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Subcellular fractionation of Cu exposed oysters, *Crassostrea virginica*, and Cu accumulation from a biologically incorporated Cu rich oyster diet in *Fundulus heteroclitus* in fresh and sea water

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ABSTRACT

In order to examine the effect of salinity on Cu accumulation from a naturally incorporated diet, oysters (*Crassostrea virginica*) were exposed in sea water for 96 days to four waterborne [Cu]: 2.9 ± 0.7 (control), 4.3 ± 0.6, 5.4 ± 0.5, and 10.7 ± 1.0 μg L^{-1}. After 96 days, the control whole body [Cu] increased from 2.1 ± 0.5 to 9.1 ± 1.1 μg g^{-1} w.w. and the highest [Cu] was 163.4 ± 27.1 μg g^{-1} w.w. in the oysters. Despite large differences in tissue [Cu], there was no effect on the fraction of trophically available metal in the oyster suggesting that trophic transfer will correlate well with tissue [Cu]. The control and highest [Cu] oysters became diet for killifish (*Fundulus heteroclitus*) in fresh and seawater for 40 days. The two diets contained 84.7 ± 5.1 and 850.5 ± 8.8 μg Cu g^{-1} d.w. Fish were fed a combined diet of oyster and a pellet supplement (20.5 ± 1.0 μg Cu g^{-1} d.w.) both at 5% body mass day^{-1}. In killifish, Cu increased ~7% in gills and 100% in intestines after 6 weeks of exposure to the high Cu diet. No other tissues accumulated Cu above control levels. An 11-fold difference free Cu^{2+} concentrations was predicted in intestinal fluid between fresh and sea water, but there was no corresponding effect of salinity on intestinal Cu accumulation suggesting that Cu is not accumulated as the free ion.

1. Introduction

Many factors are thought to affect the accumulation and toxicity of metals from dietary sources. Spiking a diet with metal salts has been demonstrated to be less toxic than a diet in which the metal has been incorporated naturally (Hook and Fisher, 2001; Bielmyer et al., 2006; De Schamphalaere et al., 2007). One reason for this is that natural incorporation may allow the metal to bind to complexes in the organism including amino acids that make the metal more available during digestion (Wapnir, 1998; Glover and Wood, 2008). This may account for the high toxicity observed in experiments using naturally incorporated diets.

Natural incorporation may also lead a change in the internal distribution of the metal in the organism that is being utilized as the diet. Subcellular fractionation techniques, such as those developed and utilized by Wallace and Louma (2003), allow for the elucidation of what portions of the organism may be most available during digestion. They and others (Seebaugh et al., 2005; Rainbow et al., 2006) have found that there is a positive correlation between trophic transfer of a metal and the amount of metal found in the enzymes, organelles, and heat stable proteins which include the metallothionein-like proteins. In addition to these three trophically available fractions, the subcellular fractionation technique of Wallace et al. (2003) separates out two other pools of metal: metal rich granules and cellular debris. Both of these pools show poor correlations with trophic transfer and are less available to the consumer organism (Wallace et al., 2003; Seebaugh et al., 2005; Rainbow et al., 2006).

Another factor that may influence the uptake of metal from a dietary source is the gut fluid chemistry itself. This chemistry is different in unfed fish depending on the salinity to which the fish had been acclimated (Wilson, 1999; Marshall and Grosell, 2005; Grosell 2006). However, a recent study using the European flounder reported similar intestinal fluid chemistry from fresh (FW) and sea water (SW) acclimated fish up to 12 h after feeding (Taylor et al., 2007). Whether these observations from European flounder apply generally across species or if ambient salinity influences gut fluid chemistry post feeding in other fish remains unknown at this time.

The gut fluid chemistry is especially important for Cu uptake in the intestine. If ambient salinity influences gut fluid chemistry post feeding, then the intestinal fluid in SW will contain high concentrations of Na^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, and HCO_3^- relative to FW (Wilson, 1999; Marshall and Grosell, 2005; Grosell, 2006). In addition, if the fish utilizes acidic digestion then the pH in FW is likely to be lower due to the high buffering capacity of the SW intestinal fluid and the low buffering in most FW. These differences in the gut fluid's physicochemical properties would cause a large difference in the speciation of Cu especially because of the high [HCO_3^-] and pH in marine fish intestinal fluids.

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This study set out to examine several of the factors that influence Cu uptake from a naturally incorporated dietary source. The first part of the experiment exposed oysters (*Crassostrea virginica*) to low waterborne [Cu] (<11 μg L^{-1}) for 96 days. This organism is generally considered a Cu hyper-accumulator with unusually high Cu body burdens even at low levels of ambient Cu (Zamuda and Sunda, 1982) with little if any toxic effect. The oysters were subcellularly fractioned in order to assess whether the distribution of Cu in the oyster changed with time or ambient [Cu]. We expected to see changes in the distribution with both time and ambient [Cu] as has been reported for other metals accumulated by invertebrates (Seebaugh and Wallace, 2004; Seebaugh et al., 2005).

The second aim of the present study was to examine the influence of gut fluid chemistry and salinity on Cu accumulation from a dietary source in killifish. To pursue this goal, we prepared a naturally incorporated Cu rich diet from the eastern oyster (*C. virginica*). These oysters were subsequently used as a diet for *Fundulus heteroclitus* which is fully euryhaline and can tolerate salinities from FW to SW (Wood and Marshall, 1994). Furthermore, Cu accumulation and its effects in this species have already been examined for waterborne exposures across a wide range of salinities (Blanchard and Grosell, 2005; Blanchard and Grosell, 2006). Fish were exposed to a high Cu diet in both FW and SW and the accumulation of Cu was measured for 40 days in gill, gonad, intestine, liver, and the remaining carcass. We expected to see Cu accumulation in both the intestine and the liver which would be higher in the FW fish due to intestinal physicochemical environment which would be more favorable to Cu uptake. The intestine was an expected site of accumulation because it is the direct site for dietary uptake while the liver is the main homeostatic organ in organisms.

