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MODULATION OF CUPRIC ION ACTIVITY BY pH AND FULVIC ACID AS DETERMINANTS OF TOXICITY IN *XENOPUS LAEVIS* EMBRYOS AND LARVAE

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Abstract—An ion-specific electrode measured cupric ion activity modulated by fulvic acid (FA) and pH in a series of modified Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX) toxicity assays. Hydrogen ion concentration was the primary determinant of cupric ion activity, while FA played a smaller but significant role. Fulvic acid was a weak copper complexing agent at pH 5.50. At pH 5.50 there was slight reduction of ionic activity and a subsequent attenuation of copper toxicity with 5.0 mg/L FA. At pH 7.50, FA also had a mild attenuating effect on copper toxicity. At pH 6.50, copper was strongly complexed by FA at total copper (TCu) concentrations below its pH-dependent solubility limit. At TCu concentrations above the solubility limit FA enhanced toxicity. There was more cupric ion activity measured in the presence of 0.5 and 5.0 mg/L FA than without it at TCu concentrations above the solubility limit. The proposed mechanism for this behavior was FA action as a nucleation inhibitor. Under the chemical conditions of the pH 6.50 experiments, a stable supersaturation of copper was formed, resulting in a more toxic aqueous matrix.

Keywords—Copper toxicity FETAX Fulvic acid pH

INTRODUCTION

Many water resources receive wastes from anthropogenic sources. Mining activities and various industrial processes produce heavy metal wastes containing copper that have created ecotoxicological problems. The application of copper to aquatic ecosystems for the control of blue-green algae is still commonly practiced today. Because copper is so widespread in both polluted and unpolluted waters, there is abundant literature on copper toxicity.

Although it is widely accepted that copper toxicity in aqueous environments is dependent on the chemistry of a given system, there is a surprising lack of literature that examines the interaction of physicochemical parameters with copper toxicity. Water hardness, pH, and dissolved organic material (DOM) exert profound influence on the toxicity of copper. For example, increasing water hardness can reduce the toxicity of copper to aquatic organisms [1]. This presumably results from calcium ions competing with metal ions for binding sites on, for example, the gills of fish. The hydrogen ion concentration in aquatic systems is the primary determinant of solubility, speciation, and availability of many heavy metals [2]. Copper solubility is extremely pH dependent, and higher solubility and toxicity are associated with low pHs. Dissolved organic materials, such as humic acids (HA) and fulvic acids (FA), are known to interact with heavy metals and in many cases reduce metal availability [3,4].

From a biological perspective, the relationship among pH, water hardness, DOM, and metal toxicity has been investigated with fish, algae, and daphnids. Quite often, biological work has not adequately characterized the chemistry of the test system, resulting in incomplete or marginally useful data. On the other hand, purely chemical approaches are often conducted under conditions that do not approach those of natural waters and may not extend to consequences of physicochemical interactions in ecosystems. This fragmentation of research into purely biological or chemical experimentation leaves gaps in our understanding of pollutant interactions in aquatic ecosystems.

Numerous chemical studies of the complexation of heavy metals by DOM including HA have been made [5–10]. Few studies, however, have quantified the degree to which complexation and changes in metal speciation by DOM affect the toxicity of these metals [11,12]. Humic substances (HA and FA) attenuate metal toxicity in some studies [13] while toxicity varies depending on the concentrations of metal and HA and other factors including hardness, alkalinity, and pH in others [14–17]. Winner [17] described enhanced cadmium toxicity with HA under certain chemical conditions but did not propose a mechanism for this unusual response.

This study was designed to investigate the toxicity of copper within defined chemical conditions. Specifically, changes in cupric ion activities ($\{Cu^{2+}\}$) resulting from physicochemical interactions of pH and FA were measured. These activities were correlated with biological responses of the South African clawed frog *Xenopus laevis* in modified Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX) bioassays [18].

