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**Copper complexation by fulvic acid affects copper toxicity to the larvae of the
polychaete *Hydroides elegans***

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1 Abstract

2 Copper toxicity is influenced by a variety of environmental factors including dissolved
3 organic matter (DOM). We examined the complexation of copper by fulvic acid (FA),
4 one of the major components of DOM, by measuring the decline in labile copper by
5 anodic stripping voltammetrically (ASV). The data were described using a one-site
6 ligand binding model, with a ligand concentration of $0.19 \mu\text{mol site mg}^{-1} \text{C}$, and a $\log K'$
7 of 6.2. The model was used to predict labile copper concentration in a bioassay
8 designed to quantify the extent to which Cu-FA complexation affected copper toxicity to
9 the larvae of marine polychaete *Hydroides elegans*. The toxicity data, when expressed
10 as labile copper concentration causing abnormal development, were independent of FA
11 concentration and could be modeled as a logistic function, with a 48-h EC_{50} of $58.9 \mu\text{g l}^{-1}$.
12 However, when the data were expressed as a function of total copper concentration, the
13 toxicity was dependent on FA concentration, with a 48-h EC_{50} ranging from $55.6 \mu\text{g l}^{-1}$ in
14 the no-FA control to $137.4 \mu\text{g l}^{-1}$ in the 20 mg l^{-1} FA treatment. Thus, FA was protective
15 against copper toxicity to the larvae, and such an effect was caused by the reduction in
16 labile copper due to Cu-FA complexation. Our results demonstrate the potential of ASV
17 as a useful tool for predicting metal toxicity to the larvae in coastal environment where
18 DOM plays an important role in complexing metal ions.

1 **Keywords:** Copper; Speciation; Toxicity, Larvae; Fulvic acid; DOM

2 **1. Introduction**

3 Copper is an essential micronutrient for aquatic animals, but can cause toxic effects at
4 elevated concentrations (White & Rainbow, 1985). Background copper concentration in
5 marine surface water is usually low (0.03 to 0.23 $\mu\text{g/L}$), but can be very high ($> 100 \mu\text{g/L}$)
6 in coastal areas severely affected by human activities (Bowen, 1985). Copper toxicity is
7 influenced by a number of environmental factors, such as salinity, pH, particulate organic
8 matter (POM) and dissolved organic matter (DOM) (Campbell, 1995; Allen & Hansen,
9 1996). The concentration of DOM in natural water is typically measured as dissolved
10 organic carbon (DOC) (Stevenson, 1982). Humic substances are the most important
11 components of DOM, which may constitute from 30 to 90% of DOC in the aquatic
12 environment (Thurman, 1985). Humic substances are biogenic refractory
13 macromolecules with functional groups such as carboxylic, phenolic, alcoholic and
14 amino groups linked in a complex branching pattern (Stevenson, 1982). These
15 functional groups react with metal ions in the water, and the binding strength to these
16 different types of ligands determines the complexation characteristics of DOM
17 (Stevenson, 1982; Turner, Varney, Whitfield, Mantoura, & Riley, 1986). Such binding
18 reduces the concentration of free metal ions, which, according to the free-ion activity

1 model (FIAM), are the most biologically available and thus toxic form of the metals to
2 aquatic organisms (Morel, 1983).

3 However, in a review of metal speciation – bioavailability relationship, Campbell
4 (1995) found only seven studies that had quantitatively related the responses of aquatic
5 organisms to free-metal ion activities in the presence of DOM; of these, three conformed
6 to the FIAM and four deviated from the FIAM. Many more recent studies of metal
7 bioavailability – DOM relationship are also divided into those supporting the FIAM (Kim,
8 Ma, Allen, & Cha, 1999; Ma, Kim, Cha, & Allen, 1999; Van Ginneken, Bervoets, & Blust,
9 2001; Lorenzo, Nieto, & Beiras, 2002; Wang, Huang, & Liu, 2002) and those indicating
10 direct interaction between DOM and organisms (Roy & Campbell, 1997; Voets, Bervoets,
11 & Blust, 2004). To account for these and other exceptions to the FIAM, Santore, Di
12 Toro, Paquin, Allen, & Meyer (2001) proposed the biotic ligand model (BLM), which
13 predicts metal toxicity based on the amount of metal accumulated at presumptive binding
14 sites on biological surfaces, such as fish gills, in aquatic organisms. The BLM has been
15 successfully applied to a variety of freshwater organisms such as cladocerans and fish (Di
16 Toro, Allen, Bergman, Meyer, Paquin, & Santore, 2001; Zhou, Nichols, Playle, & Wood,
17 2005), amphipods (Borgmann, Nowierski, & Dixon, 2005), earthworms (Steenbergen,
18 Iaccino, Dewinkel, Reijnders, & Peijnenburg, 2005) and algae (De Schamphelaere,