2. Materials and methods

2.1. Experimental animals

Oysters, *C. virginica*, were obtained from a commercial supplier (Apalachicola Bay, FL, USA) and were acclimated to aerated unfiltered Bear Cut SW (25° 43.9′ N 80° 09.7′ W, Miami, FL, USA) for 1 week prior to the exposure in 750 L tanks under flow through conditions at a rate of 750 mL min^{-1} or 1080 L day^{-1} and a natural photoperiod at a salinity of 36.0 ± 0.7 ppt (mean ± Standard error of the mean (SEM)). The physical chemical characteristics of SW are listed in Table 1 and the [Cu] in Table 2.

F. heteroclitus were obtained from Aquatic Research Organisms and were collected in Hampton, New Hampshire, USA. These fish were fed the dry pellet food described below while acclimated to the laboratory for a month in SW and then acclimated to either FW (Miami dechlorinated tap water) or Bear Cut SW at a salinity of 34.8 ± 0.2 ppt (mean ± SEM) (see Table 1 for details of water chemistry) for two weeks prior to the exposure under a 16:8 Light:Dark cycle. Fish were kept under flow through conditions at the appropriate salinity in 24 L tanks with the males and females separated. The tanks containing males held twenty one fish per tank while there were thirty five fish in each tank containing females. The difference in the number of males and females was to obtain proper breeding ratios for the planned breeding experiments at the end of the dietary exposure.

Table 1
Physical and chemical characteristics of exposure waters.

	FW	SW
Salinity (ppt)	0.16	35
pH	8.29	8.16
Na^+ (mM)	2.0	438.1
K^+ (mM)	0.2	9.6
Mg^{2+} (mM)	0.3	53.4
Ca^{2+}(mM)	1.2	10.9
SO_4^{2-}(mM)	0.3	21.5
Cl^- (mM)	2.1	451.6
TA (mM)	1.9	2.2

TA — Titratable alkalinity.

Table 2
(a) Measured total copper concentrations to which oysters were exposed (mean ± SEM; $n = 15$), (b) measured total waterborne copper concentrations during the dietary copper exposure of killifish (mean ± SEM; $n = 8$).

a. Nominal	Measured [Cu] (μg L^{-1})
Control	2.9 ± 0.7
4 μg L^{-1}	4.3 ± 0.6
5 μg L^{-1}	5.4 ± 0.5
11 μg L^{-1}	10.7 ± 1.0

b.	Male control (μg L^{-1})	Male diet (μg L^{-1})	Female control (μg L^{-1})	Female diet (μg L^{-1})
FW	3.5 ± 1.7	3.8 ± 1.2	6.7 ± 5.3	6.9 ± 2.6
SW	8.3 ± 1.6	10.9 ± 1.5	8.0 ± 1.5	6.9 ± 1.3

2.2. Oyster Cu exposure and sampling

Oysters were exposed to 4 concentrations of Cu under flow through conditions in circular outdoor tanks (~750 L) fed a continuous flow of unfiltered Bear Cut seawater at a 750 mL min^{-1}: 0 (Control), 4, 5, 11 μg L^{-1} (nominal, see Table 2 for measured concentrations). The Cu was supplied via a peristaltic pump for 96 days. Water samples were collected periodically for determination of exposure [Cu] and the oysters were sampled on days 0, 60, and 96 of exposure. Five oysters were sampled at each time point for subcellular fractionation using the method of Wallace et al. (2003) and five for whole body Cu analysis. At 96 days, the remaining oysters were sacrificed and kept at −20 °C until used for the dietary exposure.

2.3. Diet preparation and dietary exposure

Two diets were prepared from the oysters that were exposed to 11 μg L^{-1} for 96 days and from the controls (only two diets were prepared due to a lack of substantial change in the subcellular fractionation of the oysters). The [Cu] in these diets is listed in Table 3. The oyster diet was prepared by homogenization in a blender followed by "pelleting" in an agarose matrix (see below) for a final composition of 50% oyster, 46% water, and 4% agarose (Sigma-Aldrich, St. Louis, MO, USA) by weight which gave a final total water content of 87.6 ± 0.8% (mean ± SEM). The diets were prepared by homogenizing oysters adding half of the water to facilitate the homogenization. An agarose gel was prepared at the proper concentration to obtain 4% agarose in the diet with the remaining water and allowed to cool to ~37 °C. This was added to the homogenized oysters, thoroughly mixed, and stored at 4 °C. Fresh diet was prepared approximately every 7 days.

This diet was fed to killifish for 40 days at a rate of 5% body weight per day in the morning and was supplemented with a dry pellet food which was composed of 50% protein, 3% fiber, 16% fat, 0.35% Na, 2.2% Ca, and 1.3% phosphorous (Aquatic Eco-systems, Apopka, FL, USA; see Table 3 for [Cu]) at the same rate in the evening. The killifish were kept in either FW or SW and the males (4.1 ± 0.1 g) and females (4.5 ± 0.1 g) were kept separated under flow through conditions. The light cycle was 16:8 light:dark and the temperature was 26.0 ± 0.2 °C. Fish were sampled at day 4, 10, and 40 post exposure for gill, liver,

Table 3
Measured [Cu] (μg Cu g^{-1} dry weight) in the prepared oyster diets and the commercial pellet food ($n = 4$).

