Bioavailability and acute toxicity of copper to rainbow trout (*Oncorhynchus mykiss*) in the presence of organic acids simulating natural dissolved organic carbon

J.C.A. Marr, J. Lipton, D. Cacela, J.A. Hansen, J.S. Meyer, and H.L. Bergman

Abstract: Copper bioavailability and toxicity to early life stage rainbow trout (*Oncorhynchus mykiss*) were evaluated by laboratory toxicity testing performed using organic acid mixtures. Geochemical modeling was used to design exposure solutions that simulate dissolved organic carbon (DOC) of a natural aquatic system and to determine the fractions of total Cu present as inorganic species (e.g., Cu^{2+}) and as individual Cu–organic complexes. Failure time modeling indicated that mortality was best predicted by a combination of total inorganic Cu and distinct Cu–organic complexes. The Cu–organic complexes that contributed to toxicity are characterized as low-affinity Cu–ligands, and our results support the hypothesis that Cu toxicity in nature is a function of the binding characteristics of individual ligands. Estimates of time-independent median lethal concentration thresholds determined at widely varying equivalent concentrations of DOC (0–16 mg/L) were constant (7.9–8.6 µg Cu/L) when modeled using the sum of inorganic Cu and Cu bound to the two low-affinity ligands as predictors of toxicity. Our results indicate that Cu bound to organic complexes may be available to fish and that acute toxicity of Cu is determined by the binding affinities of specific DOC components relative to Cu-binding affinity of fish gill.

Résumé : Nous avons évalué la biodisponibilité du cuivre et sa toxicité pour les premières phases du cycle biologique de la truite arc-en-ciel (*Oncorhynchus mykiss*) par des essais de toxicité en laboratoire à l'aide de mélanges d'acides organiques. La modélisation géochimique a servi à composer des solutions d'exposition simulant la teneur en carbone organique dissous (COD) d'un système aquatique naturel et à déterminer les fractions de Cu total présentes sous la forme d'espèces inorganiques (p.ex. Cu^{2+}) et de complexes organiques avec Cu. La modélisation de la durée jusqu'à défaillance a indiqué que la prédiction de la mortalité était optimale avec une combinaison de Cu inorganique total et de complexes organiques avec Cu. Les complexes organiques qui contribuaient à la toxicité se caractérisaient comme des ligands du cuivre à faible affinité, et nos résultats confirment que la toxicité de ce métal dans la nature est fonction des caractéristiques de liaison de chaque ligand. Les estimations des seuils médians de concentration létale indépendante de la durée, calculées pour des concentrations équivalentes très diverses de COD (0–16 mg/L) étaient constantes (7,9–8,6 µg Cu/L) lorsqu'on les modélisait avec la somme du Cu inorganique et du Cu lié aux deux ligands à faible affinité comme prédicteurs de la toxicité. Nos résultats indiquent que le Cu lié aux complexes organiques peut être biodisponible pour le poisson, et que la toxicité aiguë du Cu est déterminée par les affinités de liaison de composantes spécifiques du COD par rapport à l'affinité de liaison des branchies de poissons pour le Cu.

[Traduit par la Rédaction]

Introduction

The chemical speciation of Cu is likely to influence its toxicity to fish and therefore may have important environmental implications. The cupric ion, Cu^{2+}, is generally accepted as toxic (Howarth and Sprague 1978; Chakoumakos et al. 1979; Laurén and McDonald 1986). Other Cu–inorganic species that have been reported to contribute to toxicity include: $CuOH^{+}$ (Pagenkopf et al. 1974; Howarth and Sprague 1978; Chakoumakos et al. 1979), $CuCO_3^0$ (Shaw and Brown 1974), $Cu(OH)_2^0$ (Chakoumakos et al. 1979; Laurén and McDonald 1986), and $Cu_2(OH)_2^{2+}$ (Howarth and Sprague 1978; Chakoumakos et al. 1979).

A number of authors have reported that dissolved organic carbon (DOC) can reduce Cu toxicity through formation of less bioavailable Cu–organic species. Numerous ligands with differing metal complexation strengths have been used in laboratory bioassays to evaluate metal bioavailability,

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J.C.A. Marr[1]. Caribbean Marine Research Center, 250 Tequesta Drive, Suite 304, Tequesta, FL 33469, U.S.A.
J. Lipton and D. Cacela. Hagler Bailly Services, Inc., P.O. Drawer O, Boulder, CO 80306, U.S.A.
J.A. Hansen, J.S. Meyer, and H.L. Bergman. Department of Zoology and Physiology, University of Wyoming, Laramie, WY 82071, U.S.A.

[1]Author to whom all correspondence should be addressed. e-mail: jmarr@gate.net

including various sources of synthetic organic compounds (e.g., glycine, oxalic acid, salicylic acid, nitrilotriacetic acid, ethylenediaminetetraacetic acid), purified soil extracts, and natural waters (Winner 1985; Daly et al. 1990; Playle et al. 1993*a*; Erickson et al. 1996; Welsh et al.1996; Roy and Campbell 1997; MacRae et al. 1999*a*).

A majority of the toxicological literature supports the concepts of attenuated toxicity and bioavailability of Cu and other metals due to metal–ligand complexation. Brown et al. (1974) found that survival time for rainbow trout (*Oncorhynchus mykiss*) exposed to Cu increased linearly at humic acid concentrations of 0.9–4.5 mg DOC/L; the survival time at 4.5 mg humic acid/L increased more than twofold compared with the treatment without humic acid. Welsh et al. (1993) found that 96-h median lethal concentration thresholds (LC_{50}s) for larval fathead minnow (*Pimephales promelas*) increased in relation to concentration of naturally occurring DOC in low-pH soft water over DOC concentrations ranging from 0 to 16 mg DOC/L. Using the DOC derived from algal and daphnid blooms of a microcosm, Cu-induced mortality in *Daphnia magna* was also reduced in the presence of DOC (Meador 1991). In concept, these example studies are in agreement showing attenuation of acute Cu toxicity through the formation of Cu–organic complexes. However, certain studies have concluded that some Cu–organic complexes may be bioavailable and toxic (e.g., Guy and Kean 1980; Borgmann and Ralph 1984; Daly et al. 1990; Florence et al. 1992; Erickson et al. 1996).

Critical factors that determine Cu bioavailability in the presence of organic ligands may include the specific complexation characteristics of ligands for Cu, such as Cu-binding affinity and capacity. For instance, Cu-binding affinities determined for fish gills by Playle et al. (1993*a*, 1993*b*) and MacRae et al. (1999*a*) suggest that some forms of organically complexed Cu may be available to fish because the Cu-binding affinity of fish gills is greater than that of some organic ligands.

The ability to measure or control experimental variables (e.g., relative concentrations of free and bound metal species) and the complexation characteristics of ligands are critical to the interpretation of toxicity data and therefore important to our understanding of metal bioavailability and conclusions about metal toxicity moderation in the presence of various DOC sources. The issue of DOC mediation of metal effects is relevant to the problem of determining thresholds for metal toxicity in natural systems. For example, in the Panther Creek Basin, located in the Northern Rocky Mountain physiographic province in central Idaho, metals are released from a large inactive Cu and Co mining operation. We conducted a laboratory bioassay in which rainbow trout were exposed to Cu and a mixture of commercially available organic acids to assess Cu bioavailability and to determine remediation targets for site waters containing DOC. The mixture of organic acids was selected to simulate conditions in Panther Creek by characterizing the Cu-binding properties of site-specific DOC using the methods and rationale described by MacRae et al. (1999*b*). Further details about the geochemistry and physiography of Panther Creek are provided in the companion paper by MacRae et al. (1999*b*). We evaluated bioavailabilty of Cu with respect to acute toxicity and differences in the Cu speciation characteristics of exposure waters.

