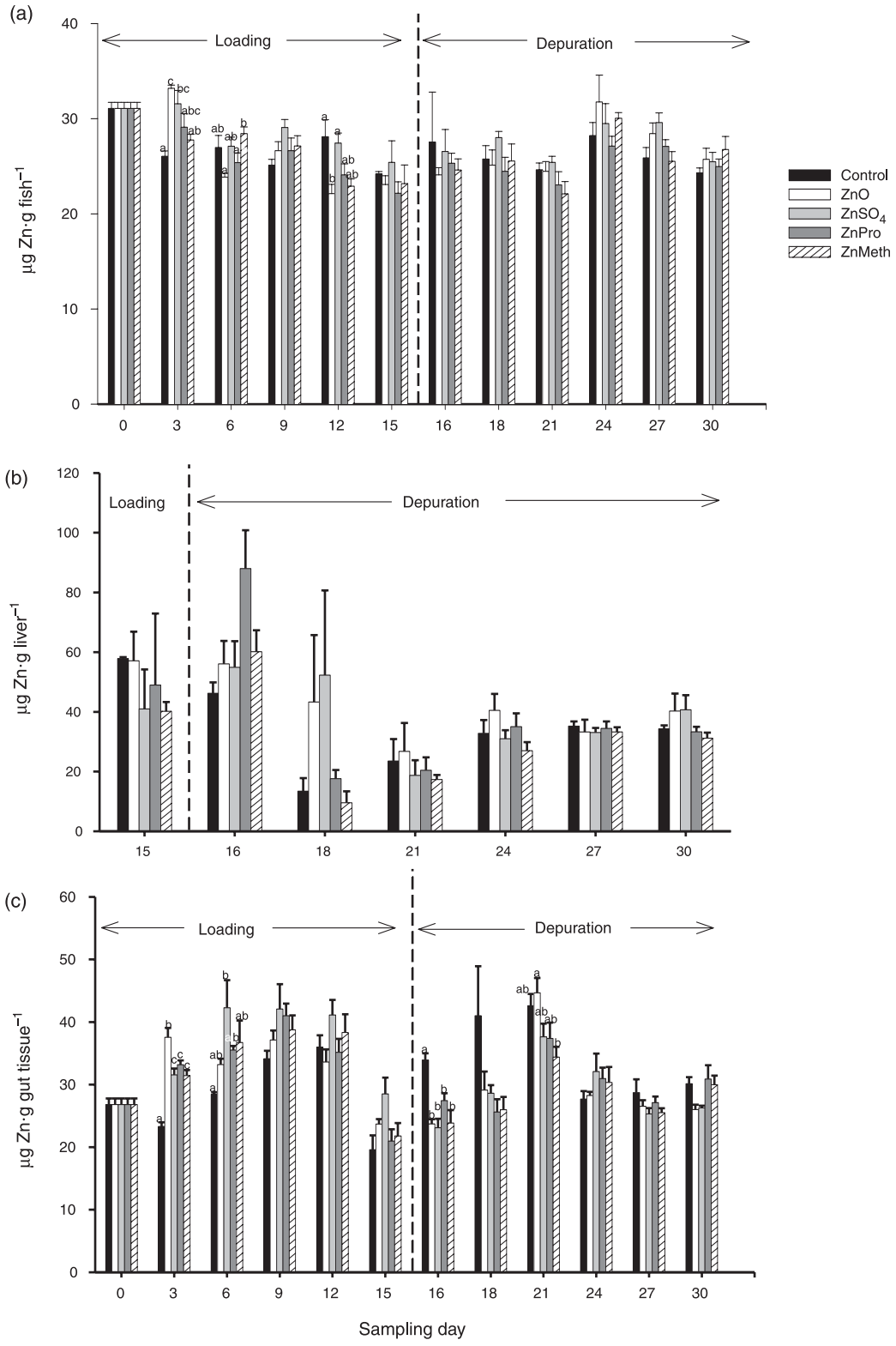

Table 3. Daily doses of Cu (μg Cu·g fish^{-1}·day^{-1}) or Zn (μg Zn·g fish^{-1}·day^{-1}) fed to juvenile rainbow trout (*Oncorhynchus mykiss*).

	Experiment 1		Experiment 2	
Treatment	Loading	Depuration	Loading	Depuration
Control	0.36	0.36	0.94	0.94
CuO	23.42	0.36	16.48	0.94
$CuSO_4$	25.88	0.36	16.06	0.94
Cu-proteinate	28.72	0.36	16.14	0.94
Cu-lysine	25.02	0.36	15.19	0.94
Control	7.3	7.3		
ZnO	61.8	7.3		
$ZnSO_4$	82.9	7.3		
Zn-proteinate	52.3	7.3		
Zn-methionine	79.1	7.3		

Note: In Experiment 1, the Cu- or Zn-loading phase lasted 2 weeks, followed by a 2-week depuration phase during which all groups were fed control food at a ration of 8% body mass·day^{-1}. In Experiment 2, the Cu-loading phase lasted 4 weeks, followed by 2 weeks of depuration at a ration of 4% body mass·day^{-1} throughout.

Cu-lysine peak was not as great as that in Experiment 1. Both experiments indicated that fish in the latter three groups needed a longer time to remove excess Cu from their tissues than the time required to upload Cu; this pattern was likely influenced by Cu stores in the liver.

In both experiments, livers showed the highest [Cu] among the observed tissues; this is not surprising, as the liver is well-known to be the major sink for excess Cu stored in the body (e.g., Taylor et al. 2000; Grosell et al. 2001; Kamunde et al. 2002). In Experiment 1, liver [Cu] during the depuration phase was relatively variable among treatment groups and among sampling times, perhaps reflecting the difficulty in sampling this organ from very small fish. However, in Experiment 2 with larger fish, differences were very obvious and lasted for the duration of the depuration period. This suggests that Cu is very persistent in the liver and, following a delayed uptake, requires a relatively long time to return to baseline levels. Liver [Cu] of the CuO group followed the same general patterns as that of the controls, again indicating that Cu was not readily taken up or stored in this form.