Diet	[Cu]
Oyster control	84.7 ± 5.1
Oyster high Cu	850.5 ± 8.8
Pellet	20.5 ± 1.0

intestine, gonad, and the rest of the carcass. Prior to sampling fish were starved for 24 h and were killed with an overdose of MS-222.

2.4. Gut fluid sampling

Ten fish (FW 6.9 ± 0.4 g, SW 6.3 ± 0.7 g) were acclimated to both FW and SW for at least 2 weeks prior to the experiment and were fed a single meal consisting of dry pellet food and sampled 8 h later. The fish were killed with an overdose of MS-222 prior to sampling and the entire contents of the gut were removed. The gut contents were measured for pH, ionic composition, and total CO_2 by the methods listed below.

2.5. Analyses

All [Cu] were measured by graphite furnace atomic absorption spectroscopy (Varian Model 220 Z, Mulgrave, Australia) under manufacturer recommended conditions. Tissue samples were digested in 1 N trace metal grade nitric acid (Fisher Scientific, Pittsburg, PA, USA) overnight at 80 °C and all samples were appropriately diluted prior to measurement. National Institute of Standards and Technology (USA) standard reference material 2976 (mussel tissue) was processed using the same tissue digestion procedure. We measured the tissue at 3.74 ± 0.04 mg Cu kg^{-1} which falls within the uncertainty reported and is 93% of the reported value for Cu in the tissue (4.02 ± 0.33 mg Cu kg^{-1}). All values presented are total Cu measurements as there was no difference between total and dissolved values. For the SW water samples, the Cu was removed from the matrix and resuspended in 1% trace metal grade nitric acid before dilution using a solvent extraction (Kinrade and Van Loon, 1974) and back extraction (Danielsson et al., 1978). All values were corrected based on extraction efficiency which was >90% in all cases.

Other cations were measured using flame atomic absorption spectroscopy (Varian Model 220 FS) under standard operating conditions after appropriate dilution and anions were measured on a Dionex DX 120 ion chromatograph (Sunnyvale, CA, USA). Total CO_2 was measured using a Corning 965 total CO_2 analyzer (Corning, NY, USA). In the exposure tanks, pH was measured using a Radiometer analytical PHM 201 portable pH meter fitted with a pHC 3005 combined pH electrode (Lyon, France) while the gut fluid pH was measured using a Radiometer analytical PHM 220 lab pH meter with an Accumet micro combination electrode (Fisher Scientific). Salinity was measured using a refractometer.

2.6. Statistical analysis

Sigmastat software (3.0) was used for statistical analyses. The oyster data were analyzed by a two way ANOVA using the Holm-Sidak pairwise comparison or an ANOVA on ranks with a Dunn's test for paired comparisons for data that were not normally distributed or have equal variances. The dietary data were analyzed using a four way ANOVA on ranks because of the lack of homoscadicity, followed by a least significant difference test for the paired comparisons. The gut fluid comparisons were two-tailed *t*-tests. All values presented are means ± the standard error of the mean and a *p*-value of <0.05 is accepted as significant throughout.

3. Results

3.1. Oysters exposure

The exposure [Cu] remained fairly constant over time as reflected by the small error associated with the measured [Cu] (Table 2). However, the control water was fairly high in Cu (2.9 ± 0.7 μg Cu L^{-1}) and exceeded the current EPA chronic guideline of 1.9 μg L^{-1} (USEPA, 2003) in our oyster exposure tanks throughout the exposure period.

3.1.1. Cu accumulation in oysters

The oysters, including controls, accumulated a significant amount of Cu in all of the exposure concentrations (Fig. 1). This accumulation

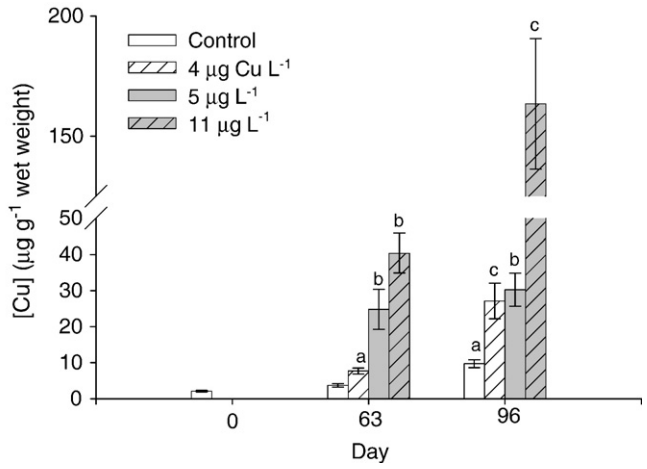

Fig. 1. [Cu] in oyster tissue after 0, 63, and 96 days of exposure to control (clear bar), 4 μg Cu L^{-1} (cross hatched bar), 5 μg Cu L^{-1} (gray bar), 11 μg Cu L^{-1} (gray cross hatched bar). Note the break in the y-axis. See Table 1 for physical chemical characteristics of the water and Table 2 for the measured [Cu]. "a" indicates a significant difference from day 0, "b" indicates a significant difference from day 0 and the simultaneous control, and "c" indicates a significant difference from day 0, day 63, and the simultaneous control ($n = 5$).

increased with both time and exposure Cu concentration. The oysters had an initial [Cu] of 2.1 ± 0.5 μg Cu g^{-1} w.w. at day 0 in the control water and significantly increased to 9.7 ± 1.1 μg Cu g^{-1} w.w. The whole body concentrations also increased in 4, 5, and 11 μg Cu L^{-1} from the initial concentration (2.1 ± 0.5 μg Cu g^{-1} w.w.) to 27.1 ± 4.9, 30.2 ± 4.6, and 163.4 ± 27.1 μg Cu g^{-1} w.w. respectively. It is interesting to note that the oysters at 5 μg Cu L^{-1} did not significantly increase their whole body Cu from day 60 to Day 96 and that the oysters at 4 μg Cu L^{-1} did not significantly accumulate Cu until after day 60 and yet still accumulated a similar amount of Cu compared to the oysters at 5 μg Cu L^{-1}.