MATERIALS AND METHODS

Nominal concentrations of 0.50 mg/L FA and 5.00 mg/L FA, representing relatively low and high surface water levels, were used to simulate lake, stream, and river water environments [19]. Copper concentrations were chosen to target nonlethal endpoints, though some mortality occurred. Growth inhibition and teratogenesis were the primary endpoints examined. Measurements of total copper (TCu) and $\{Cu^{2+}\}$ were correlated with toxicity.

Dilution water

Reconstituted water was used for all experiments, with the following constituents: 0.676 mM $CaCl_2$, 0.304 mM $MgSO_4$,

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1.19 mM $NaHCO_3$ [20]. A 1 mM phosphate buffer controlled the hydrogen ion concentration for all exposure regimes. The reconstituted water more closely approximated naturally occurring water than FETAX solution, which is an idealized saline solution for embryonic development.

Preparation of metal stock solutions

Copper was purchased from Sigma Chemical Company (St. Louis, MO, USA) as the anhydrous dichloro salt. Stock solutions of 10 mg/L were prepared with the dilution water described above. The dilution water was filtered through a Nucleopore^R 0.45-μm mixed ester cellulose filter prior to the addition of metal. One milliliter of concentrated Baker^R nitric acid was added per liter of dilution water to prevent the precipitation of copper hydroxides.

Preparation of FA stock solutions

Suwanee River FA reference material was purchased from the International Humic Substances Society (IHSS) (Denver, CO, USA). Stock solutions of 20 mg/L were prepared with the filtered dilution water as described above. Elemental content (ash free) of the FA was 53.5% carbon, 4.3% hydrogen, 41.0% oxygen, 0.7% nitrogen, and 0.6% sulfur, and the average molecular weight was 800 daltons [21].

Preparations of treatment groups

For each treatment group, copper and FA stock solutions were pipetted into 280-ml Nalgene^R polycarbonate containers. Filtered dilution water was added to equal 270 ml of solution. Averaged total copper concentrations for exposure groups were as follows: 0.042, 0.077, 0.165, 0.440, and 1.180 mg/L. Prior to pH adjustment, each solution was acidified with concentrated HCl to prevent precipitation and stirred well to obtain 10-ml samples for total metal analysis by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). All solutions were then brought to the desired final pH with NaOH.

Frog culture and egg preparation

Eggs were obtained from the *Xenopus* colony at the U.S. Environmental Protection Agency's (EPA) Environmental Research Laboratory–Corvallis, Oregon. Adult frogs were kept in glass aquaria in an environmentally controlled chamber. A temperature of 24.0 ± 1.0°C was maintained with a 16:8 h light : dark photoperiod. Frogs were fed a diet of beef liver twice weekly, and their water was changed after feeding. Well water from the EPA Willamette Research Station in Corvallis was used in frog holding and breeding tanks.

Adult frogs were induced to breed via an injection of human chorionic gonadotropin (Sigma, St. Louis, MO, USA) in the dorsal lymph sack, as per American Society of Testing and Materials (ASTM) guidelines [18]. The eggs were then harvested, and the jelly coats were removed with a 2% L-cysteine solution in distilled water. This solution was adjusted to pH 8.10 with 10% NaOH. This procedure was performed as specified in ASTM [18], with the exception that dilution water was used rather than FETAX solution. The dejellied eggs were then sorted under a dissection microscope and only normally cleaving eggs were selected and transferred to a 100-mm-diameter petri dish with dilution water. Only stage 8½ blastulae were used to start each experiment [18].