Materials and methods

The experiment was conducted at the Red Buttes Environmental Biology Laboratory, University of Wyoming, Laramie, Wyo., U.S.A.

Experimental fish

Rainbow trout eggs were obtained from Dubois Fish Hatchery, Wyoming Game and Fish Department; the fish were hatched and cultured in the laboratory using well water (hardness 220 mg/L as $CaCO_3$, alkalinity 180–210 mg/L as $CaCO_3$, pH 7.2–7.8, temperature 7°C). After hatching, rainbow trout were transferred to circular holding tanks and acclimated for >14 days to the control water used in the bioassay (hardness 25–30 mg/L as $CaCO_3$, alkalinity 22–28 mg/L as $CaCO_3$, pH 7.3–7.6, temperature 10°C). Fish condition and health were monitored daily, and fish were kept on a 12 h light : 12 h dark light cycle.

At swim-up, rainbow trout were fed frozen BioDiet® once daily at a ration in excess of 4.5% (wet weight food per wet weight body), which was a sufficient ration for growth. Fish were deprived of food 48 h before and throughout the bioassay. Fry used in the bioassay were similar in size (mean wet weight 0.36 g (SD = 0.13 g) and mean total length 35.0 mm (SD = 3.4 mm), n = 41).

Exposure water and monitoring

Exposure waters for the bioassay were formulated to simulate conditions in the Panther Creek Basin during spring runoff periods when metal concentrations are elevated. Waters were formulated by continuously mixing well water and deionized water (well water treated with sediment filtration, reverse osmosis, and separate-bed deionization) and automatically adjusted to the desired pH, as described in Marr et al. (1996). Concentrated Cu stock solutions were prepared by mixing reagent-grade chloride salt ($CuCl_2 \cdot 6H_2O$) in deionized water, stored in Mariotte bottles, and delivered to exposure aquaria via continuous-flow proportional diluters.

Organic acid (DOC) stock solutions contained malonic acid disodium salt ($C_3H_2O_4Na_2$), oxalic acid disodium salt ($Na_2C_2O_4$), and dipicolinic acid ($C_7H_5NO_4$) in deionized water mixed at constant molar ratios of 1.0:0.4:0.02, respectively, to reflect the Cu-binding characterization of natural DOC. Nominal total DOC concentrations were 0, 0.11, 0.21, 0.42, 0.84, and 1.68 mg/L, which were selected to provide Panther Creek DOC Cu-binding capacities equivalent to 0, 2, 4, 8, 16, and 32 mg/L (denoted as DOC_{eq}), respectively (MacRae et al. 1999*b*). The Cu-binding characteristics (conditional stability constants and complexation capacities) of natural DOC were determined through nonlinear regression analysis of bound versus free Cu concentraions in site waters following titration with Cu and measurement with a cupric ion selective electrode. The influence of other site-water constituents and parameters, such as competing ions, suspended particles, iron oxide particles, and storage, were evaluated and did not have an appreciable influence on Cu–DOC complexation. Furthermore, the analysis indicated that the Cu-binding characteristics of the natural DOC did not change qualitatively as shown by similarities in Cu-binding affinity and complexation capacity of site waters collected at various locations in the drainage and during low and high flows.

DOC_{eq} stock solutions for the bioassay were stored in Mariotte bottles covered with black plastic and refrigerated (4°C) to reduce bacterial growth and bio- or photo-degradation. Stock solutions were used for a maximum of 7 days. Exposure dilutions of the DOC_{eq} were achieved by metering each stock solution into the headbox of separate continuous-flow diluters so that each diluter independently maintained a constant concentration of the DOC_{eq} mixture. The average water delivery to each aquarium (8 L water

volume) was 0.2 L/min, providing a flow rate of 288 L/day (36 water volume renewals per day) and a 90% volume replacement time of <1.5 h, well within the recommendations of Sprague (1969).

Temperature and pH in headtanks were monitored and recorded continuously with a Hewlett Packard® (model 3497A) data acquisition and alarm system. Exposure and control waters were analyzed daily in water samples from randomly selected aquaria to ensure that the water quality parameters were within 10% of desired levels for hardness, alkalinity, conductivity, pH, dissolved oxygen, and temperature. Alkalinity was determined by titration using H_2SO_4 and Gran plot calculations. Hardness was determined by the EDTA titrimetric method (APHA 1992), and pH was measured using a calibrated Beckman® Omega 12 pH/ISE meter and Orion® Ross Sure Flow combination electrode with a Beckman® temperature compensation probe. Temperature was measured using a centigrade thermometer, conductivity was measured using a VWR® digital conductivity meter, and dissolved oxygen was measured using a YSI® (model 58) meter and probe.

Exposure and control waters were sampled every 3 days from randomly selected aquaria for analysis of Cu, major cations, and major anions. Water samples (25 mL) for total Cu and cations were acidified with 25 µL of 70% HNO_3 (Instra-Analyzed grade) and concentrations were determined by atomic absorption spectrophotometry using either a graphite furnace (Perkin-Elmer® model 2380 or Varian SpectrAA® model 600) or a flame (Perkin-Elmer® model 372). Water samples (25 mL) for anion analyses were stored at 4°C in the dark and analyzed using a Dionex® ion chromatograph (model 2110i). Method detection limits were as follows: Cu, 0.9 µg/L; Ca, 0.09 mg/L; K, 0.1 mg/L; Mg, 0.01 mg/L; Na, 0.06 mg/L; Cl, 0.12 mg/L; SO_4, 0.34 mg/L.

Water samples for DOC_{eq} analysis were collected in amber glass bottles, acidified with 200 µL of concentrated H_2SO_4, sealed with tinfoil under the plastic cap, and stored at 4°C in the dark until analysis. DOC was measured with a Shimadzu® TOC-5000 organic carbon analyzer. Because the DOC exposure concentrations were less than the method detection limit (2.2 mg/L), DOC concentrations were measured in the stock solutions, and the exposure concentrations were calculated based on DOC concentrations in stocks, measured stock delivery rates, and measured dilution water flow rates.

Mortality was monitored every 2 h for the first 48 h and every 6 h thereafter. Dead fish were removed upon observation and measured for length. At the end of the bioassay, all surviving fish were measured for total length and wet weight.

Exposure design

A randomized block design was used to assign exposure and control waters to aquaria. Fish were exposed to nominal Cu concentrations of 0, 5, 10, and 20 µg/L and DOC_{eq} concentrations of 0, 2, 4, 8, 16, and 32 mg/L for a total of 24 different treatments. Nominal water quality conditions were fixed as described above. Each treatment was replicated in three separate aquaria with initial density of 20 fish per aquarium. The initial loading of the fish in each aquarium was <1.5 g fish/L.

Data analysis

Calculations to determine the various inorganic and organic chemical species of Cu were performed using the geochemical speciation computer program MINEQL$^+$ (Schecher and McAvoy 1991), as described in greater detail in MacRae et al. (1999*b*).