3.1.2. Subcellular distribution

Cu had a significant effect on the distribution of Cu in the oysters in the cellular debris, the metallothionein-like fraction, and the enzymes (results from two-way ANOVA, $p<0.05$). There was a significant effect of time and the interaction between time and Cu on the organelle fraction. There was no effect of either time or Cu on the metal rich granules. There are few paired comparison differences between the fractions within time or between times and most of these differences are from the higher exposure concentrations (Table 4). In particular, oysters exposed to 11 μg Cu L^{-1} had a significantly greater proportion of Cu in the metallothionein-like fraction than in the simultaneous control while the proportion of Cu in the enzymes was reduced.

However, in spite of significant differences in the organelle, enzyme, and metallothionein-like protein fraction, which make up the trophically available metal, there were no differences in the proportion of trophically available metal in the oysters (Table 4). The trophically available metal fraction was also unaffected by Cu exposure, time, or their interaction (results from two-way ANOVA, $p>0.05$). The metal sensitive fraction, which consists of the organelle and the enzyme fractions, was affected by Cu, time, and their interaction while the biologically detoxified metal was affected only by the interaction of time and Cu. The paired comparisons, suggest that, as the exposure concentration and therefore [Cu] in the organism increases, the proportion of Cu in the metal sensitive fraction may decrease while the proportion detoxified may increase.

3.2. Dietary Cu exposure

3.2.1. Whole body ions

There was no effect of dietary Cu exposure on either whole animal cations (Na^+, Ca^{2+}, Mg^{2+}, and K^+) or Cl^- (data not shown).

Table 4
Subcellular fractionation of oysters exposed for 63 and 96 days to the indicated waterborne [Cu] (mean ± SEM).

		Metallothionein-like proteins (%)	Cellular debris (%)	Metal rich granules (%)	Enzymes (%)	Organelles (%)	Biologically detoxified metal (%)	Metal sensitive fraction (%)	Trophically available metal (%)
Day 63	Control	14.9 ± 4.4	19.8 ± 3.1	41.2 ± 2.3	15.4 ± 4.4	8.7 ± 2.4	56.0 ± 4.4	24.2 ± 5.3 a	39.0 ± 5.0
	4 μg Cu L^{-1}	24.4 ± 2.2	24.9 ± 3.3	38.6 ± 3.1	6.7 ± 1.2	5.4 ± 0.6	63.0 ± 4.0	12.1 ± 1.0 b	36.5 ± 1.9
	5 μg Cu L^{-1}	24.6 ± 5.2	19.9 ± 1.9	46.8 ± 6.4	3.1 ± 0.7 a,b,c	5.5 ± 1.1	71.4 ± 3.1 b	8.7 ± 1.8 a,b,c	33.3 ± 6.4
	11 μg Cu L^{-1}	27.3 ± 3.3 a,b	20.4 ± 3.3	36.2 ± 7.7	4.1 ± 0.9 b,c	12.0 ± 1.7 c,d	63.4 ± 4.9 a	16.1 ± 2.2 a,d	43.4 ± 4.6
Day 96	Control	23.0 ± 10.3	20.1 ± 4.0	43.2 ± 6.7	9.4 ± 1.9	4.3 ± 0.8	66.2 ± 5.2	13.7 ± 1.7	36.8 ± 9.5
	4 μg Cu L^{-1}	23.3 ± 4.0	31.3 ± 3.7 b	35.5 ± 5.2	5.6 ± 1.2 b	4.4 ± 0.3	58.8 ± 5.0	9.9 ± 1.4 b	33.2 ± 4.2
	5 μg Cu L^{-1}	29.0 ± 3.5	27.8 ± 2.7	31.1 ± 2.5	6.3 ± 1.4	5.8 ± 0.4	60.1 ± 3.1	12.1 ± 1.1	41.1 ± 2.9
	11 μg Cu L^{-1}	36.7 ± 1.5 b,c	15.8 ± 3.0 c,d	39.1 ± 2.8	3.7 ± 0.7 b,d	4.7 ± 0.2	75.8 ± 3.1 c,d	8.4 ± 0.7 b,d	45.1 ± 1.4

"a" indicates a significant difference between the two times. "b" indicates a significant difference from the control within time, "c" indicates a significant difference from 4 μg Cu L^{-1} within time, and "d" indicates a significant difference from 5 μg Cu L^{-1} within time ($n = 5$).