Exposures

A series of five copper concentrations, plus controls were prepared with dilution water. Exposure chambers were 60 × 15-mm plastic petri dishes. Nine exposure chambers were used for each TCu concentration. Fifteen eggs were placed in each chamber. For each concentration of copper, three replicates were run without FA, three with 0.50 mg/L FA, and three with 5.00 mg/L FA. This array was run at nominal pHs of 5.50, 6.50, and 7.50. Two separate assays were run from each group of exposure media for each pH. An additional experiment was conducted with a single assay at pH 6.50. The pH was measured with an Omega PHB-70X water analyzer (Stamford, CT, USA), and adjustments in exposure media pH were made daily with HCl and NaOH as needed. Previous investigation determined that between the pH 5.50 and 7.50, *Xenopus* embryos and early larval stages developed without observed hydrogen ion toxicity. Exposures occurred at 24 ± 1.0°C in an environmental chamber with a 16:8 h light : dark photoperiod. Because of limitations in the number of eggs available, each FA- metal treatment was conducted at one pH block at any time with all experiments replicated for each pH.

All exposure media (inclusive of FA, TCu, and dilution water) were equilibrated for 5–8 d prior to exposure. During this time, the pHs were adjusted daily with NaOH or HCl as needed. When pH drift in the treatment groups was less than 0.1 unit per 15 min, exposures were initiated. The pH of every exposure group was taken daily prior to the transfer of media to individual petri dishes. Mortalities were recorded and removed daily. After living eggs and larvae were transferred to new media, the old media were pooled from the three dishes, and the pHs were recorded. The eggs were transferred to a new dish containing 10 ml of exposure medium every 24 h. All exposures lasted 96 h.

Biological responses

After exposure, surviving larvae were examined with a dissection microscope for developmental abnormalities. For control groups and the lowest exposure groups it was necessary to anesthetize the larvae with MS-222 [18]. Terata, or developmental abnormalities, were recorded for each dish. Growth inhibition was examined by measuring the individual larvae with the Sigma Scan^R scientific measurement system (Jandel Scientific, Corte Madera, CA, USA). A photographic darkroom enlarger was used to project the image of the larvae onto a digitizing pad, and the individual images were traced with a mouse to read lengths into data files.

Total metal analysis

Copper concentrations of test solutions were determined with a Bausch and Lomb/Applied Research Laboratory model 3580-vacuum ICP-AES. Dilution water used for control groups was also analyzed by ICP-AES to measure background metals.

Cupric ion activity

An Orion (Boston, MA, USA) cupric ion-specific electrode (ISE) (model 94-29) was used with an Orion (model 90-02) double junction reference electrode, with 10% KNO_3 as the outer chamber filling solution to obtain millivolt measurements to ±0.1 mV. The slope of the probe response was checked prior to experimental measurements to insure the response was within the 25–30 mV/decade range as described by Orion [22].

Millivolt measurements were taken in unstirred solutions, and values were not recorded until readings were stable for at

least 3 min (approximately 20–35 min). After all solutions were measured, pH measurements were taken again to insure that all pHs were within ±0.04 pH units. Ionic strength adjustment solution was not used.

The MINTEQA2/PRODEFA2 [23] geochemical assessment model simulated inorganic equilibria for copper in each zero FA treatment. These simulations established a speciation profile for the five metal concentrations at each pH. Model-generated estimates of $\{Cu^{2+}\}$ were available in conjunction with cupric ISE data. The simulations provided (1) a method for determination of $\{Cu^{2+}\}$ in organic-free solutions, and (2) a tool to complement the ISE in determining $\{Cu^{2+}\}$ in the FA treatments.

Cupric ion activity was estimated from MINTEQ model simulations of zero FA treatment groups at pH 5.50 and 6.50. The corresponding millivolt measurements taken from those groups were used to generate a calibration curve from which all other millivolt measurements could be assigned a corresponding activity. A plot of measured millivolt values versus the log of modelled $\{Cu^{2+}\}$ had the form: mV = 30.21(log activity) + 289.39, with a correlation coefficient (r) of 0.992.

RESULTS

Probe response

Quadratic regression models for millivolt measurements at pH 5.50 versus TCu were simultaneously fit for the zero, 0.5, and 5.0 mg/L FA treatment groups using indicator variables [24]. The only significant difference between the groups was that the equation for the 5.0-mg/L FA was shifted 5.3 mV lower than the 0.0-/and 0.5-mg/L FA (p = 0.036). The 5.3 mV shift corresponded to a decrease in log $\{Cu^{2+}\}$ of 0.1726.