We used parametric and nonparametric methods of data analysis to evaluate relationships between Cu toxicity and DOC_{eq} and to assess Cu species in exposure waters that contributed to lethality responses. LC_{50}s were determined by maximum likelihood estimation of linear functions relating log metal concentration (as micrograms of measured total Cu and modeled Cu species per litre) to probit transformations of percent mortality (Finney 1971; McCullagh and Nelder 1989). For these analyses, a single aquarium holding 20 fish was treated as the experimental unit and time-specific percent mortality within individual aquaria were considered independent replicates. The time-independent LC_{50} estimates, or incipient lethal levels (ILLs), were determined by inspection of curves of LC_{50} values plotted versus time (Sprague 1969, 1970; Newman 1994).

Time to death (hours) of individual fish was used to estimate survivorship curves and to investigate relationships between exposure chemistry and survival. Mortality among control aquaria (0 µg Cu/L) was observed beginning at about 168 h, so statistical analyses were based on complete censoring at 168 h. Differences in survivorship between individual aquaria were statistically compared using log-rank tests for differences in Kaplan–Meier estimates of survivor functions (Kalbfleish and Prentice 1980). The geometric mean of Kaplan–Meier point estimates bounding 50% survival was determined as the median lethal time (LT_{50}). All such nonparametric analyses were conducted using nominal Cu concentration and DOC_{eq} exposure regimes as categorical strata. Preliminary analyses of this type were used to assess the efficacy of pooling fish from different aquarium replicates within a treatment for subsequent time to death analyses (Newman 1994). No significant difference ($p > 0.05$) between replicates was detectable in 23 of the 24 different treatments. This result was considered to be strong evidence for the absence of position effects, so all subsequent time to death analyses were based on pooled data.

Parametric curve fitting was used to examine the performance of different Cu species concentrations and organic acid levels as independent variables in time to death (failure time) models. The primary purpose of parametric modeling was to discern the bioavailable Cu species, which was done by identifying model(s) in which coefficients associated with DOC_{eq} were nonsignificant. Several candidate functional forms (exponential, Weibull, lognormal, and loglogistic) were compared using graphical inspection of linearizations, log likelihoods, and deviance (i.e., using goodness-of-fit criteria). These comparisons indicated that a lognormal model performed the best with the goodness-of-fit criteria, although differences in the candidate forms were minor. The lognormal failure time model asserts that the log of survival time for an individual organism is related to exposure levels. The model uses the assumption that the mean of log(survival time) can be described as a function of various stressors (e.g., Cu exposures) and that variability in specific survival times follows a normal distribution with constant variance. Mathematically, if T is a random variable representing survival times of individual organisms, then the mean of $\log(T)$ is estimated by $\hat{\mu} = \hat{\beta}\mathbf{x}$, where $\hat{\beta}$ are estimated coefficients and $\mathbf{x}$ are selected levels of modeled stressor variables (e.g, [Cu], [DOC]). The estimated survivor function $\hat{F}(t) = 1 - \hat{S}(t) = (P(T \le t)$ is given by

$$(1) \qquad \hat{F}(t) = 1 - \phi((\log(t) - \hat{\mu})/\hat{\sigma})$$

where $\hat{F}$ is frequency distribution and t is a particular nonnegative time, $\hat{\sigma}$ is the estimated standard deviation, and $\phi(\ )$ is the Gaussian distribution (Kalbfleish and Prentice 1980). The instantaneous probability of dying at t is given by the hazard function

$$(2) \qquad \hat{h}(t) = \hat{f}(t)/\hat{S}(t)$$

where $\hat{f}(t) = \exp((-\log(t) - \hat{\mu})^2/2\hat{\sigma}^2)/\hat{\sigma}t\sqrt{2\pi}$. Hence, $\hat{S}(t)$ describes the survivorship of a population of exposed organisms and $h(t)$ describes the probability that a particular organism will die at a particular time. The relative magnitude and statistical significance levels of the estimated coefficients $\hat{\beta}$ can be used, along with additional biological criteria, to assess the biological significance of the stressors of interest. Measured total Cu concentration, modeled concentrations of inorganic Cu species, Cu–organic com-

Table 1. Water chemistry conditions during the bioassay.

	pH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Dissolved oxygen (mg/L)	Temperature (°C)	Ca (mg/L)	K (mg/L)	Mg (mg/L)	Na (mg/L)	Cl (mg/L)	SO_4 (mg/L)
Mean	7.6	33	24.2	8.3	10	5.4	5.7	2.5	2	0.7	3.2
SD	0.08	6.6	1.9	0.3	0.3	0.62	2.99	0.22	1.22	0.12	1.32
n	69	62	77	56	69	196	196	196	196	196	193

Table 2. Mean (SD) measured Cu and geochemical species of Cu determined with the computer program MINEQL$^+$.

Nominal DOC_{eq} (mg/L)[a]	Nominal Cu (µg/L)	Total Cu (µg/L)[b]	Cu_{INRG} (µg/L)	Cu^{2+} (µg/L)	Cu_{MAL} (µg/L)	Cu_{OX} (µg/L)	Cu_{DIP} (µg/L)
0	0	<0.9 (0.36)	—	—	—	—	—
0	5	4.90 (0.60)	4.9	0.79	0	0	0
0	10	9.71 (0.58)	9.72	1.58	0	0	0
0	20	18.98 (0.90)	19.01	3.09	0	0	0
2	0	<0.9 (0.35)	—	—	—	—	—
2	5	5.17 (0.56)	2.07	0.34	0.36	0.95	1.79
2	10	10.27 (0.68)	4.96	0.81	0.86	2.17	2.29
2	20	20.01 (0.85)	10.89	1.77	1.9	4.64	2.57
4	0	<0.9 (0.51)	—	—	—	—	—
4	5	5.76 (0.54)	1.28	0.2	0.44	1.15	2.88
4	10	11.23 (1.64)	3.21	0.51	1.11	2.82	4.1
4	20	20.82 (1.00)	7.27	1.16	2.49	6.21	4.87
8	0	<0.9 (0.52)	—	—	—	—	—
8	5	5.73 (0.39)	0.58	0.09	0.41	1.06	3.67
8	10	10.75 (0.59)	1.35	0.22	0.95	2.48	5.96
8	20	20.48 (0.74)	3.45	0.56	2.42	6.21	8.36
16	0	<0.9 (0.35)	—	—	—	—	—
16	5	4.59 (0.32)	0.21	0.03	0.29	0.77	3.32
16	10	9.95 (0.43)	0.53	0.08	0.73	1.91	6.84
16	20	20.97 (0.75)	1.45	0.23	2.02	5.29	12.21
32	0	<0.9 (0.37)	—	—	—	—	—
32	5	4.83 (0.39)	0.1	0.02	0.29	0.78	3.66
32	10	10.04 (0.58)	0.23	0.04	0.66	1.74	7.41
32	20	21.05 (0.79)	0.57	0.09	1.61	4.28	14.59

[a] DOC_{eq} (simulated natural DOC source) concentrations, which consisted of a combination of malonic acid, sodium oxalate, and dipicolinic acid.
[b] $9 \geq n \geq 7$.

plexes, and nominal DOC_{eq} were used as independent continuous variables in these procedures. In summary, parametric curve fitting provided a means to evaluate combinations of Cu fractions that produced nearly identical hazard function curves (i.e., see Figs. 3*e* and 3*f*) and the best fit of predicted survival to observed survival (i.e., as in Fig. 4).

