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# The role of dissolved organic carbon in moderating the bioavailability and toxicity of Cu to rainbow trout during chronic waterborne exposure☆

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**Abstract**

We examined the influence of dissolved organic carbon (DOC) on the bioavailability of waterborne Cu to rainbow trout (*Oncorhynchus mykiss*) during chronic sublethal exposure. Juvenile rainbow trout were exposed to Cu (as $CuSO_4$) and DOC as humic acid (HA, as sodium salt) for one month in synthetic soft water to give treatments with varying combinations of free ionic and HA complexed Cu. The total Cu concentration was 7 μg/l for all treatments (except controls) and HA was added at levels of 0, 2.5 and 7.5 mg/l which corresponded to DOC levels of 1.2, 2.2 and 4.0 mg/l. Fish grew well in all treatments and no mortalities occurred. Cu was highly bioavailable in the treatment with no added HA; gill and liver Cu accumulation occurred as well as a disruption of $Na^+$ regulation. In Cu treatments with additions of both 2.5 and 7.5 mg/l HA, there was no significant tissue accumulation of Cu. The addition of HA alleviated and delayed the disruption of iono-regulatory mechanisms. A recovery of plasma $Na^+$ losses was observed and this was associated with an increase in gill $Na^+/K^+$ ATPase activity by the end of the exposure. Following the month of chronic exposure the uptake and turnover rates of Cu at the gills and into various tissue compartments were measured through radioisotopic techniques ($^{64}Cu$). While chronic Cu exposure did not result in acclimation (i.e. increased LC50), the uptake rate and extent of Cu uptake into the gills and liver was increased. This study demonstrates that growth and tissue accumulation of Cu are poor predictors of the chronic effects of Cu, and illustrates that HA moderates chronic Cu bioavailability. The lack of a link between Cu bioaccumulation and Cu impact and the role of organic matter in reducing the bioavailability of Cu are important considerations in the context of ecological risk assessment.


*Keywords:* Copper; Bioaccumulation; Bioavailability; Organic matter; DOC; Physiology; Metal uptake; Ion regulation; Chronic toxicity; Biotic ligand model

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## 1. Introduction

There have been dramatic advancements in the understanding of acute Cu toxicity and of relationships between the aquatic geochemistry of Cu, its bioaccumulation, and toxic response. These advances have built on the conceptual framework of Pagenkopf (1983) and the applied geochemical and biological approaches of Playle et al. (1993a,b), Campbell (1995) and MacRae et al. (1999) to produce biotic ligand models (BLM, see review in this issue). The BLM approach to predicting acute toxicity on a site specific basis has proven to be successful for Cu (Di Toro et al., 2001; Santore et al., 2001) as well as Ag (Paquin et al., 1999; McGeer et al., 2000c) and continues to be developed for other metals. Extension of the BLM principles to chronic toxicity through the development of longer term tissue-specific metal residue approaches has been identified as a research priority (Bergman and Dorward-King, 1997) and recent studies with Cu such as Kamunde et al. (2002), Grosell et al. (1997, 1998, 2001), Taylor et al. (2000) and McGeer et al. (2000a,b) have begun to explore approaches. However, much remains to be elucidated, particularly with respect to the effect of water chemistry on Cu bioavailability during chronic exposure. In this regard the work of Taylor et al. (2000) has illustrated the effects of chronic Cu exposure in hard water vs. soft water on a number of whole animal and physiological endpoints.

As expressed through the BLM, the uptake and toxicity of Cu is influenced by complexation reactions in the water column as well as competitive binding at the site of uptake (MacRae et al., 1999; Marr et al., 1999; Santore et al., 2001). In acute exposure experiments, complexation of Cu by dissolved organic carbon (DOC) has been shown to reduce Cu binding to the gills (Playle et al., 1993a; Hollis et al., 1997; Marr et al., 1999) and thereby reduce acute toxicity in fish via reductions in free ionic waterborne $Cu^{2+}$ (Erickson et al., 1996). In addition to reductions in toxicity and gill Cu accumulation, the study of Richards et al. (1999) illustrated that DOC complexation (in exposures to waterborne Cu and Cd mixtures) reduced the effects of acute exposure, particularly those related to ion regulation (e.g. plasma $Na^{+}$) and stress (plasma glucose) in rainbow trout (*Oncorhynchus mykiss*). In that study, DOC from natural organic matter sources and commercially available DOC (humic acid) were both effective in reducing physiological impacts. The alleviation of the sublethal effects of chronic Cu toxicity by DOC has been less well studied, particularly in fish. However, fresh water invertebrate studies on chronic Cu exposure have shown that both toxicity and bioaccumulation of Cu is reduced as DOC concentration increases (Winner, 1985; Kim et al., 1999). Given the work of Ma et al. (1999) it is possible that a number of the prior studies on the effects of DOC on waterborne Cu have underestimated Cu–DOC interactions due to the extended equilibration time needed for these interactions to occur. While the influence of DOC on acute toxic responses is clear, its role in modulating chronic sublethal effects, metal accumulation and the physiological response to long term exposure in fish is not well understood.

The general process of acclimation to Cu during chronic sublethal waterborne exposure has been characterized by a 'damage-repair' model (McDonald and Wood, 1993) comprising three phases: an initial 'shock' phase, a recovery phase, and then acclimation itself, the latter including an increased tolerance which persists indefinitely during continued exposure. The initial 'shock' phase corresponds to a period of physical damage, primarily at the gill, and assorted disturbances of internal physiological homeostasis (McGeer et al., 2000a). The damage phase is usually short-lived (a few days). Thereafter, recovery starts coincident with increased biosynthetic processes (mitosis, enhanced protein synthesis) which help repair the damage and correct the physiological disturbances. Inherent within the recovery phase is mobilization of metal binding proteins such as metallothionein (Bradley et al., 1985; McCarter and Roch, 1983) and an up-regulation of other pathways to counteract or compete with the deleterious effects of the metal, including those related to ion regulation (Laurén and McDonald, 1987a,b; McGeer et al., 2000a). Ultimately, the internal physiology of the animal either returns to the pre-exposure condition or, a new equilibrium is established during the final period of increased tolerance. In these circumstances, the deleterious effects of long term exposure may be minimal (Taylor et al., 2000; McGeer et al., 2000a).