At pH 6.50 ISE measurements indicated complexation at TCu concentrations below the solubility limit of copper (Fig. 1a). Voltages at both 0.5 and 5.0 mg/L were lower than for the 0.0-mg/L FA groups up to TCu = 0.44 mg/L. At that concentration, where MINTEQ modelling output suggested the system was essentially saturated with copper, and at 1.20 mg/L, the trend of complexation and lower activities reversed. Higher millivolt readings were recorded for the higher FA groups. This was contrary to observations made throughout the pH 5.50 experiments and readings taken below saturation concentrations at pH 6.50. The ISE readings were taken again the following day, with good replication of voltage values. As these results were unusual, the experiments were repeated, and the same patterns were observed (Fig. 1b). Note the influence of FA as it increases $\{Cu^{2+}\}$ beyond its normal saturation point at high total copper (shown by flat region of the zero FA curve).

Regression models for millivolt measurements at pH 7.50 versus TCu for the 0-, 0.5-, and 5.0-mg/L FA were simultaneously fit using indicator variables [23]. No significant differences were found between the three groups. Because the measured voltages were so low at this pH, and response becomes nonlinear approaching the detection limits of the probe, no attempt was made to use the linear calibration curve to convert these measurements to activities. Too few data were available to produce an alternative model to fit the probe response. All biological responses were reported in terms of TCu.

Biological responses

Mortality was highly correlated with $\{Cu^{2+}\}$ over all pH treatments. No significant copper concentration-dependent

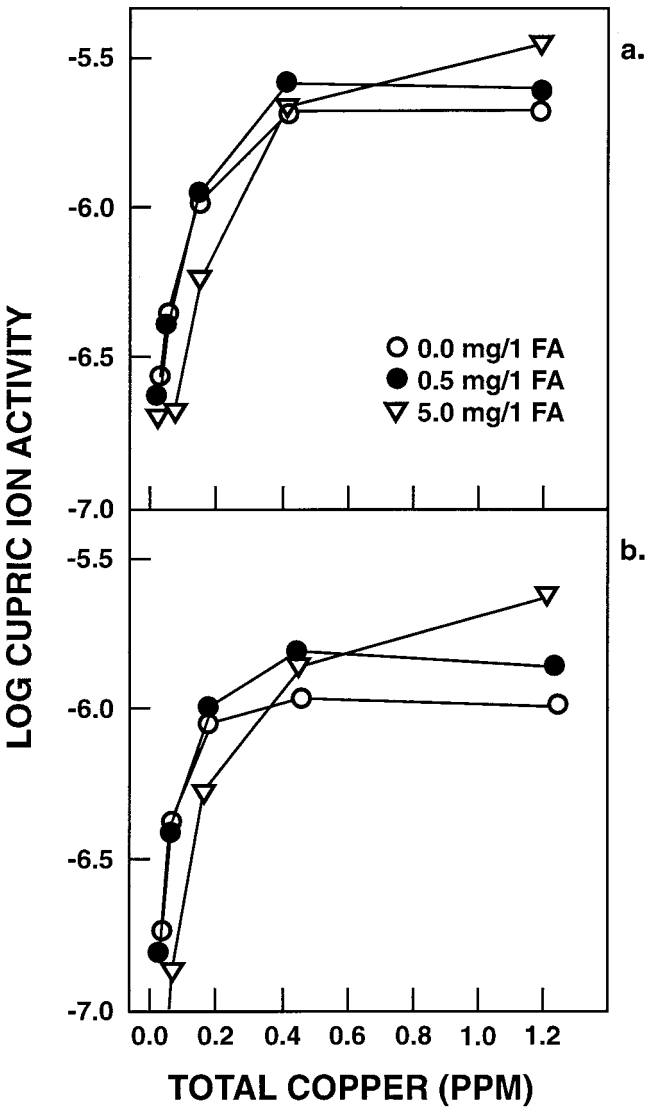

Fig. 1. Fulvic acid (FA)-modulated copper activity ($\{Cu^{2+}\}$) at pH 6.50. (**a**) A profile of FA-modulated $\{Cu^{2+}\}$ measured directly from exposure media with a cupric ion-specific electrode (ISE). (**b**) The FA-modulated $\{Cu^{2+}\}$ measured directly from exposure media in a separate pH 6.50 experiment.