All statistical calculations were performed using SAS (SAS Institute Inc. 1990) or S-PLUS (Statistical Sciences Inc. 1993).

Results

Water chemistry

Measured concentrations of water quality parameters and major cations and anions were used in all data analyses and interpretations (Table 1). In the text that follows, we use the following abbreviations for expressing the various modeled inorganic and organic Cu species: total inorganic Cu, Cu_{INRG}; ionic Cu, Cu^{2+}; Cu bound to malonic acid, oxalic acid, and dipicolinic acid, Cu_{MAL}, Cu_{OX}, and Cu_{DIP}, respectively. The measured total Cu concentrations, which fell within 92–115% of the nominal concentrations, and the calculated inorganic and organic species of Cu are shown in Table 2. All calculated values for DOC concentrations in exposure waters (based on measured concentrations in stock solutions) were between 81 and 90% of nominal. Based on the agreement between the calculated concentrations and the nominal concentrations, the nominal concentrations were deemed accurate and were used in geochemical speciation calculations and data interpretation.

Dose–response relationship

Mean survival in each of the DOC_{eq} concentrations at 0 μg/L Cu (controls) was greater than 90% at the end of the 336 h, with the exception of the 2 mg DOC_{eq}/L exposure, which had 73% survival. Control survival rates were indicative of good laboratory conditions, healthy fish, and no toxicity from the DOC_{eq} alone, but due to the possible confounding effects of starvation occurring beyond 7 days, survivorship analyses were not conducted on data collected beyond 168 h. Survival curves for controls were not significantly different ($p > 0.69$) between different DOC_{eq} exposures, except for fish exposed to 2 mg DOC_{eq}/L, which had slightly reduced survival due to unidentified causes. Results from control Cu exposures were not used in statistical modeling.

Survivorship at 168 h among fish exposed to 32 mg DOC_{eq}/L was >90% at all Cu levels, indicating that mortality response to Cu exposure was insufficiently different from controls (0 μg Cu/L). Therefore, results from exposures with 32 mg DOC_{eq}/L were not included in lethality endpoint and failure time analyses.

We observed an inverse relationship between Cu toxicity and DOC_{eq}; percent survival decreased with increasing Cu concentration and increased with increasing DOC_{eq} concentration over the range of concentrations tested (Fig. 1). In the absence of DOC_{eq} (0 mg/L), survival was exposure dependent: the 20, 10, and 5 μg Cu/L exposures reduced survival to about 0, 20, and 60%, respectively (Fig. 1). Similarly for the cells containing DOC_{eq}, survival showed a monotonically increasing dose–response relationship of mortality with Cu concentrations, with the exception of the highest DOC_{eq} level, 32 mg/L, which showed no increase in mortality (Fig. 1). Within a given Cu treatment, DOC_{eq} concentrations between 0 and 4 mg/L had no effect on survival. Survival improved as DOC was increased further (Fig. 1).

Assessment of Cu bioavailabilty

Acute lethality estimates (LC_{50}) were determined for total Cu and the modeled Cu species. LC_{50} values commonly decreased through 96 h at each DOC_{eq} level (Fig. 2). The LC_{50} curves were relatively stable for time periods beyond 96 h, with slopes approximately parallel to the time axis and minimal to no differences between 96-h estimates and 168-h estimates. This indicates that the LC_{50} values provided good predictors of ILL's, and we used the 168-h LC_{50} estimate as an ILL for comparing effects of DOC_{eq} levels within the various Cu species. When expressed as total Cu, ILL's, as well as LC_{50}'s for all time periods >48 h, increased in relation to DOC_{eq} concentration (Table 3; Fig. 2*a*). This relationship shows that total Cu overpredicted toxic Cu species over the range of DOC_{eq} concentrations tested. In contrast, Cu_{INRG}, Cu^{2+}, and $Cu_{INRG} + Cu_{MAL}$ all showed a decreasing trend in relation to DOC_{eq} concentration (Figs. 2*b*–2*d*; Table 3), showing that these Cu species underpredicted toxic Cu fractions. After 72 h, the different LC_{50} curves for DOC_{eq} concentrations began to converge towards equivalent values for both $Cu_{INRG} + Cu_{OX}$ and $Cu_{INRG} + Cu_{MAL} + Cu_{OX}$ (Figs. 2*e* and 2*f*). Consequently, ILL values determined for both $Cu_{INRG} + Cu_{OX}$ and $Cu_{INRG} + Cu_{MAL} + Cu_{OX}$ were consistent (within 2 μg/L among the DOC_{eq} levels; Table 3). These results indicate that a combination of inorganic Cu species and organic complexes (i.e., complexes with malonic acid and oxalic acid) contributed to toxicity.

Pooled survival data were used to derive failure time (parametric) models and further evaluate bioavailability of Cu species by including coefficients for DOC_{eq} and for fractions of total Cu (comprising Cu species), with both characteristics treated as continuous variables. Models were compared based on the magnitude and statistical significance of coefficient estimates (Table 4). There was a significant residual effect of DOC_{eq} on failure time in all the models considered, except in the cases with the following combination of Cu species: (*i*) $Cu_{INRG} + Cu_{OX}$ and (*ii*) $Cu_{INRG} + Cu_{OX} + Cu_{MAL}$ (Table 4). As with the ILL results, this indicates that both inorganic and organic Cu species contributed to observed toxicity.

Hazard functions corresponding to fitted models that included DOC_{eq} and various Cu fractions as parameters were distinctive for each separate DOC_{eq} concentration except for the two models containing the above Cu species (i.e., $Cu_{INRG} + Cu_{OX}$ and $Cu_{INRG} + Cu_{OX} + Cu_{MAL}$ (Fig. 3)). We tested several Cu stressor levels, and the pattern and relative density of DOC_{eq} curves within each Cu species plot were consistent regardless of Cu stressor level input in the model. Figure 3 presents comparisons of $h(t)$ at different DOC_{eq} levels for the failure time model fitted using the concentration of DOC_{eq} and alternative Cu species as predictors. In each panel of Fig. 3, $h(t)$ is shown where the concentration of Cu is set at the 96-h LC_{50} for Cu derived from the 4 mg DOC_{eq}/L treatment level. This stressor level was chosen because it is a sufficient concentration of Cu to determine $h(t)$ for each of the respective Cu species (i.e., indicative of a lethal Cu concentration within the range of DOC_{eq} tested). Results from the parametric modeling were consistent with those from the nonparametric modeling (i.e., ILL/LC_{50} comparisons) in showing those Cu species demonstrating the least amount of variability to lethality across DOC_{eq} levels. Various survivor functions, $S(t)$, were plotted using different Cu species fractions to express the lognormal failure time model in comparison with observed survival. Observed survival was reasonably well predicted by modeling survivorship as a function of $Cu_{INRG} + Cu_{OX} + Cu_{MAL}$, although predicted survival tended to exceed observed survival for treatments with low Cu and low DOC_{eq} concentrations (Fig. 4).