3.2.2. Tissue [Cu] in killifish

Cu did not accumulate in the gonad (2.44 ± 0.47 μg Cu g^{-1}, $n = 120$), liver (34.09 ± 2.25 μg Cu g^{-1}, $n = 120$), or carcass (0.41 ± 0.01 μg Cu g^{-1}, $n = 120$) during the dietary exposure. Dietary [Cu] had a significant effect ($p < 0.05$, results from the four way ANOVA) on [Cu] in the gill although there were few paired comparison differences (Fig. 2a and b). There was also significant accumulation in the intestine which showed different patterns in males and females (Fig. 3a and b). In females, there was a difference between the FW and SW fish whereas in males there was no difference between the two salinities. In males, accumulation increased over time. In females the [Cu] in FW was elevated at day 4 and returned to control levels by day 10 (Fig. 3a). At day 40, the females fed the high Cu diet had significantly lower [Cu] in their intestine then their

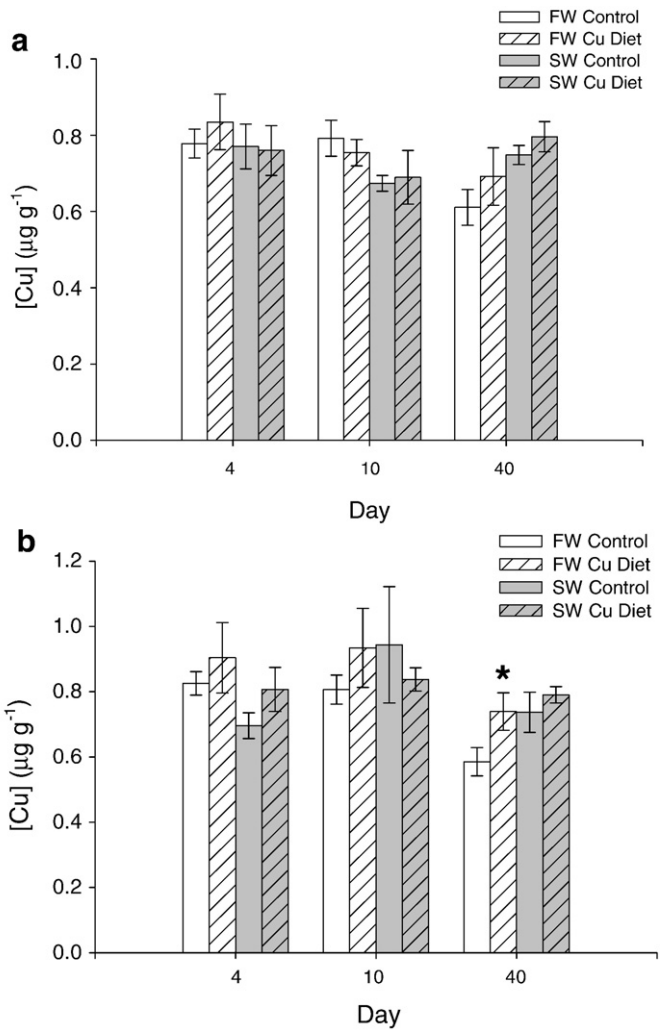

Fig. 2. Gill [Cu] in female (a) and male (b) killifish exposed to a control oyster diet and a high Cu diet (hatched bars, see Table 3 for [Cu]) for 40 days in FW (clear bars) and SW (grey bars). "*" indicates significant differences from the simultaneous control ($n = 5$).

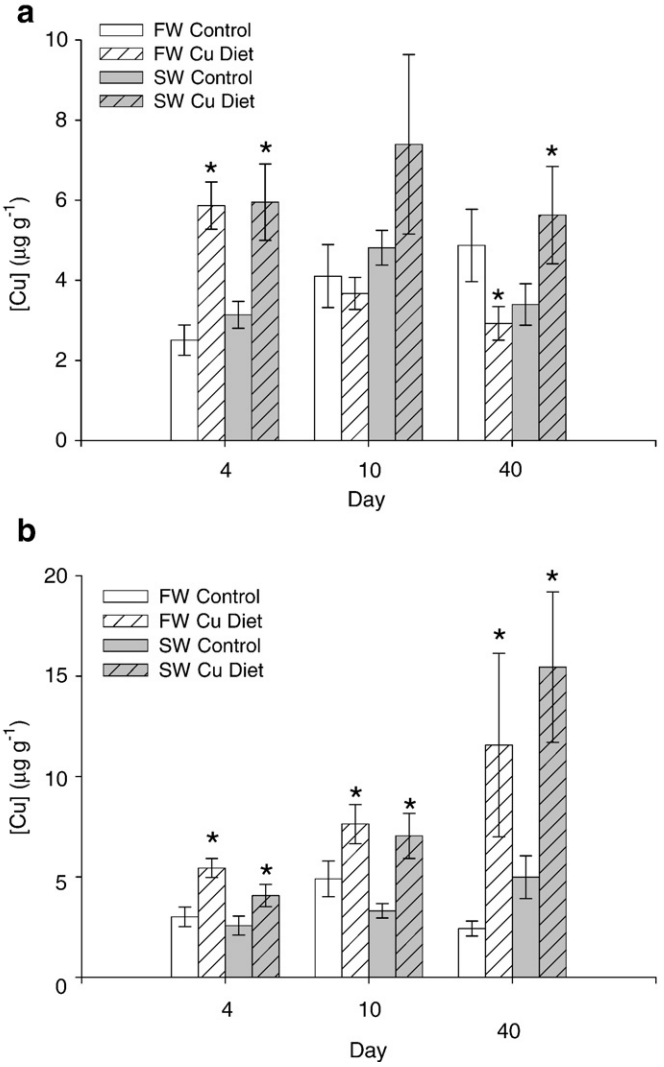

Fig. 3. Intestinal [Cu] in female (a) and male (b) killifish exposed to a control oyster diet and a high Cu diet (hatched bars, see Table 3 for [Cu]) for 40 days in FW (clear bars) and SW (grey bars). "*" indicates significant differences from the simultaneous control ($n = 5$).

corresponding control. In SW, the [Cu] were elevated at all days and the concentration was similar, however they were only significantly elevated relative to their control at day 4 and 40.