mortality occurred in any of the treatment groups at pH 7.50 (data not shown). A Weibull regression model [25] was fit for activity vs. mortality data for two replicates at pH 5.50, and three replicates at pH 6.50 (Fig. 2). The model has the form:

$$\% \text{ mortality} = \underset{(24.60)}{139.85} \cdot \exp[-\log \text{ activity}/-\underset{(0.08)(3.71)}{5.29^{15.98}}]$$

Values in parentheses are standard errors of regression coefficients.

Length was also highly correlated with ionic activity (Fig. 3). No statistically significant growth inhibition occurred at pH 7.50 (data not shown). Weibull regression models were individually fit for pH 5.50 and each 6.50 data set.

A common model was fit for all data, with R^2 = 0.81, but a test for the adequacy of a common model rejected the hypothesis of no difference between the full regression model and the individual models described above.

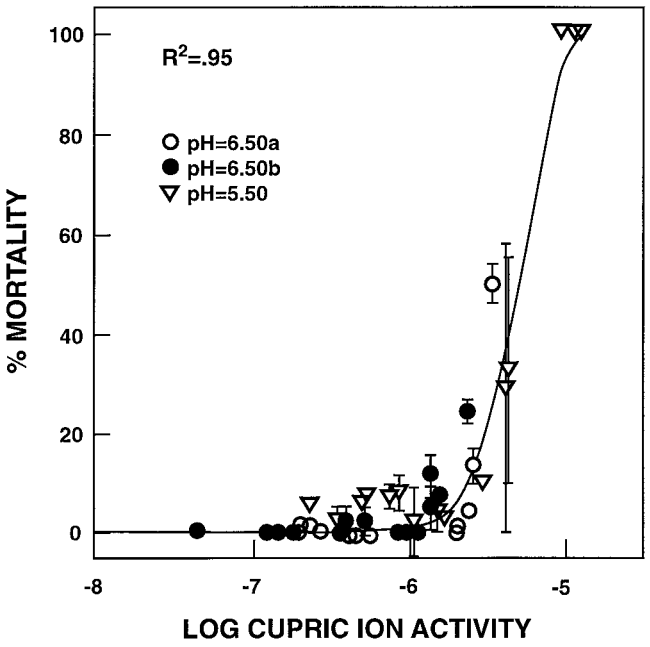

Fig. 2. Mortality. A Weibull regression model was fit for pooled mortality data over the pH range of 5.50 and two replicates at 6.50.

Malformations

Slight facial malformations and incomplete gut coiling were observed for high TCu at pH 7.50 and were statistically significant only in a few cases. At pH 5.50 and 6.50, malformation rates were highly correlated with $\{Cu^{2+}\}$ (Fig. 4). Weibull regression models were fit for data over these pHs (these were bound to a lower limit of 10% to be realistic biologically, as control malformation rates were approximately 10%).

DISCUSSION

The hydrogen ion concentration played a pivotal role in the activity and toxicity of copper. Not only was pH the primary determinant of $\{Cu^{2+}\}$ in the zero FA treatments, but it also determined FA behavior. Interactions between FA and pH were associated with FA having a varying role in modulating $\{Cu^{2+}\}$ and toxicity.

At pH 5.50, FA was a mild cupric ion complexing agent. No measurable difference in $\{Cu^{2+}\}$ occurred between the 0- and the 0.5-mg/L FA treatment groups, but at 5.0 mg/L FA there was a statistically ($p = 0.036$) and biologically (note steepness in all dose–response curves) significant lowering of activity and a corresponding decrease in toxicity. At high TCu however, sufficient $\{Cu^{2+}\}$ for 100% mortality was present, regardless of FA. Possibly $\{Cu^{2+}\}$ was not as good a competitor for binding sites on the FA, when a high concentration of protons was present at pH 5.50.