Timing of mortality

For treatments where ≥50% mortality occurred within 168 h, LT_{50} estimates were within a narrow range (45–75 h), with no apparent trend relating timing of mortality to DOC_{eq} level (Fig. 1). LT_{50} was estimated to be >168 h in the two lowest Cu treatments regardless of DOC_{eq} and in the Cu exposures of 10 and 20 μg/L with $DOC_{eq} > 4$ and >8 mg/L, respectively.

Discussion

Our results show that the bioavailable fraction of Cu is best predicted by considering the various inorganic and organic fractions of Cu. Analysis of both failure time models and acute toxicity endpoints (ILL/LC_{50}) consistently showed that Cu–inorganic species (i.e., Cu_{INRG}, Cu^{2+}) do not appear

Fig. 1. Cumulative survival curves (with 95% confidence intervals) estimated by the Kaplan–Meier method for each of the Cu–DOC_{eq} treatments. Curves depicted are based on censoring at 336 h.

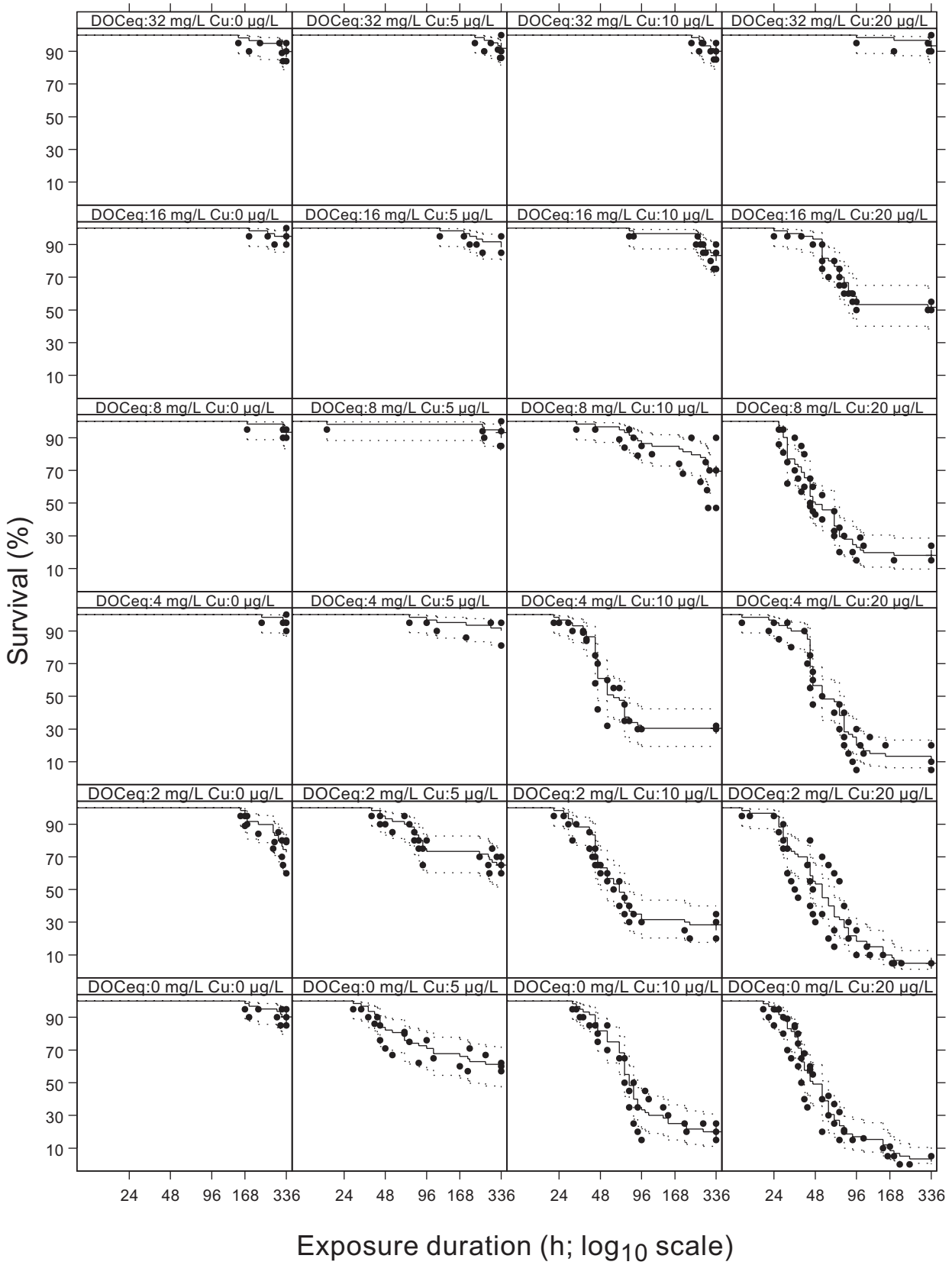

Fig. 2. Median lethal concentrations (LC_{50}) determined using (*a*) measured total Cu and (*b–f*) modeled Cu species fractions. Plotted lines are the LC_{50} values over time estimated separately for DOC_{eq} treatment levels (0–16 mg DOC_{eq}/L).