The objectives of this study were to develop an understanding of the possible role of DOC in moderating the physiological effects of chronic sublethal waterborne Cu exposure. Juvenile rain-

bow trout were exposed to a total Cu concentration of 7 μg/l (0.11 μmol/l) in synthetic soft water with different concentrations of DOC, provided as commercially available (Aldrich) humic acid (HA). The Cu exposure levels were environmentally relevant (USEPA, 1985; CCME, 1999) and the experimental design resulted in treatments with varying exposure concentrations of free $Cu^{2+}$ and HA-complexed Cu (HA–Cu) under a constant total copper exposure regime. Physiological variables related to growth, ion regulation, stress response and bioaccumulation were selected to track the relative effects of the different treatments within the context of the damage—repair—acclimation response.

## 2. Methods

Juvenile rainbow trout (approx. 10 g) were purchased from a local supplier and reared in 400 l polyethylene tanks supplied with flowing dechlorinated Lake Ontario water. Prior to experimentation, trout were acclimated to flowing soft water (pH of 6.7, $Na^+ = 0.06$ mM, $Cl^- = 0.06$ mM, $Ca^{2+} = 0.12$ mM and DOC = 1 mg·$l^{-1}$) at 14 °C. Synthetic soft water was produced by mixing reverse osmosis water (BW3030 membranes, Osmonics Inc.) with Lake Ontario water in an approximate ratio of 10:1. Following a two month acclimation to synthetic soft water (see Taylor et al., 2000), trout ($n = 450$) were non-selectively distributed to the experimental system. The experimental system consisted of nine 200 l polyethylene tanks ($n = 50$ trout per tank), each of which was supplied with a 1 l per min flow of soft water. During acclimation and experimental exposure periods trout were fed a commercial dry ration (Martin's Feed Mill Ltd. Elmira ON) at 2% of body weight per day, and tanks were cleaned and checked for mortalities daily.

Following distribution to the experimental system but prior to Cu exposure, 10–15 individual fish were non-selectively removed from each tank, lightly anaesthetized (75 mg/l MS222 buffered with $NaHCO_3$), wet weight was measured, and then trout were each implanted with a passive integrated transponder (PIT) tag (Destron Fearing, St. Paul, MN). PIT tags were implanted in the peritoneal cavity and the adipose fin removed so that tagged fish could be easily identified at future weighing events. Fish were allowed to acclimate to the experimental system and recover from tagging for 6 days before Cu exposure began. Of the 115 PIT tagged fish, all survived the tagging procedure, and no moribund individuals were observed.

### 2.1. Experimental treatments

All trout, with the exception of controls, were chronically exposed to a nominal concentration of 7 μg/l (0.11 μmol/l) Cu for 30 days. Copper exposures were achieved by continually mixing appropriate volumes of concentrated Cu (as $CuSO_4 \cdot 5H_2O$) into the flowing soft water via a metering pump or from a mariotte bottle (see below for further details). The Cu exposure level was chosen to provide a physiological challenge with minimal acute mortalities in these softwater exposures. The selection of 7 μg/l was based on the results of a challenge test just prior to initiating the chronic exposure. The challenge test exposed the soft water acclimated fish to a nominal concentration of 12 μg/l (measured concentration was 11.5 μg/l) for 96 h and resulted in a mortality rate of 70%.

Within Cu exposure regimes, trout were exposed to DOC through the addition of either 0, 2.5 or 7.5 mg/l HA (Aldrich, as a sodium salt). Therefore, treatments consisted of controls (no added Cu and no added HA), Cu exposed with no added HA, Cu exposed with 1 mg/l added DOC and Cu exposed with 3 mg/l added DOC. Additions were adjusted assuming HA is 40% DOC and thus, additions of 2.5 mg/l HA delivered 1 mg/l DOC and 7.5 mg/l HA delivered 3 mg/l DOC. For each of these four treatments groups of 50 trout were exposed, in duplicate. Additionally an extra control group consisting of one tank of 50 fish was exposed to 7.5 mg/l HA without added Cu. The HA exposures were achieved as described for Cu (above) and for combined HA and Cu exposures the concentrated solutions were mixed together for at least half a day and then up to 7 days to ensure sufficient time for complexation interactions (see Ma et al., 1999) prior to delivery into the fish tanks.

The HA levels were chosen to provide reduced levels of $Cu^{2+}$ through complexation (see Table 1). HA was chosen based on pragmatic considerations related to three factors. The first was the large amount of material required for the single pass continuous flow exposure conditions. The second was the need for a reliable and consistent

Table 1
Measured total Cu and dissolved organic carbon (DOC) concentrations as well as pH and modeled* Cu speciation in experimental exposure treatments of Cu and humic acid (HA)

| | Control | Control + 7.5 mg/l HA | Cu-no added HA | Cu + 2.5 mg/l HA | Cu + 7.5 mg/l HA |
|---|---|---|---|---|---|
| Total Cu (nmol/l) | 2.7 | 3.9 | 108 | 111 | 111 |
| DOC (mg C/l) | 1.2 | 4.0 | 1.2 | 2.2 | 4.0 |
| pH | 6.71 | 6.81 | 6.72 | 6.71 | 6.75 |
| $Cu^{2+}$ (nmol/l) | 0.016 | 0.009 | 1.08 | 0.61 | 0.31 |
| CuDOC (nmol/l) | 2.03 | 3.55 | 63.9 | 86.3 | 98.4 |
| $Cu(OH)_2$ (nmol/l) | 0.62 | 0.32 | 41.3 | 23.2 | 11.8 |
| $CuOH^+$ (nmol/l) | 0.025 | 0.013 | 1.7 | 0.95 | 0.48 |

* *Note*: The number of binding sites for DOC was set at 100 nmol binding sites per mg C. Concentrations of $Cu^{2+}$, CuDOC, CuOH and $Cu(OH)_2$ were modeled using MINEQL+ (ver 4.0 Schecher and McAvoy, 1992) using the additional conditional equilibrium constants from Hollis et al., 1996; (Cu–DOC of 9.1 and H–DOC of 5.4) and the inorganic copper constants of De Schamphelaere and Janssen (2002). Note that other minor Cu complexes are not reported and have concentrations below $10^{-12}$ M.

source of organic carbon over the 30 days of exposure and the third factor was cost. As such the choice of Aldrich HA as a source of DOC was consistent with understanding the effects of DOC complexation on the chronic impact of Cu but it must be recognized that HA may not be representative of a naturally occurring organic material. Measured Cu levels were $6.87 \pm 0.04$ μg/l (mean ± S.E.M., $n = 17$) for Cu exposures without added HA, $7.04 \pm 0.04$ μg/l ($n = 17$) for Cu exposures with 2.5 mg/l added HA and $7.04 \pm 0.03$ μg/l ($n = 17$) for Cu exposures with 7.5 mg/l added HA. The waterborne Cu concentrations in control waters were $0.17 \pm 0.01$ μg/l ($n = 15$) with no added HA and $0.25 \pm 0.02$ μg/l ($n = 15$) when 7.5 mg/l HA was added. Background DOC concentrations in control water was $1.2 \pm 0.06$ mg/l ($n = 6$) while with 2.5 mg/l added HA it was $2.2 \pm 0.22$ mg/l ($n = 6$) and for 7.5 mg/l added HA it was $4.0 \pm 0.10$ mg/l ($n = 6$). A listing of treatments and the associated Cu speciation is given in Table 1.