Contrary to the attenuation of toxicity by FA observed at pH 5.50, enhanced $\{Cu^{2+}\}$ and resulting enhanced toxicity were observed at high TCu at pH 6.50. The ISE measurements showed $\{Cu^{2+}\}$ well above the solubility limit at high TCu in the FA treatment groups. Figure 1 (a and b) shows a variety of chemical interactions. In the 0 FA treatment groups, ionic activity tended toward saturation. This is represented by the flatness of the curve where little change occurred in $\{Cu^{2+}\}$ as excess copper precipitated. In the FA treatment groups at low TCu, there was some complexation of cupric ions. The amount of complexation was positively correlated with FA concentration. There was not, however, an attenuation of toxicity in these low TCu treatments. Elevated biological responses occurred in these groups. The mechanism for this was not identified.

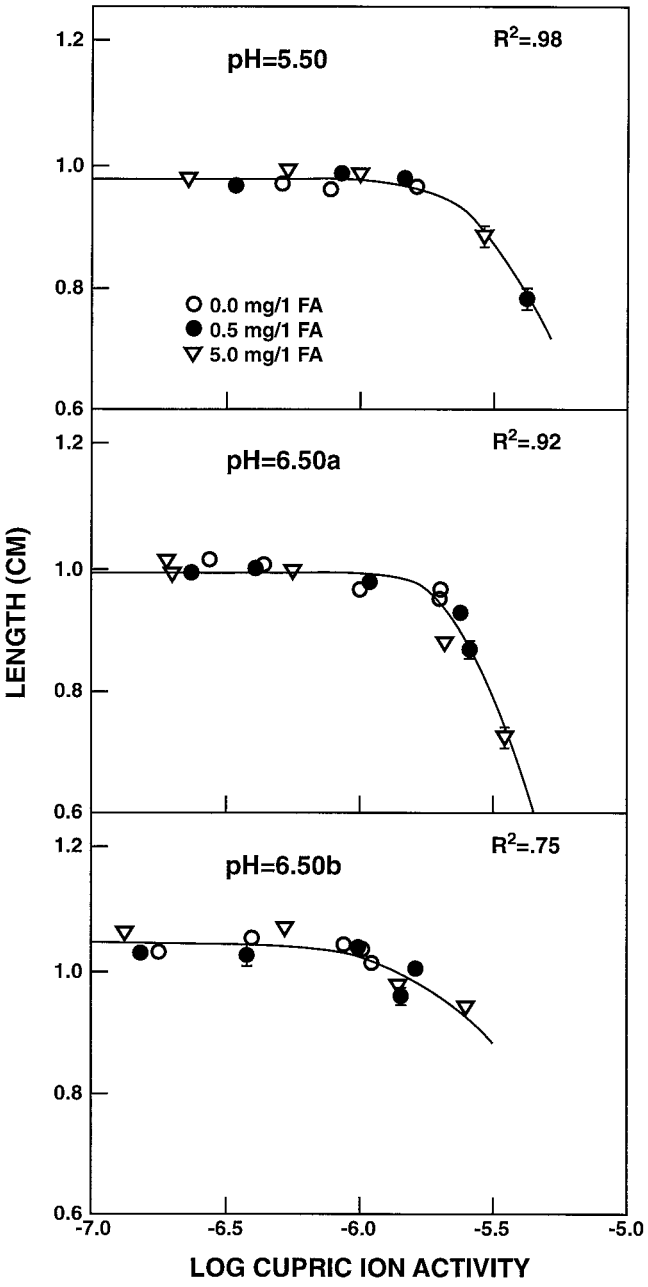

Fig. 3. Growth inhibition. Comparison of growth inhibition data for pH (**a**) 5.50, (**b**) 6.50a, and (**c**) 6.50b. Error bars are the standard errors of the means. The fit curves are Weibull regression models of the form: Length = a − exp[−(log activity/b)^c, where a = asymptotic to no effect lengths; b = log activity associated with 37% response; c = a shape parameter.