3.2.3. Gut fluid chemistry

The pH, [Cl^-], and [K^+] of the gut fluids were not different in the two salinities (Table 5). However, the total CO_2, [SO_4^{2-}], [Ca^{2+}], and [Mg^{2+}] were significantly higher in the SW acclimated fish's gut fluid than the FW acclimated fish's gut fluid, while the [Na^+] was higher in FW fish gut fluid.

4. Discussion

4.1. Oysters

The oysters accumulated Cu even at low ambient Cu concentrations (2 fold increase in tissue concentration in the control oysters at 2.9 ± 0.7 μg Cu L^{-1}), and internal concentrations increased with increasing ambient Cu. The accumulation rate was higher in the control, 4, and 11 μg Cu L^{-1} groups in the final thirty days than during the first sixty which could potentially be explained by an increase in temperature over this period as the experiment was started in May and ended in August.

The subcellular fractionation of the oysters changed over 90 days, however there are no clear trends with time or concentration. It appears that at high whole body [Cu] the organism may detoxify a proportionally larger fraction of Cu in metallothionein-like protein, while the fraction detoxifying as metal rich granules fraction remains unchanged (Table 4). However, this increase in the proportion of Cu in the metallothionein-like protein fraction has no effect on the trophically available metal due to a simultaneous decrease in the proportion of Cu in the enzymatic fraction. This response is consistent with an organism trying to protect itself from the deleterious effects of a high Cu body burden. At higher exposure concentrations or longer time periods, the proportional increase in the metallothionein-like protein fraction and decrease in the enzyme fraction may not offset each other directly leading to a potential increase in the trophically available metal fraction as proposed by Wallace et al. (2003). However, at [Cu] below 11 μg L^{-1}, total [Cu] and the trophically available metal fraction are well correlated in these experiments. Therefore, the [Cu] in the trophically available fraction will be no better at predicting toxicity or accumulation than whole body [Cu] in this organism at low ambient [Cu].

4.2. F. heteroclitus accumulation

In this study, Cu accumulated in the intestine and to some extent the gill. The gill has been noted to be a site of accumulation for dietary Cu in several studies (Miller et al., 1993; Kamunde et al., 2001, 2002a). Branchial Cu accumulation could have come from the water but the [Cu] in the control and high Cu diet tanks did not differ (Table 2). An alternate explanation for branchial Cu accumulation during dietary exposure is that Cu is accumulated from the diet via intestinal absorption and subsequent transfer via the blood to the gills. In support of this proposal is a previous study demonstrating Cu accumulation in the gill from the blood side (Grosell et al., 2001).

The intestine is also a site in which dietary Cu accumulates commonly, however there are two distinct patterns of accumulation. In some cases, the intestine rapidly accumulates Cu and then the Cu remains at the same level throughout the exposure (Berntssen et al., 1999a,b; Kamunde et al., 2001, 2002a,b; Kamunde and Wood, 2003). At other times, the Cu concentrations increase throughout the exposure period (Berntssen et al., 1999a,b; Kamunde et al., 2001, 2002a; Kjoss et al., 2005) as seen in the male intestinal tissue from the present study.

The liver is also a site in which many studies have seen accumulation of dietary Cu (Miller et al., 1993; Berntssen et al., 1999a,b; Kamunde et al., 2001, 2002a,b; Kamunde and Wood, 2003; Kjoss et al., 2005), but, as seen in this study, there are also instances in which this does not occur (Handy, 1992). Therefore, the tissue specific patterns of accumulation of dietary Cu observed here with a naturally incorporated Cu diet are not dissimilar to those seen for diets spiked with Cu salts. Although the patterns are similar, there appear to be no relationships between Cu dose, exposure duration, or salinity in regards to the amount of Cu accumulation. A review of the existing literature revealed that over a wide range of exposure times and concentrations no trends in accumulation exist between studies for the intestinal, hepatic, or branchial [Cu] (Fig. 4a, b, and c). The studies used in this analysis utilized different species, life stages, and exposure regimes which could account for some of the observed variability. But overall, the accumulation of Cu does not appear to be a good indicator of dietary Cu exposure and diets spiked with Cu salts appear to behave similarly to the naturally incorporated Cu diet used in the present study in that accumulation appears to be highly variable and occurs in the same tissues.

From the patterns of accumulation seen here it appears that Cu from dietary sources is dealt with differently by fish when compared to waterborne Cu (Blanchard and Grosell, 2005). During waterborne exposure Cu accumulates in the liver in both FW and SW, the gill in FW, and in the intestine in SW (Blanchard and Grosell, 2005) while liver accumulation is not always seen in dietary Cu exposures (Handy, 1992). The reason for the difference in liver Cu accumulation could be that the form of Cu taken up in waterborne versus dietary exposures is different. During waterborne exposures, free ionic Cu is the form which is most likely to be taken up (Paquin et al., 2002), whereas in dietary studies the Cu could likely be taken up complexed to amino acids (Wapnir, 1998; Glover and Wood 2008). Also because fish can receive a large portion of their nutritive Cu from their diet (Kamunde et al., 2002a), there is the potential that the cellular pathways used to transport this Cu are different from the pathways utilized by the gill perhaps leading to different internal dispositions of the Cu in the various tissues. Regardless of what leads to the differences in the pattern of accumulation, there are differences in the physiological effects of the Cu suggesting that there truly is a difference between Cu obtained through the diet and from the water. This is exemplified in a study by Miller et al. (1993) which demonstrates a difference increased resistance to waterborne Cu exposure after pre-exposure to waterborne Cu but not after pre-exposure to dietary Cu. This occurred even though gill Cu accumulation was attributable to the dietary Cu exposure.