The contribution that Aldrich HA made to waterborne $Na^+$ levels was slight in the 2.5 mg/l added HA treatment. However, the addition of 7.5 mg/l HA resulted in waterborne $Na^+$ concentration that were increased by approximately 20% (by 0.01 mmol $Na^+$/l) over the background soft water $Na^+$ level. Even though this increase in waterborne $Na^+$ concentration was minor, sufficient $Na^+$ (as NaCl) was added to the stock solutions of the control with no added HA and the Cu exposure with no added HA treatments. This ensured that trout were exposed to similar $Na^+$ concentrations (0.07 μmol/l).

### 2.2. Sampling protocol

On the day prior to the exposure (day 0) and then on days 7, 14, 21 and 28, all of the fish in each tank were bulk-weighed and individual PIT tagged fish were separated from the rest of the fish and lightly anaesthetized (see above). The PIT tag was then electronically identified and the fish was weighed to the nearest 0.1 g before being returned to the tank. Weighing was done before the first feeding of the day. At regular intervals water samples (10 ml) were collected and acidified with 100 μl of $HNO_3$ (trace metals grade, Fisher Scientific, Nepean ON) for Cu analysis. Sample collection was frequent (daily) over the first week to ensure a reliable exposure regime and then every 2–3 days for the rest of the exposure. Separate water samples were collected weekly and frozen at −20 °C for subsequent DOC analysis.

Tissue and blood samples were collected on days 2, 5, 15 and 29 of exposure. Trout were non-selectively netted from tanks (equal numbers from each replicate), anaesthetized in 200 mg·$l^{-1}$ buffered MS222 and euthanized. Eight trout from each treatment were usually sampled but in one case, the controls with 7.5 mg/l added HA, only six fish were sampled and only on day 29. A sample of blood was drawn from the caudal vasculature into a heparin-rinsed 1 ml syringe via a 22G needle. The blood sample was centrifuged at $10\,000 \times g$ rpm for 3 min, plasma separated and then frozen for further analysis. Gills were excised, vigorously rinsed in deionized water for 10 s, blotted dry and then two separate samples were saved. One gill sample was saved for total Cu

measurement, and the other was immediately frozen in liquid $N_2$ and stored at −70 °C for subsequent $Na^+/K^+$ ATPase activity measurements. Liver and kidney samples were also collected and along with gill samples were weighed and saved for later Cu analysis. Note that due to the shortage of fish, tissue and blood sampling for the extra control group (7.5 mg/l HA without added Cu) was conducted only on day 29.

Beginning on day 30, isotopic methodologies were applied to measure the uptake of 'new' Cu into chronically exposed trout. Groups of 24 fish were removed from each treatment and placed into a 150 l recirculating system initially containing 36 μCi/l of $^{64}$Cu with nominal total Cu concentrations of 12 μg/l. When adding the isotope (specific activity 5 μCi/μg Cu), its contribution to the total waterborne Cu concentration was accounted for and confirmed by measuring Cu content of the system after addition of isotope and before addition of fish. The recirculating system consisted of a 20 l header tank which distributed water evenly to six 18 l fish tanks and a 20 l sump tank that collected outflow from the fish tanks and subsequently fed water back to the head tank via a submersible pump (Little Giant). Aeration was provided in the head tank and in each of the six tanks with fish. The $^{64}$Cu was obtained by irradiation of solid $CuCl_2$ at the McMaster University Nuclear Research Reactor. Fish were maintained in one of six 18 l tanks in the exposure system and at 8, 20, 32 and 50 h, samples of 6 fish were removed, rinsed in clean water for 5 min, and then euthanized prior to sampling of gills and liver as previously described. Three sequential series of $^{64}$Cu trials were done, the first being in soft water with 13.5 μg/l Cu (measured, no HA added) where controls and Cu only exposed trout were tested. A second trial was in soft water with 13.4 μg/l Cu (measured) and 7.5 mg/l HA where the fish that were similarly exposed and the high HA controls were tested. The final trial was in soft water with 12.4 μg/l Cu (measured) and 2.5 mg/l HA where trout chronically exposed to Cu and 2.5 mg/l HA were tested. The Cu level chosen for these trials was designed to match the EC50 value for Cu in the most bioavailable conditions (no added HA, see below) and permit an evaluation of chronic Cu exposure within similar DOC treatments, particularly for the no added HA (but also at 7.5 mg/l added HA). This experimental design also allowed an assessment of the effect of DOC within similar chronic Cu exposure treatments.

After the month of chronic exposure, responses to acutely lethal levels of Cu were tested for determination of 96 h LC50 values. LC50 values were measured: in water with no added HA (controls and 7 μg/l Cu with no added HA were tested at concentrations of 7, 9, 12, 18 and 25 μg Cu/l) or in water with 2.5 mg/l added HA (the group exposed to 7 μg/l Cu with 2.5 mg/l added HA was tested at 7, 25, 50 and 75 μg Cu/l) or in water with 7.5 mg/l HA added (high HA controls groups and 7 μg/l Cu with 7.5 mg/l added HA groups were tested at 7, 50, 75, 100 and 150 μg Cu/l). Therefore, LC50 tests were done using the underlying exposure water and at least 6 fish were exposed to each concentration. In all cases, LC50 tests were done under flow-through conditions (see Taylor et al., 2000 for details) and mortality checks were done twice per day. As with the isotopic trials, the design allowed for an assessment of the effect of Cu exposure within DOC treatments as well as the effect of DOC within Cu exposures.

### *2.3. Measurements*

Plasma total ammonia (Tamm) concentration was measured with a commercial kit (Sigma 171 UV) and plasma cortisol was measured via radioimmunoassay (Immunocorp). Plasma $Na^+$ concentrations were determined by atomic absorption spectrophotometry (AA1275 Varian) and plasma $Cl^-$ via the colorimetric method of Zall et al. (1956). In some cases, there was insufficient plasma to allow all samples to be measured for each parameter. Frozen gill filament samples were thawed, homogenized and then assayed for $Na^+/K^+$ ATPase activity using the methods of McCormick (1993) and associated protein content was assayed using the dye binding technique of Bradford (1976). Spectrophotometric assays were modified for use with 96- well microtitre plates and a microplate reader (Spectramax 340, Molecular Devices). DOC content of water samples was measured via a total organic carbon analyzer (Shimadzu TOC 5050A, Mandel Scientific Co. Ltd., Guelph).