Gut [Cu] followed the same trends, although the overall [Cu] in the gut tissue was intermediate between liver and whole-body. Differentiation between the low Cu groups (controls, CuO) and the high Cu groups ($CuSO_4$, Cu-proteinate, Cu-lysine) occurred relatively early, suggesting a fast uptake in this compartment. Depuration also occurred rapidly, which indicates that Cu acquired through the diet does not reside in the gut over long periods; rather, it is mobilized to other compartments, notably the liver.

It is interesting to note that fish showed similar whole-body and liver [Cu] between experiments (≤~3 μg·g^{-1} and ~20–60 μg·g^{-1}, respectively). In contrast, fish in Experiment 2, which were fed a lower food ration but for twice as long, had lower overall gut [Cu] than fish in Experiment 1. This suggests that ration and duration of feeding do not affect [Cu] in long-term storage organs such as the liver, whereas organs with a high Cu turnover rate, such as the gut, will respond to changes in ration or duration of feeding.

Claims of growth promotion achieved by supplementing commercial feeds with high levels of essential elements are often advanced by manufacturers. However, in both the short-term and long-term experiments, diets supplemented with Cu (regardless of the chemical form or ration level) to levels well above standard nutritional requirements neither stimulated nor inhibited growth. Clearwater et al. (2002) calculated that a threshold daily dose of 35–45 μg Cu·g^{-1}·day^{-1} is likely to cause chronic toxic effects. In both experiments, the daily doses of Cu ingested by fish in this study fell far below this threshold (Table 3).

Unlike Cu, we observed no consistent differences in Zn uptake among any of the treatments, including the controls. This indicates not only that the form of Zn supplementation was of no consequence to these juvenile trout, but more importantly that 8- to 10-fold elevation in dietary [Zn] had no effect relative to controls. This suggests very strong homeostatic regulation of Zn. Spry et al. (1988) similarly reported that juvenile rainbow trout were able to maintain homeostasis over a wide range of waterborne and dietary [Zn] over a 16-week exposure. Their results indicated that Zn uptake from the water was independent of dietary uptake; in fact, the fish were able to obtain all required Zn from the water via the gills.

Apines et al. (2001) noted that while weight gain may not be a useful index of Zn bioavailability, tissue Zn accumulation could be used to ascertain differences among treatments. However, unlike Cu, we found no consistent differences in Zn uptake among groups, regardless of the form in which Zn was complexed. Maage et al. (2001) similarly found $ZnSO_4$ and Zn-gluconate (an organic form) to be equally efficient as Zn supplements for juvenile Atlantic salmon (*Salmo salar*). The whole-body [Zn] of all their experimental fish ranged from ~25 μg·g^{-1} to 55 μg·g^{-1}, while in our study whole-body [Zn] was ~30 μg·g^{-1}, which falls into this range. (Note, however, that the dietary Zn levels used in the study by Maage et al. (50–180 μg Zn·g^{-1} food) were much lower than those in our study (1000 μg Zn·g^{-1} food).) Maage et al. (2001) suggested that, overall, the bioavailability of dietary Zn is relatively low.

Relative bioavailability of Zn showed the same pattern as Cu did in Experiment 1 (i.e., higher percent bioavailability for controls during the loading phase and similar percent

bioavailability for all groups during depuration). Bury et al. (2003) similarly reported that as dietary Zn load increases, the proportion of Zn absorbed from the diet decreases. Thus, our results of both metals combined illustrate a general trend that metals are relatively more bioavailable when the metal concentrations are lower (i.e., a higher proportion of the metal is incorporated by the animal when less is present in the food) (see Kamunde et al. 2002 and Kamunde and Wood 2003 for comparable Cu data).

In a review on the bioavailability of diet-borne Zn to fish, Clearwater et al. (2002) concluded that in trout, most diet-borne Zn is accumulated in the digestive tract, regardless of exposure duration. While this may be true on an absolute basis, given the size of the gut compared with other tissue compartments, this was not the case in relative terms in our study; instead, we found that by the end of the loading phase, the liver had the highest [Zn], although it was only about twice that of the gut tissue. During the depuration phase, liver, gut, and whole-body [Zn] were similar to each other. Similarly, Clearwater et al. (2002) noted that as diet-borne [Zn] increases, it does not seem to accumulate selectively in the liver; rather, the intestinal tissues and the gills become equally important in Zn uptake and excretion.

Clearwater et al. (2002) estimated a threshold dietary Zn dose of 30 $\mu g \cdot g^{-1}$ body weight$\cdot day^{-1}$, above which chronic exposure might have adverse effects on fish. However, the daily doses that we calculated in this study were more than double this estimated threshold, and no outward signs of toxicity, growth deficits, or growth promotion were evident in the fish, again suggesting tight homeostatic control of this essential element, and that dietary supplementation with essential metals to well above standard nutritional values is of negligible value. In conclusion, the form in which a metal was added to commercial fish feed affected how much Cu was taken up by juvenile rainbow trout, but apparently did not affect how much Zn was taken up.

Acknowledgements

We thank D. Matsuda for excellent technical assistance in the laboratory, Dr. S. Clearwater for facilitating this study, and two anonymous reviewers for their constructive comments. This study was funded by a grant to DGM and CMW from the US Environmental Protection Agency through a cooperative agreement with the University of Delaware (P.I., Dr. H. Allen). CMW is supported by the Canada Research Chair Program.

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