Table 5
Physical and chemical characteristics of gut fluid collected from killifish 8 h after feeding in both FW and SW (mean ± SEM).

	FW (0.16 ppt)	SW (35 ppt)
pH	7.37 ± 0.14	7.69 ± 0.15
[Na^+] (mM)	115.3 ± 5.5	62.6 ± 5.4 *
[K^+] (mM)	7.5 ± 0.9	5.7 ± 0.6
[Mg^{2+}] (mMl)	3.4 ± 0.3	106.6 ± 11.5 *
[Ca^{2+}] (mM)	2.3 ± 0.3	10.9 ± 0.9 *
HCO_3^- (mM)	29.0 ± 4.9	43.8 ± 4.5 *
[Cl^-] (mM)	32.5 ± 6	30.7 ± 3.1
[SO_4^{2-}] (mM)	3.3 ± 0.9	40.3 ± 4.9 *

"*" indicates a significant difference between the two salinities ($n = 5$).

4.3. Intestinal Cu accumulation and salinity

In male fish, Cu accumulation in the intestine did not differ between the two salinities. In females, there were differences by day 40, but the amount of Cu in the intestine was much lower than in males at this point probably because female fish ate less after day 10. Food intake was not quantified, but the amount of food that had to be siphoned from the tanks containing females increased after day 10. Therefore, the changes are probably due to differential feeding rather than salinity.

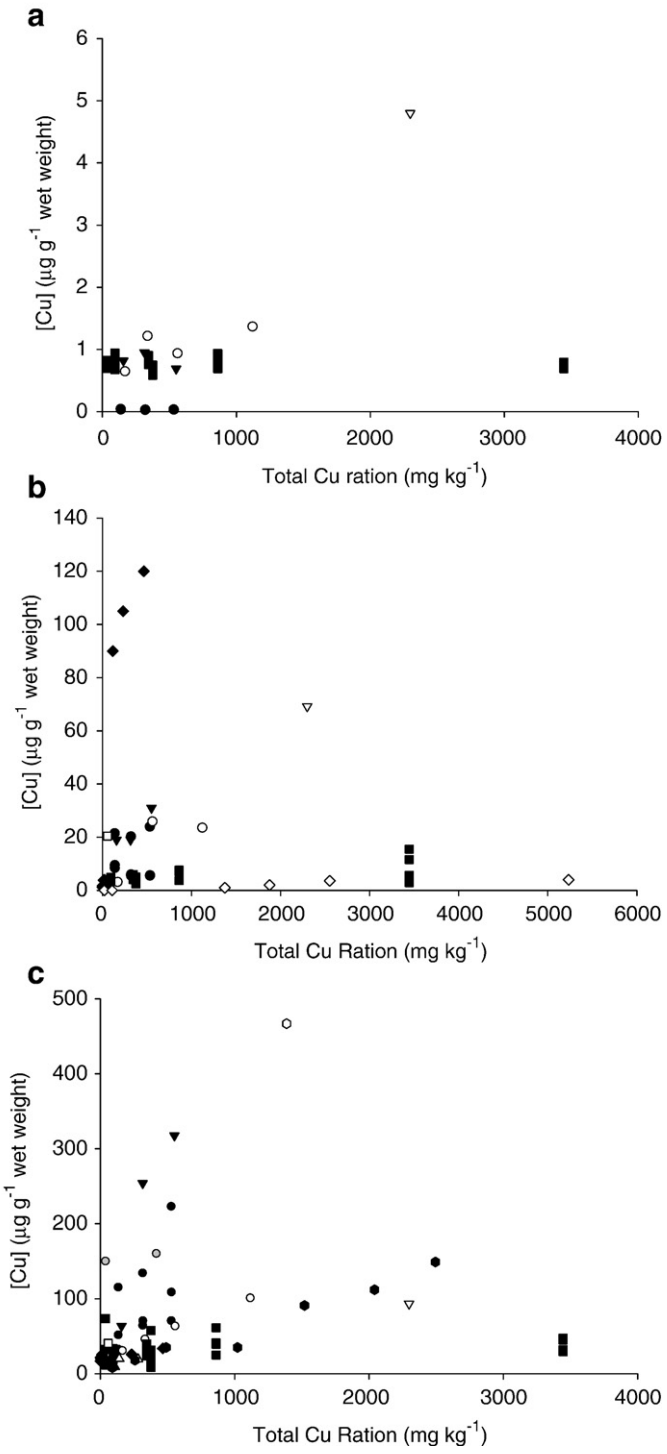

Fig. 4. [Cu] (µg g^{-1} wet mass) in the gill (a), intestine (b), and liver (c) of fish as a function of total dietary Cu ration (mg kg^{-1}) which is the product of the daily dietary dose and the days of exposure. Data were compiled from the following sources: black circle = Kamunde and Wood (2003), clear circle = Kamunde et al. (2001), black inverted triangle = Kamunde et al. (2002b), clear inverted triangle = Miller et al. (1993), black square = current study, clear square = Handy (1992), black diamond = Berntssen et al. (1999a), clear diamond = Berntssen et al. (1999b), black trangle = Murai et al. (1981), clear triangle = Overnell and McIntosh (1988), black hexagon = Julshamn et al. (1988), clear hexagon = Knox et al. (1984), grey circle = Knox et al. (1982). Dry weight values were corrected to wet weight assuming 80% water in the tissue for Berntssen et al. (1999a,b). Values from Kamunde and Wood (2003) for the intestine are the average of the values given for the four sections of the gut.