Gill, kidney and liver tissue samples were digested in 5 volumes of 1 N $HNO_3$ (Janes and Playle, 1995), and the total Cu concentrations of digested tissue and water were measured by graphite furnace atomic absorption spectrophotometry

(AA1275 and GTA-95, Varian). The gamma activity from $^{64}$Cu in tissue samples, plasma samples and water was determined using a gamma counter (MINAXI gamma Auto-gamma 5000 series, Canberra–Packard). The measured cpm value was corrected for the short half-life of $^{64}$Cu (12.4 h), a procedure done automatically by the instrument.

### 2.4. Calculations and statistics

Specific growth rate (SGR, % per day) was calculated for each individual PIT tagged fish for each weekly interval between weighing using the formula,

$$SGR = 100*[(Ln(wt._2)-Ln(wt._1))/t]$$

Where wt refers to the individual's weight at the start $wt._1$ and end $wt._2$ of the interval and t is the length of time of the interval in days.

Cu concentrations of tissue digests were adjusted for tissue weight and expressed as $\mu g\ Cu \cdot g^{-1}$ wet weight. Branchial $Na^+/K^+$ ATPase activity was expressed as the concentration of adenosine diphosphate (ADP) produced per unit time and adjusted for the protein content of the sample ($\mu$mol ADP·mg protein$^{-1}$·h$^{-1}$). The 96 h LC50 values were determined from the acute toxicity tests results using measured waterborne Cu concentrations and the US EPA Probit Analysis Program (ver. 1.5).

The uptake of $^{64}$Cu radioactivity into gills and liver was used to determine the total uptake of 'new' Cu by using the previous compartment specific activity approach described by Grosell et al. (1997). The newly accumulated Cu was calculated by the following equation:

$$a \cdot (b \cdot c^{-1})^{-1}$$

Where $a$ is the cpm from $^{64}$Cu in the tissue per unit weight, $b$ and $c$ are the cpm and total concentration of Cu (respectively) per unit volume in the previous compartment. The previous compartment was exposure water for the gills and plasma for the liver.

Data have been expressed as mean ± S.E.M. The effects of chronic Cu exposure under different HA regimes were compared to the corresponding control values at each sampling time by ANOVA. In all cases, $P < 0.05$ was the accepted level of significance, and where appropriate, Duncan's New Multiple Range Test was used for comparisons.

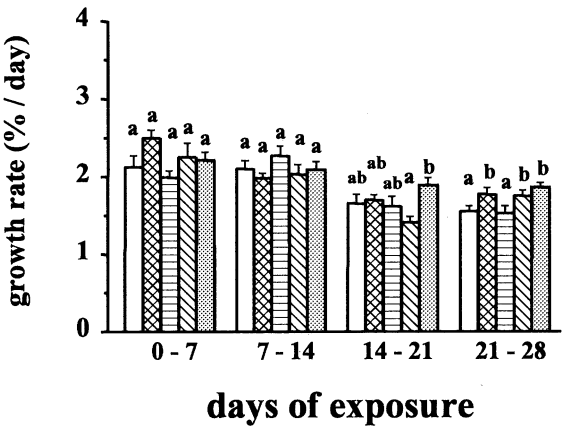


Fig. 1. The separate and combined effects of chronic waterborne Cu and HA on individual growth rates in rainbow trout. Growth rates were calculated over weekly intervals and fish were exposed to 7 μg/l Cu with either no added HA (cross-hatched bars), 2.5 mg/l added HA (horizontal lined bars) or 7.5 mg/l added HA (diagonally lined bars) for 28 days. Control groups were exposed to no added HA (open bars) or 7.5 mg/l added HA (shaded bars) and no Cu. Within each growth interval, bars labeled with the same letter are not significantly different ($P < 0.05$), mean ± S.E.M. are shown and $n \geq 23$ for each bar except for the 7.5 mg/l added HA control group where $n = 15$.

## 3. Results

### 3.1. Toxicity and growth

As a result of the chronic exposure level being at approximately 80% of the LC50 (see below) for the trout exposed to Cu without added HA, some survival and growth effects were expected. However, all fish in all treatments survived and grew well. There were some minor significant differences in SGR among the 5 treatments (Fig. 1), particularly in the final 2 weeks of exposure. In terms of the mean weight of individuals at each weekly weighing event there were no significant differences (data not shown). When the SGR values (Fig. 1) were calculated over the 28 day of exposure, there were no significant differences among the 5 treatments (SGR ranged from $1.85 \pm 0.07$, $n = 24$ to $2.00 \pm 0.07$, $n = 15$).

The response to acute challenges with Cu revealed the dramatic effect that HA can have on Cu toxicity. The 96 h LC50 values increased approximately 6-fold over the range of HA exposure (Fig. 2). There was no evidence of an acclimation response to the chronic exposure conditions as LC50 values for control and exposed fish within

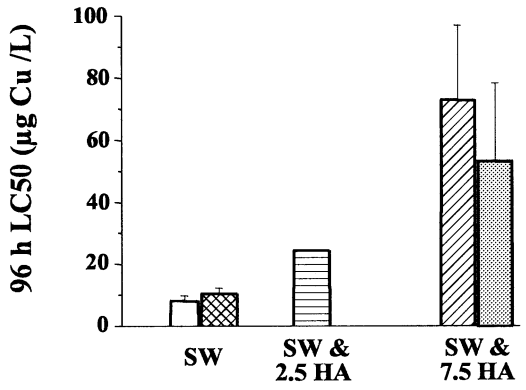


Fig. 2. Acute toxicity of waterborne Cu to rainbow trout in soft water (SW) and the effect of chronic Cu exposure and HA. The 96 h LC50 values are shown for trout previously exposed to 7 μg/l Cu with either no added HA (cross-hatched bar), 2.5 mg/l added HA (horizontal lined bar) or 7.5 mg/l added HA (diagonally lined bar) for one month. Control groups were exposed to soft water with no added HA (open bar) or 7.5 mg/l added HA (shaded bar) and no Cu. For each bar, the 95% CI is shown except for fish chronically exposed to 7 μg/l Cu with 2.5 mg/l HA added where the pattern of mortalities did not permit calculation of the interval.

the high and low HA exposure conditions were not significantly different.