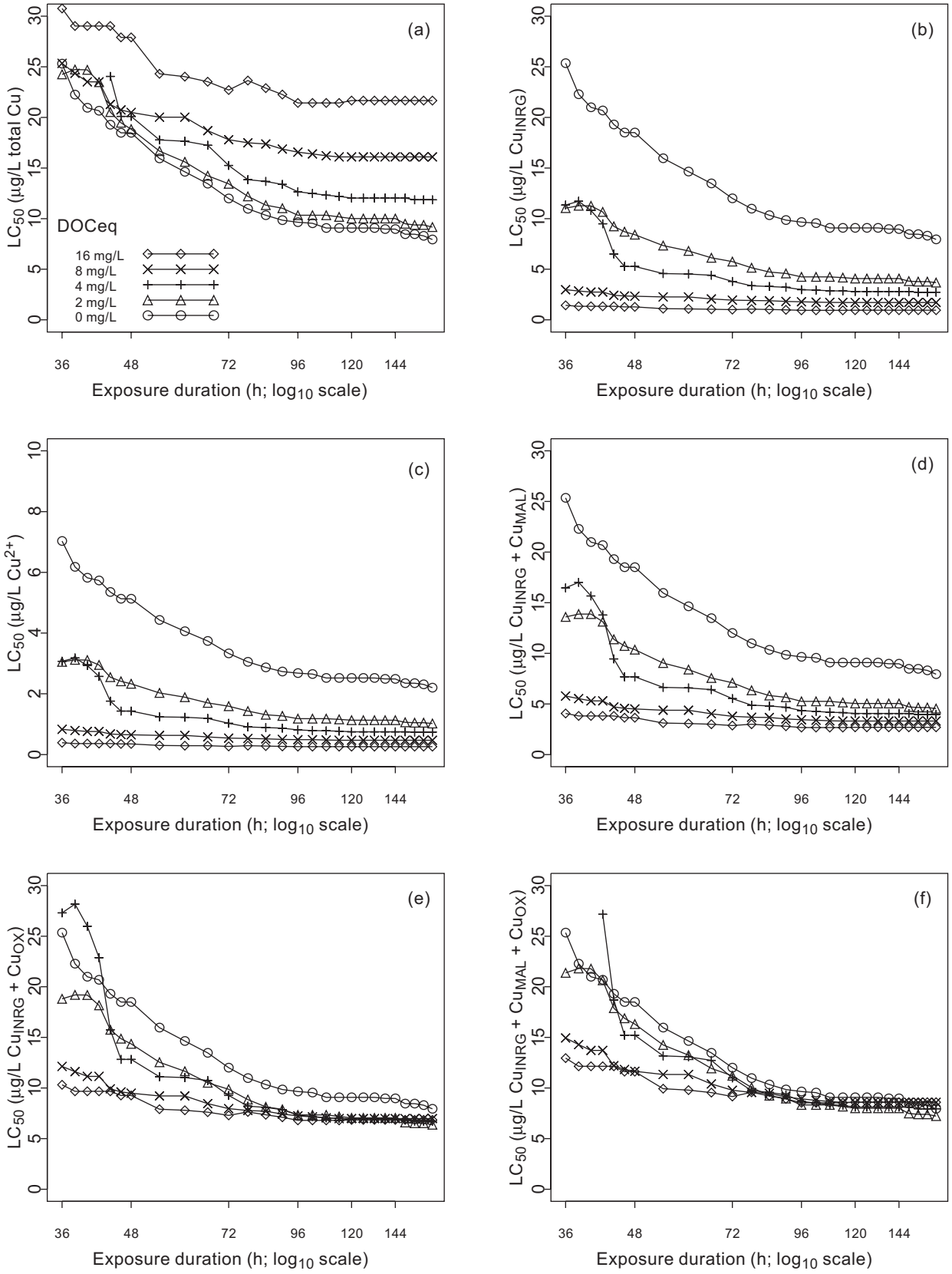

Table 3. Estimates of ILL (168-h LC_{50}) (SE) based on measured Cu and modeled Cu species.

Nominal DOC_{eq} (mg/L)	Total Cu (μg/L)	Cu_{INRG} (μg/L)	Cu^{2+} (μg/L)	Cu_{INRG} + Cu_{MAL} (μg/L)	Cu_{INRG} + Cu_{OX} (μg/L)	Cu_{INRG} + Cu_{MAL} + Cu_{OX} (μg/L)
0	7.9 (2.7)	7.9 (2.7)	2.2 (0.7)	7.9 (2.7)	7.9 (2.7)	7.9 (2.7)
2	9.2 (3.0)	3.7 (1.4)	1.0 (0.4)	4.5 (7.8)	6.4 (2.4)	7.2 (2.8)
4	11.9 (2.9)	2.7 (0.9)	0.7 (0.2)	4.0 (1.3)	6.7 (2.1)	8.0 (2.5)
8	16.1 (3.0)	1.7 (0.4)	0.5 (0.1)	3.3 (0.8)	7.0 (1.7))	8.6 (2.1)
16	21.7[a] (5.3)	1.0[a] (0.3)	0.3[a] (0.1)	2.7 (0.8)	6.9[a] (1.9)	8.6 (2.4)

[a]Value exceeds the measured or modeled exposure concentration and estimated by extrapolation.

to be the only toxic Cu fraction in the bioassay. According to the models and the comparisons of endpoints, toxicity of Cu was best explained by combinations of Cu–inorganic (Cu_{INRG}) plus the Cu bound to the two low-affinity organic ligands used in our bioassay, oxalic acid and malonic acid (Cu_{OX}, Cu_{MAL}) (see Figs. 2 and 3; Tables 3 and 4). These two organic acids had substantially lower Cu-binding affinities (log K_{Cu} = 5.58 and 6.60 for malonic acid and oxalic acid, respectively) than dipicolinic acid (log K_{cu} = 8.15) (MacRae et al. 1999*b*). The fraction of Cu bound to the high-affinity ligand (i.e., dipicolinic acid, Cu_{DIP}) did not appear to be bioavailable as indicated by both failure time models and lethality endpoints that excluded Cu_{DIP} (i.e., Cu_{INRG} combined with Cu_{OX} and Cu_{MAL}) compared with those that included Cu_{DIP} (i.e., total Cu) in the various DOC_{eq} levels. By distinguishing among combinations of inorganic and organic Cu species, we show that a portion of Cu complexed with DOC_{eq} contributed to toxicity, in addition to the toxicity caused by inorganic Cu. These general results are in agreement with Erickson et al. (1996) who concluded that organically complexed Cu was potentially available and toxic to fathead minnow and with Daly et al. (1990) who found that some portions of organically complexed Cu (Cu bound to glycine) contributed to the observed Cu toxicity in freshwater shrimp.

Our conclusions regarding the differential toxicity of Cu bound to high- versus low-affinity organic ligands may be explained by considering binding characteristics of the different organic acids relative to those of the fish gill. The Cu-binding constants determined by Playle et al. (1993*b*) and MacRae et al. (1999*a*) suggest that some forms of DOC-bound Cu may be available to fish. The conditional stability constants for the two low-affinity organic ligands in our study, malonic acid and oxalic acid, are lower than the values empirically derived for fish gill tissue (log K_{Cu} = 7.2–7.5) (Playle et al. 1993*b*; MacRae et al. 1999*a*). Thus, Cu complexed with organic ligands for which log K_{Cu} values are lower than gill tissue may be available to the organism and contribute to toxicity, whereas Cu complexed with organic ligands for which log K_{Cu} are greater than fish gill appears to be nontoxic (Table 5).

As with natural sources of DOC, gill tissue is composed of individual ligands, primarily consisting of phospholipids with amino, phospho, and carboxylate functional groups (Seimiya and Ohki 1973; Bolis et al. 1984) and an associated external mucus layer composed of polyanionic mucopolysaccharides and glycoproteins (Wold and Selset 1977; Van de Winkel et al. 1986). These functional groups each have characteristic binding affinities and capacities for metals and protons that collectively determine the complexation characteristics of gill tissue (Miller and Mackay 1982; Part and Lock 1983; Reid and McDonald 1991). Given the heterogenous and changing composition of gill epithelia and associated mucus, estimating the specific Cu binding of individual component ligands may be both difficult and physiologically inappropriate. Therefore, reasonable estimates of Cu complexation characteristics of gill tissue likely will derive from competition bioassays to determine Cu-binding constants determined for the whole gill (e.g., Playle et al. 1993*b*; MacRae et al. 1999*a*). Additional research in this arena will help determine whether this mechanistic explanation can be verified and generalized.

Our conclusions indicate that an evaluation of Cu bioavailability in the presence of DOC should consider the Cu-binding characteristics of the individual ligands comprising the DOC. The conclusion that the nature of the ligand is important in evaluating Cu bioavailability and determining Cu toxicity is supported by Azenha et al. (1995). Their study used Cu–ligands covering a broad range of stability constants and found that Cu bound to weak to moderate ligands was available and toxic to bacterial cells. In a followup study, these researchers showed that Cu bound to organic ligands is displaced by bacteria and chitin, and as expected with equilibrium theory, the nature of both the abiotic and biotic ligands was important in Cu distribution (Vasconcelos et al. 1997).

Although we were able to distinguish among Cu species that contributed to overall toxicity in the bioassay, we cannot conclude whether the Cu_{OX} and (or) Cu_{MAL} fractions contributing to toxicity were directly available through adsorption/uptake or indirectly available through dissociation/association. Research into distinctions between direct and indirect biological availability of various Cu fractions deserves greater attention. New information in this research area will continually improve assessments in bioavailability and determining underlying mechanisms that explain relative contributions to toxicity from various Cu fractions bound by DOC.

