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Bioavailability of Apparent Fulvic Acid Complexed Copper to Fish Gills

S. Tao, S. Xu, J. Cao, R. Dawson

Department of Urban and Environmental Sciences, Peking University, Beijing 100871, People's Republic of China

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It is well recognized that only the bioavailable species, rather than all metals, are of concern with regard to bioaccumulation by and toxicity to aquatic organisms. It would be desirable to establish the relative bioavailability of various chemical species of metals and to understand the fundamental interactions that control the speciation and bioavailability of inorganic contaminants to organisms (Hudson 1998). It is generally known that the dominant bioavailable species of trace metals available for fish in aquatic environments include the hydrated metal ions along with the metal hydroxo complexes, although these account for only a small fraction of the total metal content surface waters (Andrew *et al.* 1977).

In natural waters containing various amounts of suspended solids and humic substances, a large portion of metals are those adsorbed on to particulate matter and those that are complexed to naturally occurring organic ligands as a result of the relatively high affinity metals have for these carriers, Sufficient evidence exists indicating that the addition of humic substances to aquatic systems can significantly reduce the bioavailability of metals to aquatic organisms (Hollis *et al.* 1996). It was, therefore, generally understand that organic metal complexes were unavailable to fish via uptake by the gills, Recently, however, Tao and colleagues evaluated the bioavailability of particulate copper to fish gills by exposing *Paracheirodon innesi* to copper in a synthetic river water spiked with kaolin clay. The concentrations of 'free metal' were maintained at a constant level while the particulate copper concentrations varied from one tank to another based on the measured isomer of the copper-kaolin system and the equilibrium calculation. The results indicated that although the kaolin clay could reduce fish uptake of copper, a stoichiometric analysis indicated that a fraction of the kaolin clay adsorbed copper was available for fish uptake via gills (Tao *et al.* 2000a). As such, a mechanism of particulate metal uptake by the fish gills was suggested, with the key point of the proposed mechanism being the desorption of the metal from the particles within the gill microenvironment where the particles adhered to mucus (Tao *et al.* 1999a). Additional strong support for this hypothesis was provided by evidence collected in another experiment where *Carassius auratus* and gibbsite adsorbed lead were used (Tao *et al.* 1999b).

It was assumed, therefore, that the mechanism for particulate metal uptake via fish gill mucus might also be applicable to naturally occurring organic compound complexed

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Correspondence to: S. Tao

metals. In order to test this assumption, a specially designed experiment was conducted in which *Paracheiroadon innesi* were exposed to copper along with fulvic acid. It is the purpose of this paper to highlight the results of the exposure experiment and to discuss the bioavailability of fulvic acid complexed copper to fish gills.

MATERIALS AND METHODS

The exposure experiments were conducted in reconstituted water synthesized by adding reagent-grade salts to deionized water. The ionic composition of the major rivers of China was adopted as the basis for the synthetic water used in this study. The major cation and anion concentrations of the water were $[Na^{+}]$ 31 mg/l, $[Ca^{2+}]$ 65 mg/l, $[K^{+}]$ 14 mg/l, $[Mg^{2+}]$ 16 mg/l, $[Cl^{-}]$ 115 mg/l, $[CO^{2}_{3}]$ 103 mg/l, $[NO^{-}_{3}]$ 5 mg/l, and $[SO^{2-}_{4}]$ 58 mg/l, respectively. The pH was 8.9. Fulvic acid (FA) was collected from the Amur River at Mohe. A column filled with Amberlite XAD-8 resin was used to isolate the fulvic acid. The filtrated (0.45µ) river water was acidified to a pH level of 2 using HCl and passed through the column. The sorbed fulvic acid was eluted with NaOH and de-ionized using a dialysis membrane (Tao *et al.* 1988). De-ionized water was used throughout for copper determination. All glassware was soaked in 10% nitric acid (v/v) for 24 hrs and rinsed with de-ionized water before use.