### 3.2. Metal burden

Chronic exposure to waterborne Cu resulted in an increased tissue burden but only in gills and liver (Fig. 3). Gill Cu content was increased only for Cu exposures without added HA on days 15 and 29. The Cu content of kidney tissue was not significantly different from controls (Fig. 3b). Liver Cu content increased for fish chronically exposed to 7 μg/l Cu with no added HA and was significantly higher on day 15. Treatments where HA was added were associated with no increase in gill (Fig. 3a) or hepatic Cu content (Fig. 3c). Thus, the effect of HA on reducing the bioavailability of Cu was clearly evident.

### 3.3. Physiological effects

Chronic waterborne Cu exposure resulted in a significant reduction in plasma $Na^+$ content (Fig. 4a) on day 5. Plasma $Na^+$ declines were greatest for trout exposed to Cu without added HA, intermediate at 2.5 mg/l HA and not significant at 7.5 mg/l HA. Although not significantly different from controls, fish exposed to Cu at 7.5 mg/l HA showed a trend for reduced $Na^+$ that was delayed compared to other treatments. Plasma $Na^+$ declines were temporary in nature. Plasma $Cl^-$ showed trends of a similar pattern to plasma $Na^+$ but these were not significant (Fig. 4b) and occurred primarily at day 2. Gill $Na^+/K^+$ ATPase

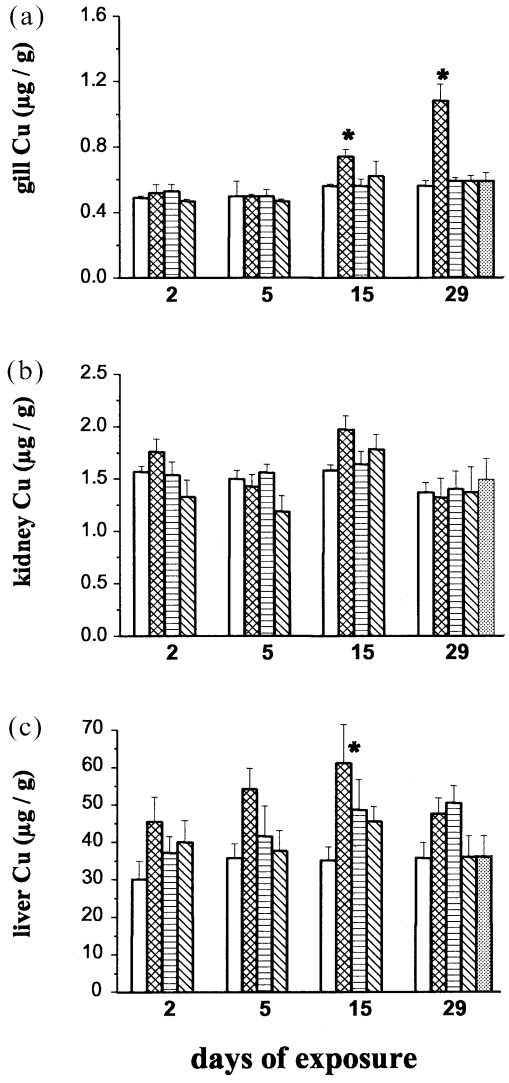


Fig. 3. The separate and combined effects of chronic Cu exposure and HA on Cu burden in gills (a), kidney (b) and liver (c) of rainbow trout. Mean tissue burdens are shown for fish in soft water exposed to 7 μg/l Cu with either no added HA (cross-hatched bars), 2.5 mg/l added HA (horizontal lined bars) or 7.5 mg/l added HA (diagonally lined bars) for up to 29 days. Control groups were exposed to soft water with either no added HA (open bars) or 7.5 mg/l added HA (shaded bar, day 29 only). A * indicates means significantly different from controls (open bar, $P<0.05$) on that day, error bars show S.E.M. and $n \geq 6$.

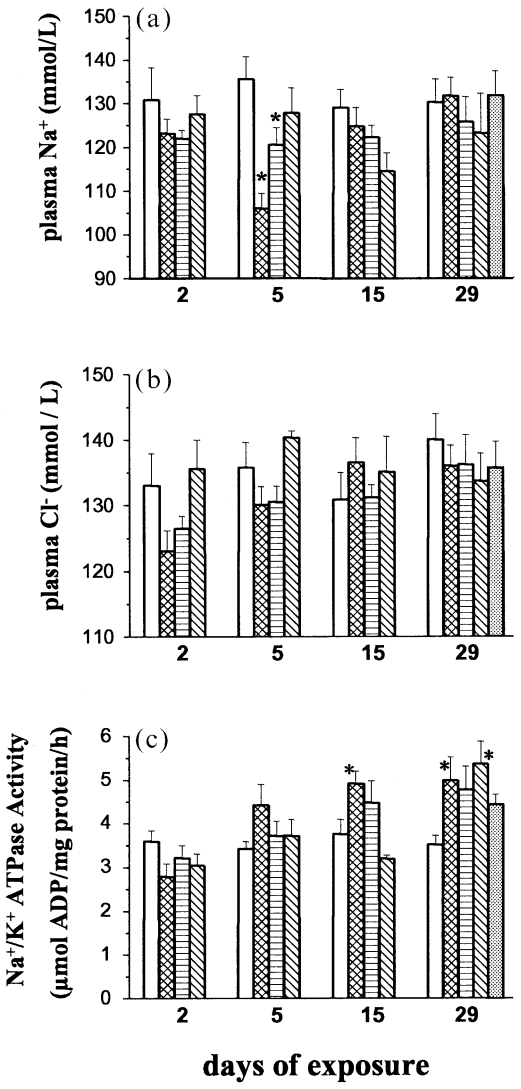


Fig. 4. Effect of chronic Cu exposure and HA on plasma $Na^+$ (a), plasma $Cl^-$ (b) and gill $Na^+/K^+$ ATPase activity (c) of rainbow trout. Mean values are shown for fish in soft water exposed to 7 μg/l Cu with either no added HA (cross-hatched bars), 2.5 mg/l added HA (horizontal lined bars) or 7.5 mg/l added HA (diagonally lined bars) for up to 29 days. Control groups were exposed to soft water with either no added HA (open bars) or 7.5 mg/l added HA (shaded bar, day 29 only). A * indicates means significantly different from controls (open bar, $P<0.05$) on that day, error bars show S.E.M. and $n\geq 6$.

activity was not significantly inhibited by Cu exposure (Fig. 4c) but a response to the plasma ion imbalances was observed. By day 15 trout exposed to Cu without added HA experienced an increase in $Na^+/K^+$ ATPase activity (Fig. 4c). Compared to the controls with no added HA, $Na^+/K^+$ ATPase activity was also significantly increased in fish exposed to Cu with 7.5 mg/l added HA. However, when compared to the high HA controls there was no difference in $Na^+/K^+$ ATPase activity levels in Cu exposed fish at high HA.