At higher TCu concentrations, however, the trend of complexation and subsequent lower ISE voltage measurements for FA reversed. Where the 0 FA system became saturated, the 0.5 and 5.0 FA treatment groups actually exhibited higher millivolt measurements. Increased toxicity corresponded with the higher ISE measurements. Enhanced mortality, malformation, and growth inhibition were observed for the FA treatment groups in comparison to the 0 FA groups.

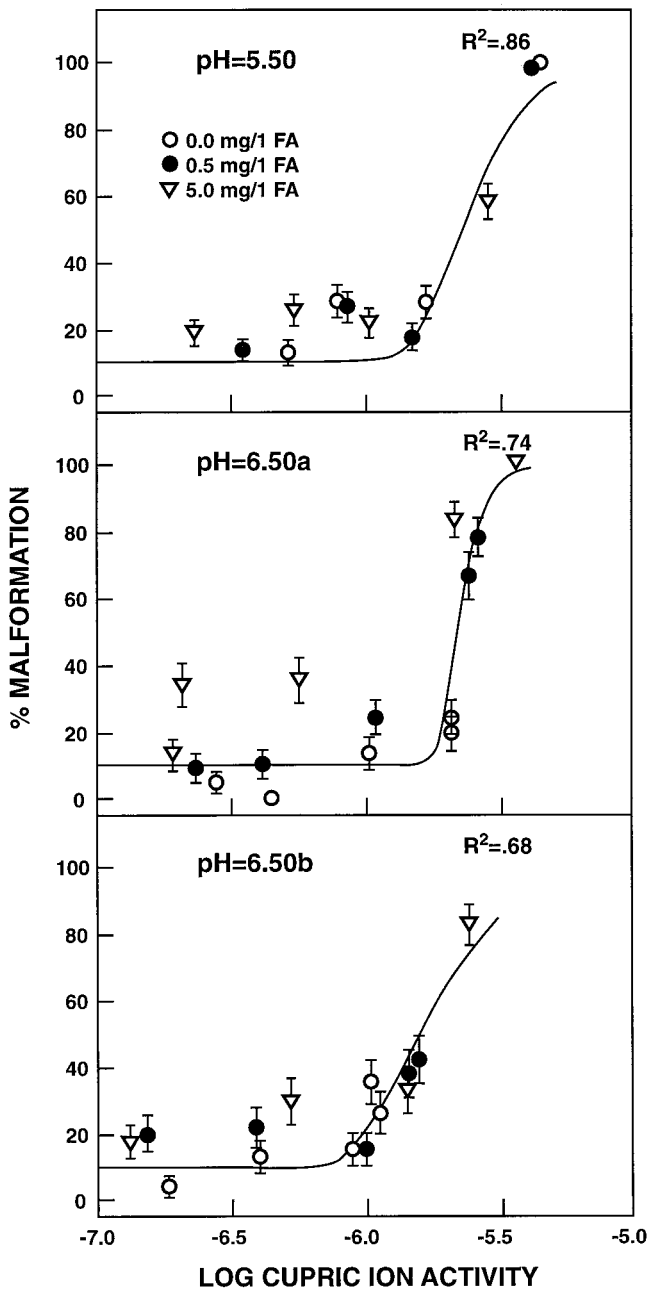

Fig. 4. Malformation. Weibull type regression models were fit for malformation data from pooled pH 5.50 results and two experiments at pH 6.50. These models were bound so that the curves were asymptotic to 10% malformation as a lower limit and 100% as an upper limit.