Neon Tetras *Paracheirodon innesi* (size ca. 15 mm) were obtained from a fish market in Beijing. On arrival in the laboratory, the fish were placed in the synthetic water solution for a one-week acclimation period prior to the exposure experiments. Exposures were initiated by transferring twelve fish to each of eleven separate glass tanks holding 1000 ml of synthetic. The water was spiked with copper of various concentrations with and without fulvic acid as shown in Table 1. The first tank served as control. The total number of fish used was then 132.

Table 1. Protocol of the exposure experiment

No.	1	2	3	4	5	6	7	8	9	10	11
Cu (mg/l)	0	0.3	0.6	0.9	1.2	1.5	1.5	1.5	1.5	1.5	1.5
FA (mg Carbon/l)	0	0	0	0	0	0	2	5	10	17	28

Preliminary results of the experiment indicated that there was no significant decrease in the metal concentration of the solution after 200 hours. It was not necessary to replace the experimental solution during the exposure experiment and non-exposed fish from the holding tank were used as the control group. The fish were not fed during the exposure experiments, avoiding the potential for a significant change in metal speciation. After exposure, the fish were randomly pooled into two groups of six individuals for copper determination purpose

A dialysis membrane tilled with cation exchange resin was employed to determine

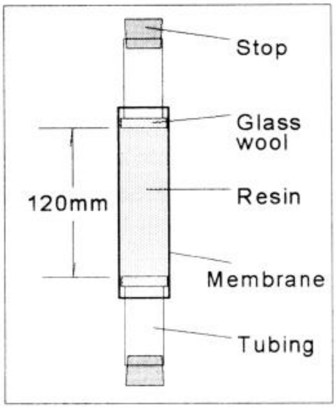

Figure 1. Schematic diagram of a resin tilled dialysis membrane
(After Tao and Liang, I997)

the free metal content of the water in which fulvic acid was added. A cellulose dialysis membrane (Spectra/Pro) with pore size of 100 MWCO was used for this purpose. Membranes with a S-mm diameter were filled with a cation exchange resin of Chelex-100 to a height of 12 mm in order to maintain a constant exposure area. The resin was initially rinsed with 7% HCl (overnight) and deionized water. Both ends of the cells were connected to polyethylene tubing with tiny rubber stoppers. The device was tested against a number of parameters (Tao *et al.* 1997) and is shown in Figure 1. The cells were rinsed with 2 ml of 3% nitric acid (v/v) and 20 ml deionized water before use. Two membranes were installed in each of the tanks. Copper accumulated on the membrane was measured after exposure. The results from tanks No. 1 to 6 (without FA added) were used to calibrate an equation for free copper calculation, while those obtained from tanks No. 7 to 11 were used to calculate the free metal concentrations in those tanks spiked with fulvic acid.

The membranes were eluted with 2 ml 3% nitric acid (v/v) and 10 ml deionized water in sequence after exposure. The elutriate was then raised to the 50 ml level before conducting the analysis, Twelve fish from each treatment group were randomly divided into two groups for purposes of duplicate analysis. After drying at 105°C and weighing, the fish samples were soaked in concentrated nitric acid overnight and then digested with nitric acid - perchloric acid - hydrofluoric acid at 200°C. The resulting residue was dissolved in de-ionized water and the solution neutralized prior to copper determination. The copper content in the samples was measured using a PAR polarograph model 384 in a differential pulse anodic stripping mode in conjunction with a model 303 hanging mercury drop electrode. The sample solution was buffered with 1.0M NaAc-HAc (1 ml sample and 9 ml buffer) and was purged for 10 minutes. A 120 s deposition time, 30 s equilibrium time, 0.5 V ~ 0.1 V scan range, 50 mV voltage pulse height, and 4 mV/s scan rate were adopted.

RESULTS AND DISCUSSION

During the experiment, both the dialysis membrane and the fish were exposed to various levels of copper in aqueous solution. The concentrations of total copper varied from 0 to 1.5 mg/l in the six treatments, Accumulated copper in both the membrane and fish were then measured and plotted against the total copper concentration of the ambient water shown in Figure 2.