The overall protective effect of total DOCeq (the composite of individual ligands comprising DOC_{eq}) observed through increased survival was only appreciable above thresholds of ≥8 and ≥16 mg DOC_{eq}/L for the 10 and 20 μg Cu/L exposures, respectively (Fig. 1). For exposures where ≥50% mortality occurred within 168 h, LT_{50} fell within a narrow range for low-DOC_{eq} treatments (45–75 h) but was >168 h for higher DOC_{eq} levels. This effect occurred at a DOC_{eq} of >4 mg/L and at a DOC_{eq} of >8 mg/L for nominal Cu of 10 and 20 μg/L, respectively. Similarly, time-dependent protective effects of DOC were reported by Brown et al. (1974) using humic acid and Andrew et al. (1977) using pyrophosphate, where increased median sur-

Table 4. Parameter estimates for lognormal models of failure time as a function of various Cu species, determined with and without DOC_{eq} as an independent variable.

		DOC_{eq} included in model						DOC_{eq} omitted from model					
		96 h			168 h			96 h			168 h		
Cu species	Variable	Coefficient	SE	*p*	Coefficient	SE	*p*	Coefficient	SE	*p*	Coefficient	SE	*p*
Total Cu	Intercept	6.519	0.15	<0.001	7.906	0.20	<0.001	6.687	0.15	<0.001	8.234	0.22	<0.001
	log(Cu species)	–0.896	0.06	<0.001	–1.376	0.08	<0.001	–0.839	0.06	<0.001	–1.304	0.08	<0.001
	log(DOC_{eq})	0.243	0.02	<0.001	0.389	0.03	<0.001						
	Scale (σ)	0.625			0.907			0.677			1.007		
	log likelihood	–601			–778			–665			–868		
Cu_{INRG}	Intercept	6.082	0.11	<0.001	7.237	0.16	<0.001	5.183	0.06	<0.001	5.912	0.08	<0.001
	log(Cu species)	–0.855	0.05	<0.001	–1.313	0.07	<0.001	–0.477	0.03	<0.001	–0.756	0.04	<0.001
	log(DOC_{eq})	–0.382	0.04	<0.001	–0.571	0.05	<0.001						
	Scale (σ)	0.610			0.884			0.660			0.969		
	log likelihood	–590			–774			–664			–835		
Cu^{2+}	Intercept	5.366	0.08	<0.001	6.150	0.11	<0.001	4.820	0.04	<0.001	5.343	0.05	<0.001
	log(Cu species)	–1.087	0.07	<0.001	–1.683	0.10	<0.001	–0.624	0.04	<0.001	–0.997	0.06	<0.001
	log(DOC_{eq})	–0.336	0.04	<0.001	–0.500	0.06	<0.001						
	Scale (σ)	0.634			0.930			0.673			0.994		
	log likelihood	–624			–815			–663			–857		
Cu_{INRG} + Cu_{MAL}	Intercept	6.076	0.11	<0.001	7.220	0.16	<0.001	5.510	0.07	<0.001	6.405	0.10	<0.001
	log(Cu species)	–0.818	0.05	<0.001	–1.253	0.07	<0.001	–0.606	0.04	<0.001	–0.948	0.05	<0.001
	log(DOC_{eq})	–0.212	0.03	<0.001	–0.310	0.04	<0.001						
	Scale (σ)	0.610			0.880			0.633			0.920		
	log likelihood	–584			–767			–611			–796		
Cu_{INRG} + Cu_{OX}	Intercept	6.124	0.12	<0.001	7.284	0.16	<0.001	6.063	0.10	<0.001	7.225	0.14	<0.001
	log(Cu species)	–0.788	0.05	<0.001	–1.203	0.07	<0.001	–0.771	0.04	<0.001	–1.186	0.06	<0.001
	log(DOC_{eq})	–0.023	0.02	0.308	–0.022	0.03	0.486						
	Scale (σ)	0.609			0.878			0.610			0.879		
	log likelihood	–581			–763			–581			–763		
Cu_{INRG} + Cu_{MAL} + Cu_{OX}	Intercept	6.142	0.12	<0.001	7.308	0.16	0.000	6.206	0.11	<0.001	7.437	0.15	<0.001
	log(Cu species)	–0.779	0.05	<0.001	–1.187	0.07	0.000	–0.795	0.05	<0.001	–1.219	0.06	<0.001
	log(DOC_{eq})	0.027	0.02	0.223	0.053	0.03	0.082						
	Scale (σ)	0.607			0.876			0.608			0.877		
	log likelihood	–578			–761			–579			–762		

Fig. 3. Hazard functions ($h(t)$) derived from the lognormal failure time model determined using (*a*) measured total Cu and (*b–f*) modeled Cu species fractions. The Cu stressor variable used for the output depicted is the 96-h LC_{50} for each Cu species at the 4 mg DOC_{eq}/L level. Plotted lines are the probability values for each time interval that were estimated separately for DOC_{eq} treatment levels (0–16 mg DOC_{eq}/L).