The similarity between the two salinities however was not because of similarities in the gut fluid chemistry (Table 4). Unlike the Taylor et al. (2007) study, we found differences in several of the intestinal parameters measured. The SW fish had higher [Mg^{2+}], [Ca^{2+}], and [SO_4^{2-}] than the FW fish because SW taken in with the food has much higher concentrations of these ions than FW (see Table 1) and these ions are not actively taken up in the gut (Marshall and Grosell, 2005). The [HCO_3^-] was also significantly higher in the SW intestine because of active HCO_3^- excretion by the gut in SW which is thought to play a role in SW osmoregulation (Wilson, 1999; Marshall and Grosell, 2005). Surprisingly, this had no significant effect on intestinal fluid pH suggesting the presence of non-HCO_3^- buffering in the intestinal lumen.

The [Cl^-] were not different in spite of a ~200 fold difference in the ambient Cl^- which suggests high rates of Cl^- uptake in the SW intestine to aid water absorption and facilitate HCO_3^- excretion through Cl^-/HCO_3^- exchange (Marshall and Grosell, 2005). The [Na^+] was higher in FW than in SW, which is caused by the active absorption of Na^+ by the SW intestine to aid water uptake (Marshall and Grosell, 2005). In FW, the gill is the site of Na^+ uptake to maintain homeostasis (Patrick et al., 1997; Marshall and Grosell, 2005) and the intestine plays a much smaller role. The difference between the [Na^+] and [Cl^-] occurs because killifish do not actively take up Cl^- at the gill (Patrick et al., 1997) and in FW the intestinal Cl^- uptake rate must be fairly high in order ionoregulate (Scott et al., 2006). Thus, some of the Cl^- from the food is taken up in FW leaving no difference between the two salinities.

Since the fish used in the present study were fed the same diet regardless of exposure salinity, the organic matrix of the intestinal fluids can reasonably be assumed to be the same regardless of salinity. However, the differences in inorganic gut fluid chemistry between FW and SW discussed above have a profound effect on the speciation of Cu. Intestinal as well as internal Cu accumulation would therefore be expected to be different if inorganic speciation and competition controlled accumulation. Inorganic speciation calculations predict an ~11 fold difference in the free ion concentrations based on the gut fluid composition (Table 6) (Millero and Pierrot, 1998). Therefore, it is unlikely that Cu is being taken up solely as the free ion. Amino acid Cu complexes have been demonstrated to be taken up at different rates than both the amino acid alone and free Cu^{2+} (Wapnir, 1998; Glover and Wood, 2008). These and similar complexes are likely to account for the Cu uptake observed due to the lack of pattern attributable to either inorganic speciation or competition.

It is also important to note that even without feeding, SW fish drink the ambient water and during waterborne exposures in SW will be exposed to waterborne Cu. The values presented in Table 4 are generally similar to the gut fluid of unfed fish (Marshall and Grosell, 2005). The exceptions to this are the pH which in an unfed fish is higher (>8.0) and the HCO_3^- which is also generally higher. Table 6 (Millero and Pierrot, 1998) shows that Cu speciation in ambient SW and the intestinal fluid differ substantially with an ~35 fold difference in the free Cu^{2+} ion concentration between these two and the differences in gut fluid composition between unfed and fed fish should increase this difference further. It is likely that intestinal Cu accumulation during waterborne Cu exposures will be better correlated with Cu speciation in the gut fluid rather than the ambient SW.

Table 6
Calculated Cu speciation in the gut fluid of killifish in FW and SW using the values presented in Table 4 (Millero and Pierrot, 1998).

	FW gut fluid	SW gut fluid	FW	SW
Cu^{2+}	1.59%	0.14%	1.34%	4.93%
$CuOH^+$	0.19%	0.04%	2.17%	4.10%
$Cu(OH)_2$	0.02%	0.02%	2.28%	2.50%
$CuHCO_3^+$	0.84%	0.08%	0.11%	0.09%
$CuCO_3$	66.83%	22.16%	84.92%	68.35%
$Cu(CO_3)_2^{2-}$	30.42%	77.50%	9.12%	19.23%
$CuHS^-$	0.00%	0.00%	0.00%	0.00%
$Cu(HS)_2$	0.00%	0.00%	0.00%	0.00%
$CuSO_4$	0.13%	0.07%	0.05%	0.79%

5. Conclusions

The proportion of Cu in the trophically available metal fraction of oysters exposed to low levels of waterborne Cu for a prolonged period did not change Because of this, total [Cu] and the trophically available metal fraction will correlate equally well with assimilation efficiency and thus trophic transfer. However, as may be expected, the proportion of Cu in the biologically detoxified metal fraction may increase while it decreases in the metal sensitive fraction. The existing data suggest that there are no apparent differences between naturally incorporated and spiked diets and that dietary copper dose, duration, and salinity are unrelated to Cu accumulation. It appears that Cu assimilated from the diet is treated differently from waterborne Cu based on patterns of accumulation and acclimatory responses and therefore may have different toxic modes of action. Finally, the uptake of dietary Cu from a natural diet is not governed by inorganic speciation and is therefore unaffected by salinity.

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