It was demonstrated in a preliminary study dealing with copper accumulations in dialysis membranes under various conditions, that copper accumulations in the membrane was positively proportional to its concentration in the water at constant temperature (Tao *et al.* 1997). A similar relationship between fish uptake and water concentration has been observed within certain concentration ranges (Liang *et al.* 1999). The results shown in Figure 2 confirm this simple linear relationship. These results were then fitted with the two linear regression functions below.

$$[\textit{Cu-membrane}]\ (\mu g/g) = 1.14 + 9.38\ [\textit{Cu-water}]\ (mg/l)\ ,\quad R^2 = 0.90 \qquad (1)$$

$$[\textit{Cu-fish}]\ (\mu g/cell) = 20.28 + 23.50\ [\textit{Cu-water}]\ (mg/l),\quad R^2 = 0.92 \qquad (2)$$

In this study the free copper concentration in the system with fulvic acid was calculated based upon the copper accumulations in the dialysis membrane exposed together with the fish using the calibration equation (equation 1). However, because the complex equilibrium might shift in the direction of complex dissociation due to weak affinity of fulvic acid to copper, the calculated result should be the concentration of “apparent” free copper instead of “true” free copper. Consequently, concentration of apparent copper complex rather than true copper complex was derived. Both apparent free and complexed copper concentrations in the system with known quantities of total copper and spiked fulvic acid, could then be calculated.

It has been reported in the literature that the addition of fulvic acid or other naturally occurring organic compounds may inhibit metal uptake by aquatic organisms [Hollis *et al.* 1996]. This in turn is often taken as evidence supporting the non-availability of the metal complex. However, there is no stoichiometric basis for such an inference, partially due to the fact that accurate measurement of aquatic species of trace metals at low concentration levels is very difficult. When the dialysis membrane technique was used in this study, it was possible to derive the apparent free and complexed copper concentrations. In Figure 3, the copper accumulations in the fish exposed to copper and Fulvic acid has been plotted as scatted circles against the apparent free copper concentration (derived based on the metal sorbed in the dialysis membrane and equation 1). In the same figure, a straight line representing fish uptake from the system without fulvic acid complexed copper is shown according to equation 2.

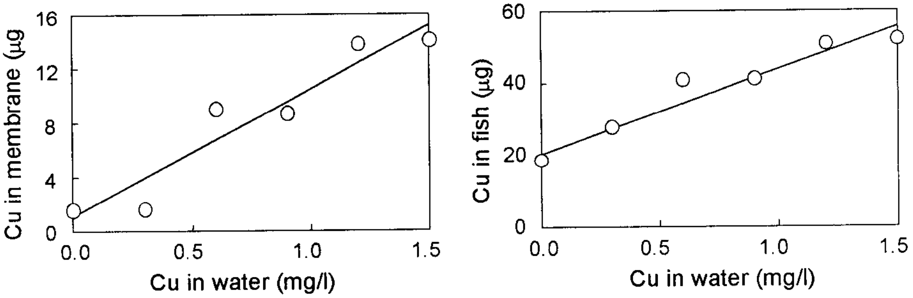

Figure 2. Copper accumulated in the membrane and the fish as functions of copper concentrations in the ambient water

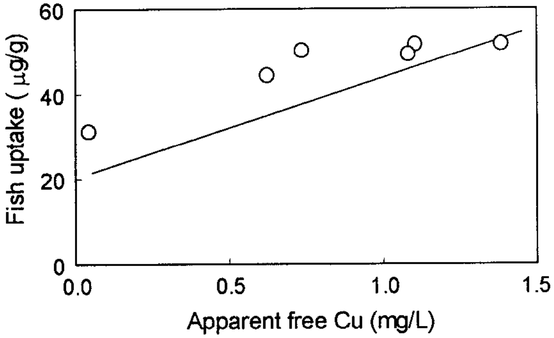

Figure 3. Copper uptake by the fish vs. free copper concentration with (scatted circles) or without (straight line) fulvic acid complexed copper in the system

If the fulvic acid complexed copper is totally non-available to fish for uptake, the scatted data points should appear more or less grouped around the straight line. However, as shown in Figure 3, all of the scatted circles are above the line indicating the apparent free copper was not the only species involved in the uptake of metal by the fish. The contribution of the apparent fulvic acid complexed copper has on fish uptake was derived by examining the difference in copper uptake with and without fulvic acid present in the system. The results were plotted against the fulvic acid complexed copper and presented in Figure 4.