Plasma cortisol measurements were variable and all Cu exposed groups showed a trend towards higher levels, particularly on day 5, but there were no significant differences compared to controls (Fig. 5a). Plasma total ammonia (Tamm) content was not significantly different from controls for trout chronically exposed to Cu (Fig. 5b). There was a trend toward elevated plasma Tamm levels in trout exposed to Cu with no added HA but this suggestion was evident only up to day 5 (Fig. 5b).

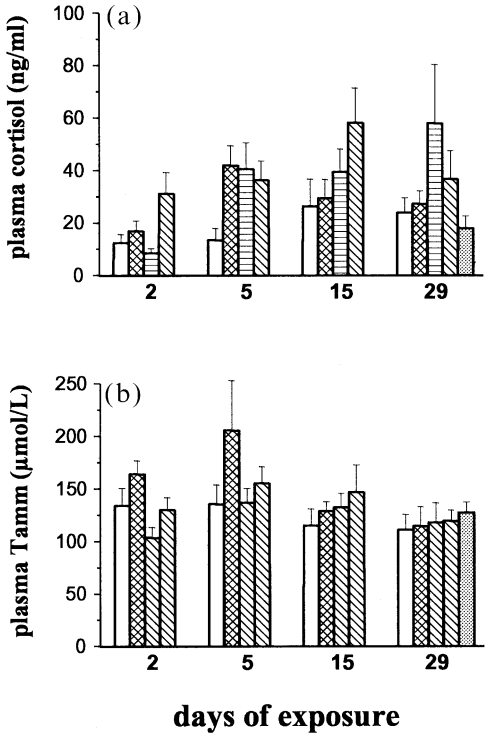


Fig. 5. Effect of chronic Cu exposure and HA on plasma cortisol (a) and Tamm (b) of rainbow trout. Mean values are shown for fish in soft water exposed to 7 μg/l Cu with either no added HA (cross-hatched bars), 2.5 mg/l added HA (horizontal lined bars) or 7.5 mg/l added HA (diagonally lined bars) for up to 29 days. Control groups were exposed to soft water with either no added HA (open bars) or 7.5 mg/l added HA (shaded bar, day 29 only). There were no significant differences between means ($P<0.05$), error bars show S.E.M. and $n\geq 6$.

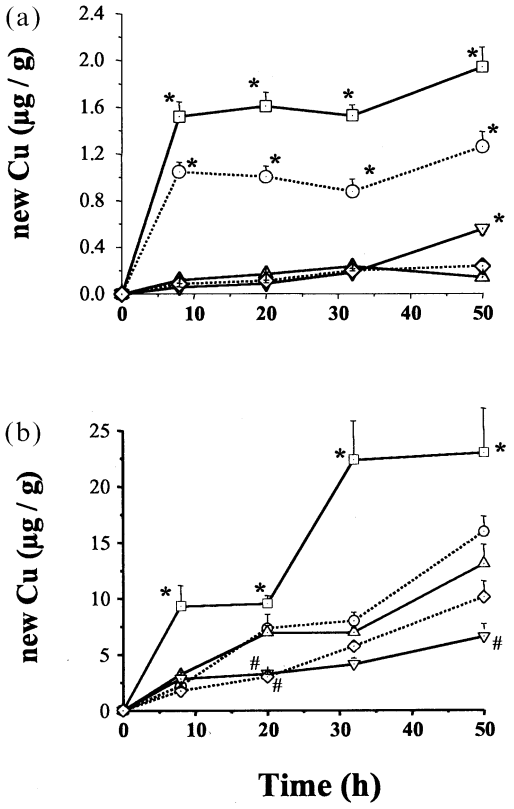


Fig. 6. Effect of chronic Cu exposure and HA on uptake of new Cu into the gills (a) and liver (b) of rainbow trout over 50 h of exposure to $^{64}$Cu. Mean values (with S.E.M.; $n=6$) are shown for fish which had previously been exposed in soft water to 7 μg/l Cu with either no added HA (squares and solid line), 2.5 mg/l added HA (upright triangles and solid line) or 7.5 mg/l added HA (inverted triangle and solid line) for a month. Control groups ($n=6$) were exposed to soft water with either no added HA (circle and dotted line) or 7.5 mg/l added HA (diamond and dotted line) and no Cu. Within each sampling time, a * indicates means significantly different from all other means and a # significant difference from controls with no added HA ($P<0.05$).

### *3.4. New Cu uptake*

Uptake of 'new' Cu into the gill, measured by radioisotopic uptake, illustrated both the impact of chronic Cu exposure and the effect of HA on Cu bioavailability. In Cu solutions without added HA, both unexposed and chronically exposed fish experienced a rapid uptake of Cu that stabilized at its maximum by 8 h (Fig. 6a). For these 2 groups, those chronically exposed to Cu showed a higher saturation concentration. When either 2.5 or 7.5 mg/l HA was added to the exposure water with Cu, metal bioavailability to the gill was significantly reduced compared to uptake of Cu from water with no added HA (Fig. 6a).

Uptake of 'new' Cu into the liver varied significantly among treatment groups with trout acclimated to 7 μg/l Cu with no HA having the highest uptake (Fig. 6b). This high uptake of new Cu was significantly greater than the corresponding controls. For trout exposed to 7.5 mg/l HA with 7 μg/l Cu, chronic exposure to Cu for a month produced a significantly reduced rate of uptake of new Cu into the liver relative to those exposed to 7.5 mg/l HA alone. The effect of HA on Cu bioavailability was clearly evident with progressive reductions in new Cu uptake into the liver as HA concentration increased.

## 4. Discussion

This study clearly illustrates the effect that complexation of waterborne Cu by HA has on reducing the chronic bioavailability (Fig. 3) and physiological impacts of exposure in rainbow trout (Fig. 4 and Fig. 5). The lack of Cu accumulation with the addition of HA at the relatively low level of 2.5 mg/l (1 mg/l added DOC) was unequivocal. Elevated HA also alleviated the acute effects of waterborne Cu as the 96 h LC50 increased by a factor of six (Fig. 2) over the range of added HA. The modifying effect that HA had on reducing acute toxicity was associated with reduced uptake of Cu into the gills and liver (Fig. 6).