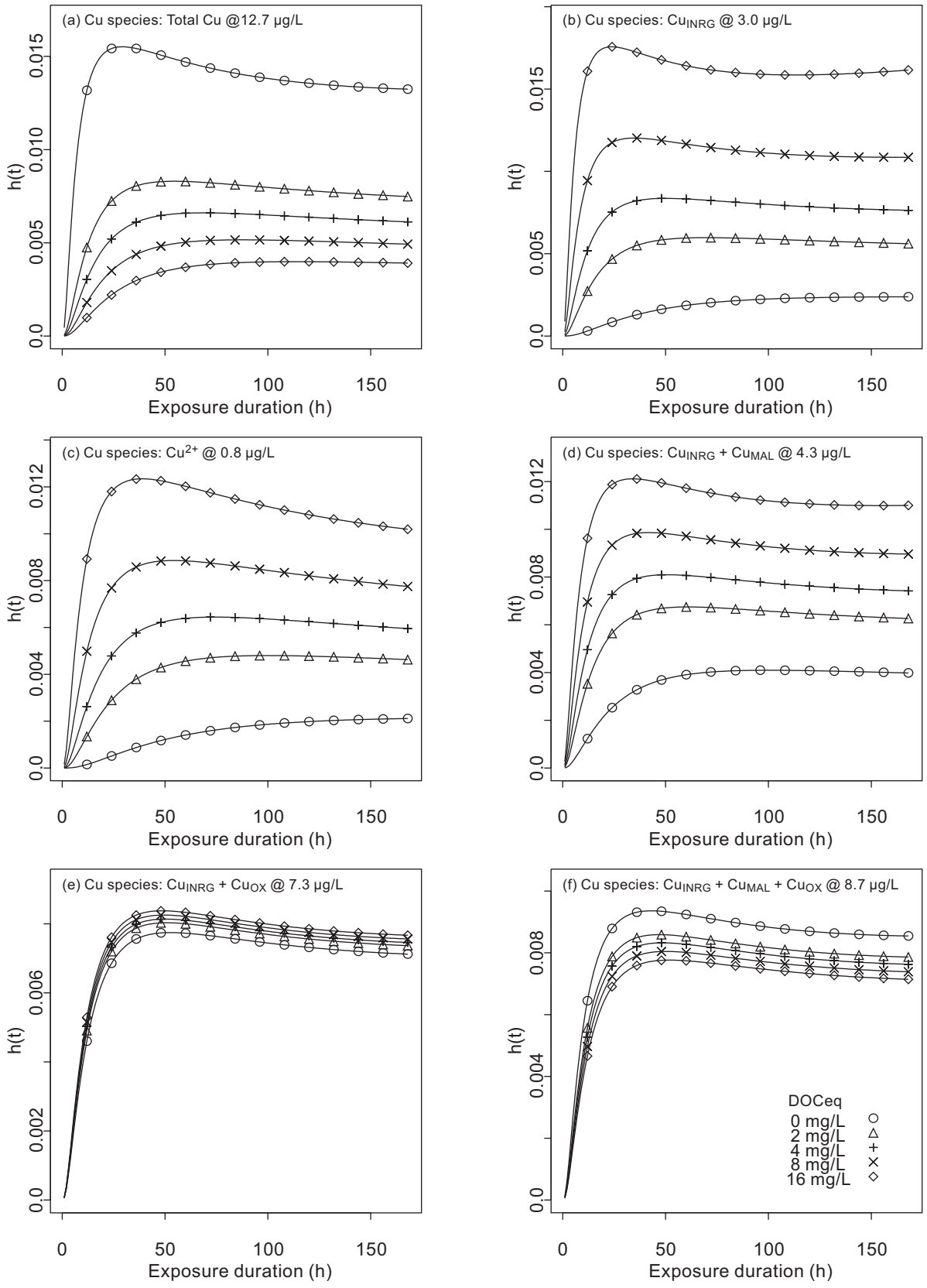

Fig. 4. Observed survival and estimated survival functions ($S(t)$) derived from the lognormal failure time model expressed as a function of the Cu species fraction, $Cu_{INRG} + Cu_{OX} + Cu_{MAL}$.

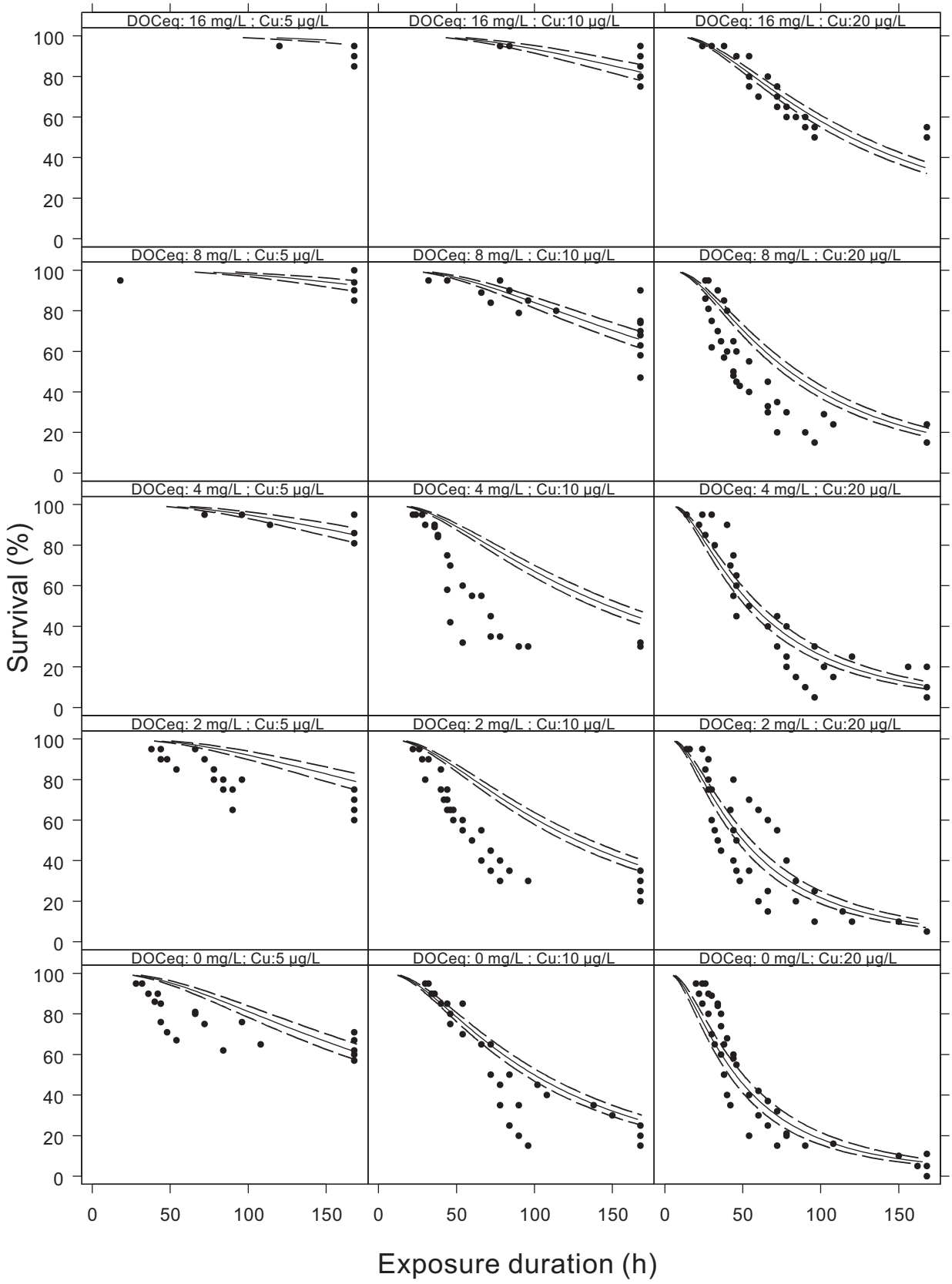

Table 5. Cu-binding affinities (K_{Cu}) of organic acids used in the bioassay and reported for fish gills.

Ligand	log K_{Cu}	Source
Malonic acid	5.58	MacRae et al. 1999*b*
Oxalic acid	6.6	MacRae et al. 1999*b*
Dipicolinic acid	8.15	MacRae et al. 1999*b*
Rainbow trout (*Oncorhynchus mykiss*) fish gill	7.5	MacRae et al. 1999*a*
Brook trout (*Salvelinus fontinalis*) fish gill	7.25	MacRae et al. 1999*a*
Fathead minnow (*Pimephales promelas*) fish gill	7.4	Playle et al. 1993*b*

vival times were directly related to the total concentration of the Cu ligand.

Conclusions

Our study demonstrates that Cu bound in low-affinity organic complexes similar to those found in nature contributes to acute toxicity in rainbow trout. These results are consistent with the hypothesized model wherein (*i*) Cu toxicity is proportional to the effective bioavailable concentration of Cu and (*ii*) a threshold for Cu bioavailability is determined by the Cu-binding affinity of the fish gill, which may be greater than or less than the affinity of particular organic ligands present in natural waters. Furthermore, the results presented show that studies to assess specific conditions of Cu toxicity should include analysis of specific Cu–ligand complexes to determine their relative concentration, bioavailability, and toxicity. This includes studies that establish water quality criteria intended to be protective of fish because toxicity thresholds cannot be accurately determined without considering site-specific geochemical characteristics that determine the bioavailability of Cu.

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