It seems clear that the contribution of the apparent fulvic acid complexed copper on fish uptake increases with increases in its concentration. As indicated previously, the calculated concentration is apparent copper complex rather than true copper complex due to kinetic dissociation of the weakly binding between copper and the naturally occurring fulvic acid. This dissociation, however, occurred not only during the copper translocation into the resin-filled dialysis membrane, but also during the fish gill uptake process [Playle 1993; Compbell 1995]. Therefore, the difference of copper accumulations in the dialysis membrane and the fish gills

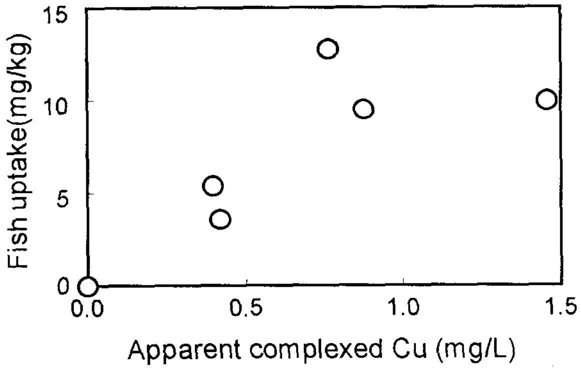

Figure 4. Fish uptake of apparent fulvic acid complexed copper

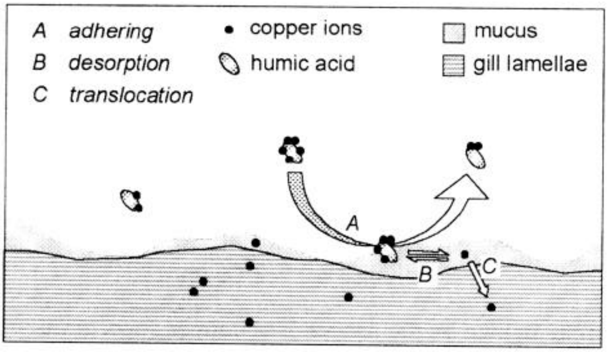

Figure 5. Translocation of fulvic acid complexed copper from water to the gills

indicates that the fulvic acid complexed copper is available to fish gill uptake to certain extent.

Naturally, the rate of uptake of complexed copper is much lower than that of free copper, For example, at a concentration of 0.8 mg/l, fish uptake of free copper was approximately 40 mg/kg, while for fulvic acid complexed copper of similar concentrations, the quantity of accumulation during the same period of exposure was about 10 mg/kg. Obviously, the presence of fulvic acid is a key factor inhibiting the uptake of the metals in fish.

It is hypothesized that fulvic acid complexed copper is not able to transfer directly across the epithelial cell membrane of fish gills. Therefore, the adsorption most likely occurs in an indirect manner. Tao and colleagues have suggested a mechanism that allows for particulate metal uptake via fish gills [Tao *et al.* 2000a]. A similar process might occur with regard to fulvic acid complexed copper as well. It is proposed, as shown in Figure 5, that the complex may be adhering to the mucus of the epithelial cell surface during fish aspiration. Dissociation of the complex could then release free copper which, in turn, could be transported into the gill tissue.

The uptake rate of fulvic acid complexed copper by fish gills, when compared with that of free copper is much slower. This slower rate of uptake adds further support to the concept that fulvic acid is an inhibiting factor in the uptake of metals via fish gills.

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