Although there was a lack of significant Cu accumulation during chronic exposures with added HA, the trout did experience a physiological response. In terms of responses to chronic exposure over the range of HA levels, survival and growth were unresponsive (Fig. 1), tissue metal burden (Fig. 3) was the least sensitive and isotopically measured tissue Cu uptake (Fig. 6), particularly uptake by the liver, was the most sensitive. Plasma variables related to a generalized stress response (plasma Tamm and cortisol, Fig. 5) were unresponsive while plasma ion responses (Fig. 4a,b), particularly $Na^+$ showed that Cu exposure caused physiological impacts. Declines in plasma $Na^+$ (Fig. 4a) illustrated not only the impact of Cu exposure but also the ability of trout to respond to ion imbalances, a result complemented by elevated gill $Na^+/K^+$ ATPase activity on day 29 (Fig. 4c). In this regard, plasma $Na^+$ illustrated the initial impact of Cu while gill $Na^+/K^+$ ATPase and hepatic uptake of isotopic Cu revealed adaptive

responses related to regulating and maintaining homeostasis.

Although there was a 6-fold increase in the 96 h LC50 value (reduced acute toxicity) as a result of exposure to HA, a much greater reduction in acute toxicity was expected. The predictions derived from the computational BLM of Di Toro et al. (2000) suggested a much higher level of protection from HA. In each of the three water chemistries used to derive acute toxicity values, our LC50 values were $70 \pm 5\%$ of those predicted for the rainbow trout by the BLM model for Cu. The reasons for the somewhat higher sensitivity of our trout relative to the results predicted by the model may arise from the unique water chemistry conditions in combination with the trout themselves (for example genetic differences). Alternately, the HA used in the experiments may not be representative of the natural humic material on which the BLM is based (Tipping, 1994; Di Toro et al., 2000). Given that the BLM under-prediction of measured toxicity was uniform across the HA concentrations, the latter explanation seems more likely.

The fact that fish exposed to Cu with no added HA all survived in spite of being exposed to 80% of the 96 h LC50 illustrates the ability of rainbow trout to adapt to this nutrient metal. While not sufficient to cause mortality, chronic exposure to Cu, particularly with no added HA, produced a physiological disruption that was followed by recovery. This is similar to the results of Taylor et al. (2000) and McGeer et al. (2000a) which illustrated that if trout can survive the initial shock or damage caused by Cu, then physiological adjustments can ensure that homeostatic control is re-established. Therefore, the trout in these experiments followed the damage–repair scenario for metal exposure as discussed by McDonald and Wood (1993).

A number of studies have now shown that chronic sublethal exposure of fish to Cu may have no effect on growth (Seim et al., 1984; Taylor et al., 2000; McGeer et al., 2000a), especially when ration is relatively high as it was in this study (2% per day). Although growth was not affected, it is likely that trout chronically exposed to Cu in soft water with no added HA experience an increased metabolic demand. This hypothesis is supported indirectly by the gill and liver accumulation of Cu, and by the plasma $Na^+$ loss with a subsequent recovery and elevated levels of gill $Na^+/K^+$ ATPase activity. Chronic sublethal Cu exposures that result in an increased metal burden and disruptions in ion regulation have been associated with increased metabolic demand (De Boeck et al., 1997; McGeer et al., 2000a). The fact that growth was not affected may be due to the relatively short (one month) exposure or may illustrate the adaptive ability of the trout to balance energy demands.

The acute toxicity tests after chronic exposure showed that chronic exposure did not induce a resistance to Cu challenges. This lack of an acclimation response for fish in soft water (with no HA) is similar to the results of Taylor et al. (2000) who postulated that acclimation may be a feature of Cu exposure but only in hard waters. Taylor et al. (2000) did not suggest a mechanism to explain the lack of acclimation and studies on how water chemistry influences this phenomenon would be useful. In the case of the trout chronically exposed to Cu with 7.5 mg/l HA added, the lack of an acclimation response was likely due to the low bioavailability of Cu as no metal accumulation occurred. The results from the work of Bradley et al. (1985) and Dixon and Sprague (1981) showed that the acclimation response is only induced if the chronic exposure is sufficiently challenging.

The accumulation of Cu in gills and liver, or lack thereof, illustrated the effect that HA has on reducing waterborne Cu availability during chronic exposure. In the low complexing capacity environment (no added HA), Cu accumulated in both the gills and liver but not the kidney. This is generally consistent with other studies on chronic Cu exposure which illustrate that the gills and liver in trout tend to be the major organs for accumulation during chronic exposure (Dixon and Sprague, 1981; Laurén and McDonald, 1987a,b; Grosell et al., 1997, 1998; Taylor et al., 2000). In chronic exposures with HA added at 2.5 and 7.5 mg/l, organic complexation of $Cu^{2+}$ completely eliminated accumulation (Fig. 3). These results are similar to those of Winner (1985) where additions of HA decreased both the acute and chronic toxicity of Cu in *Daphnia pulex* as well as Cu bioaccumulation in *Daphnia magna*. Our results are also consistent with the studies of Erickson et al. (1996) and Ma et al. (1999) where HA reduced Cu bioavailability as measured by acute toxicity responses in fathead minnows and *Ceriodaphnia dubia* (respectively).

The modulation of Cu accumulation by HA during chronic Cu exposure clearly supports the Free Ion Activity Model (FIAM) described by Campbell (1995) in terms of the fact that when the free ion activity of $Cu^{2+}$ in solution declines, so does accumulation. However, a lack of Cu accumulation in the gills and liver of trout chronically exposed to Cu with added HA did not indicate a lack of physiological impact. The disruption of $Na^+$ regulation in trout chronically exposed to Cu with 2.5 mg/l added HA illustrated that Cu was bioavailable and caused effects even though a net accumulation could not be detected. This may reflect a sensitivity to very low levels of $Cu^{2+}$ in trout, the bioavailability of inorganic Cu complexes (see Table 1) and/or the bioavailability for Cu when complexed to HA. The bioavailability of Cu complexes (e.g. Cu–HA or CuOH) could be in the form of uptake of the complex itself or the uptake of $Cu^{2+}$, released from HA at the gill surface. It is important to note that the $Cu^{2+}$ levels in the different exposure treatments, as provided in Table 1, are modeled estimates because $Cu^{2+}$ levels were not measured and the complexing capacity of the DOC in soft water and of the added HA was not quantified. Regardless of the form or mechanism of Cu uptake, it was bioavailable in Cu exposed trout with 2.5 mg/l added HA and the lack of accumulation in spite of ionoregulatory effects may also be due to an inherent ability to regulate internal Cu accumulation.