An explanation of this behavior requires a discussion of precipitation dynamics and more specifically nucleation. Nucleation is the ''birth'' of crystals from solutions [26]. This process controls the size, number, and structure of precipitated crystals. Prior to nucleation there is continuous formation and dissolution of ionic or molecular clusters in equilibrium with all other clusters. If the concentration of solute ions or molecules is high enough, the clusters become significantly large enough to become consolidated into small crystallites, whereupon the supposedly irreversible crystal growth ensues [26].

However, small quantities of certain compounds greatly hinder the formation of crystals and hence stabilize supersaturated solutions [26]. Fulvic acid could be functioning as a nucleation inhibitor under the chemical conditions at pH 6.50 (J.C. Westall, personal communication). This would account for the enhanced activity and toxicity as a function of increasing FA concentrations that we observed and for results reported by Winner [17].

Two assays were run from the same batches of exposure media for the pH 6.50 (a) experiment, and another assay was conducted from a new batch of exposure media for the 6.50 (b) experiment. The difference in the magnitude of $\{Cu^{2+}\}$ between the two experiments at pH 6.50 is probably a function of the kinetics of the system.

Based on the low $\{Cu^{2+}\}$ at pH 7.50, observed malformation rates were higher than expected. These malformations were subtle in comparison to malformations observed at lower pHs, and it is unknown if these slight anomalies would compromise survival outside the test system. Toxicity may be the result of the dissociation of other aqueous copper species such as hydroxides and carbonates at the low pH environment created at the gill boundary layer. Acidification of the gill boundary layer in waters of high pH occurs as a result of CO_2 excretion in fish [27]. Presumably, the same phenomenon occurs at the gills of aquatic amphibian larvae. Here precipitated copper may act as a source of aqueous copper as the equilibrium is shifted by copper uptake at the gills. Other copper species in solution such as hydroxides and carbonates may also be toxic to *Xenopus*.

Weibull regression models fit activity–response data extremely well. One problem that we faced in fitting these models was that we were unable to use zero-$\{Cu^{2+}\}$ because the predictor variable was log $\{Cu^{2+}\}$. This precluded using control groups or other measurements from treatment groups that had associated activities below the detection limit of the probe. Typical ''noise'' inherent to our test system led us to bound regression models for malformation data to 10% as the lower asymptote. The observed scatter in malformation data (Fig. 4) was not surprising. Malformations were graded quantally; an individual was either malformed or not. Because there were many types of developmental anomalies observed, malformation is not a single endpoint but a collection of endpoints.

As *Xenopus* develop at a rapid rate, they are very susceptible to perturbation by chemical insult. The natural variability inherent in any biological system would add an expected amount of scatter to the data as well.

CONCLUSION

The description of copper toxicity as a function of TCu in the literature is presently incomplete. $\{Cu^{2+}\}$ is the result of many factors that play significant roles in all aquatic systems. The measurement of $\{Cu^{2+}\}$ to estimate potential risks to aquatic life is far superior to TCu measurement, as the influences of pH and DOM are somewhat reflected in activity measurements. These experiments show that a given concentration of TCu may be lethal or irrelevant to the development of amphibian eggs and larvae (Appendix), while biological responses associated with $\{Cu^{2+}\}$ were predictable. FA can either increase or decrease $\{Cu^{2+}\}$ and resultant toxicity. Although FA can complex copper and attenuate toxicity, and probably does so under most natural conditions, there are conditions under which FAs actually enhance toxicity. By acting as nucleation inhibitors, FAs may create stable supersaturations of ionic copper and other aqueous species, by adsorbing onto crystal nuclei growth sites, resulting in a potentially more toxic aqueous matrix.

We must be critical and careful of the wholesale acceptance of the role of DOM as complexation agents of heavy metals in the environment. Work with soil pore water has shown that large organic acids such as humic and fulvic materials play significant roles in the retardation and/or prevention of inorganic precipitates such as hydroxyapatite and dicalcium phosphate dihydrate [28,29]. The same phenomenon was observed in these experiments at pH 6.50. The extent to which this may occur in natural aquatic systems remains unknown.

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