The results of our experiments in soft water without added HA showed that the initial effect of sublethal exposure to bioavailable Cu was a temporary loss of $Na^+$, which was followed by a recovery and return to control levels. The addition of HA in the chronic Cu exposure media had a significant effect on both the severity and the timing of the loss of plasma $Na^+$. Therefore, the protective effect of HA on acute toxicity (Fig. 2) and Cu accumulation (Fig. 3) also extends to the disruption of $Na^+$ balance (Fig. 4a). A loss of plasma $Na^+$ is typical for Cu-exposed trout (Laurén and McDonald, 1987a; Beaumont et al., 1995; McGeer et al., 2000a). The temporary nature of the Cu-induced osmoregulatory disturbance (Fig. 4a,b) with return to control levels has also been shown by Laurén and McDonald (1987a). It is probable that the recovery of ionic balance in metal exposed fish was aided by dietary uptake of $Na^+$ as shown recently for acid-exposed trout by D'Cruz and Wood (1998).

The initial osmoregulatory disturbance and subsequent recovery observed for Cu exposed fish in soft water with no added HA and 2.5 mg/l added HA undoubtedly reflects alterations in gill transport kinetics although this study did not address this directly. Inhibition of specific gill ion transporters by Cu has been shown for $Na^+/K^+$ ATPase (Pelgrom et al., 1995; Laurén and McDonald, 1987b). Interestingly, the ion losses experience by Cu exposed trout (Fig. 4a) were not temporally linked to gill Cu accumulation (Fig. 3a) nor to an inhibition of gill $Na^+/K^+$ ATPase (Fig. 4c). It is possible that the sampling design may have missed these two events as it is known that the inhibition of gill $Na^+/K^+$ ATPase is temporary during chronic sublethal exposure and that gill Cu accumulation can follow a biphasic (accumulation followed by clearance) pattern (Laurén and McDonald, 1987b; Grosell et al., 1997).

Ion loss during chronic metal exposure is known to result in a mobilization of mechanisms for recovery of gill ion transport (McDonald and Wood, 1993). Trout exposed to Cu without added HA clearly showed a significant increase in gill $Na^+/K^+$ ATPase activity, in agreement with previous studies (Shephard and Simkiss, 1978; Laurén and McDonald, 1987a,b; Pelgrom et al., 1995; McGeer et al., 2000a). Although variation was sufficient to obscure possible significance, there were trends indicating a delayed disruption of $Na^+$ balance (Fig. 4a, day 15) and subsequent gill $Na^+/K^+$ ATPase activity response (Fig. 4c) for trout chronically exposed to Cu with 7.5 mg/l HA added. This trend supports the concepts that HA complexation reduces Cu bioavailability and that ionoregulatory alteration is a sensitive biomarker of waterborne Cu exposure.

Plasma cortisol and Tamm content were measured as possible indicators of a generalized response to the stress of Cu exposure. The correlation between cortisol mobilization and ion loss has been documented previously in silver-exposed fish (McGeer and Wood, 1998; Webb and Wood, 1998). Increases in plasma Tamm levels can be indicative of the cortisol-induced breakdown of proteins (van der Boon et al., 1997) and is characteristic of stress. Although there was a trend suggesting some elevations in these 2 stress response parameters, there were no significant

differences among treatments in our experiments. This is in accord with the general homeostatic regulation and 100% survival of the trout at these sublethal levels of Cu.

Uptake of 'new' Cu into the gills and liver, as measured over 50 h of exposure to $^{64}Cu$, illustrated both the effect of HA on bioavailability and the effect of chronic Cu exposure on uptake dynamics. For both the gill and liver, HA complexation of Cu reduced the bioavailability of the metal. Trout chronically exposed to Cu with 2.5 and 7.5 mg/l added HA experienced very little new Cu accumulation (Fig. 6a). This was in contrast to trout exposed to Cu in soft water without added HA where a much higher uptake and accumulation occurred. When compared to controls with no previous exposure to elevated Cu, chronic Cu exposure resulted in a greater uptake of Cu into the gills. These results are consistent with those of Taylor et al. (2000) with Cu as well as studies with Cd (Hollis et al., 1999) and Zn (Alsop and Wood, 2000) which have shown that chronic exposure results in an increase in the capacity for short term uptake of the metal of exposure. In our experiments this response was only observed in exposures without added HA.

In spite of significant accumulation of new Cu in the gills (where a plateau was reached by 8 h), the rate and extent of accumulation was low relative to that of the liver, where accumulation occurred at a more or less steady rate for at least 50 h. For all treatments, levels of new Cu in the liver were at least 10-fold higher than in the gills after 50 h of exposure. These results are consistent with previous studies showing that the liver is the primary organ for Cu homeostasis and accumulation in rainbow trout (Buckley et al., 1982; McCarter and Roch, 1983; Grosell et al., 1997, 1998; Kamunde et al., 2002). As with the gill, there was a clear link between addition of HA and reductions in uptake to the liver. As well, prior chronic exposure to Cu resulted in higher Cu uptake into the liver but only in the soft water with no added HA treatment. Overall, HA reduced the short term uptake of Cu into the gills and liver, and chronic exposure to Cu without added HA increased the uptake capacity for Cu in both the gills and the liver.

In summary, the complexation of Cu by HA reduces both the acute and the chronic bioavailability. In these experiments trout were exposed to a uniform level of total waterborne Cu but with increased HA content the $Cu^{2+}$ levels decreased and the Cu–HA levels increased. Clearly Cu complexed to HA was much less toxic and much less bioavailable relative to $Cu^{2+}$. While elevated HA blocked significant Cu accumulation, the metal was still available for uptake that resulted in ionoregulatory effects. This may reflect sensitivity to very low levels of waterborne $Cu^{2+}$ or, alternately, a low chronic availability for Cu–HA complexes and/or inorganic Cu complexes. The development of BLM type approaches for chronic toxicity needs to include the moderating effects that DOC can have on Cu bioavailability, and the results from this study with HA may serve as a starting point for further work.

The conclusions of this study are also relevant in the context of ecological risk assessment as HA, which served as a surrogate for natural organic matter, was shown to have a dramatic effect on bioavailability, bioaccumulation and toxicity. In agreement with other studies such as Taylor et al. (2000), tissue accumulation of Cu has been shown to be a poor predictor of potential impact. The effects of DOC on Cu bioavailability needs to be incorporated into the risk assessment process and ideally this could be done through an integration of the chronic BLM approach.

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