1 Stauber, Wilde, Markish, Brown, Franklin et al., 2005).

2 Marine invertebrate larvae have also been widely used to assess the toxicity of
3 environmental samples because of the environmental relevancy, high sensitivity and low
4 cost (Chapman, Denton, & Lazorchak, 1995; His, Beiras, & Seaman, 1999). However,
5 the relationship between metal speciation in the presence of humic substances and the
6 toxic effects to marine organisms has been studied only in few instances (Lorenzo et al.,
7 2002; Rivera-Duarte, Rosen, Lapota, Chadwick, Kear-Padilla, & Zirino, 2005; Stauber,
8 Andrade, Ramirez, Adams, & Correa, 2005). Therefore there are scarce data to assess
9 the applicability of the FIAM and BLM as regulatory tools to predict metal toxicity to
10 coastal organisms. In this study, we examined the complexation of copper by fulvic
11 acid (FA) using anodic stripping voltammetry (ASV), and determined how the
12 complexation affected acute toxicity of copper to the larvae of *H. elegans*. Fulvic acid
13 can account for up to 90% of dissolved humic substances in coastal waters (Porta &
14 Ronco, 1993) and forms complexes with copper (Stevenson, 1982; Newell & Sanders,
15 1986). ASV labile copper, which includes cupric ion and inorganically bound copper
16 (Florence, 1986), has been shown to correlate well with cupric ion concentration (Allen
17 & Hansen, 1996; Lorenzo, Beiras, Mubiana, & Blust, 2005; Rivera-Duarte et al., 2005)
18 and considered to be the bioavailable fraction of the metal (Florence, 1986). *H. elegans*

1 is a serpulid polychaete worm ubiquitous in tropical and subtropical coastal communities.
2 The larvae of this polychaete are as sensitive as echinoderm and mollusk larvae to copper
3 stress (His et al., 1999; Xie et al., 2005) and are available throughout the year for use in
4 monitoring of water quality in tropical and subtropical waters. The larvae of another
5 species of serpulid polychaete (*Galeolaria caespitosa*), also sensitive to copper, have
6 been successfully used for this purpose in Australia (Ross & Bidwell, 2001). The result
7 of this study may help us understand the binding between FA and copper in seawater, and
8 how such binding affects the toxicity of dissolved copper to a species of this widely
9 distributed taxon of coastal animals.

10 **2. Materials and Methods**

11 *2.1. Reagents*

12 Standard copper solution (Orion ionplus™ 0.1 M cupric nitrate) was purchased from
13 Thermo Electron. Fulvic acid (FA) reference material (Suwannee River 1R101F, 1 g FA
14 = 0.5304 g C) was purchased from International Humic Substances Society. All other
15 chemicals used were of analytical grade. All solutions were prepared with Milli-Q™
16 water. Glass and polypropylene containers were soaked in 10% HCl, rinsed with
17 Milli-Q water, and dried in an oven before use. Experiments were conducted at $23 \pm 1^\circ\text{C}$
18 in artificial seawater (ASW) (34 psu, pH = 8.1) prepared according to a recipe in Lorenzo

1 et al. (2002). The background copper content, as measured by differential pulse anodic
2 stripping voltammetry (DPASV) using the standard additions method (Harvey, 2000),
3 was $1.1 \pm 0.3 \mu\text{g l}^{-1}$ ($n = 3$).

4 2.2. *Cu-FA complexation model*

5 A two-factor experiment was conducted to determine the conditional stability constant
6 and binding capacity for the Cu-FA binding reaction. The purpose was to determine if
7 we could use these binding constants and total copper concentration to predict labile
8 copper concentration. FA stock solution (0.2 g l^{-1}) was prepared by dissolving the FA
9 reference material in Milli-Q™ water, and stored at $4 \text{ }^\circ\text{C}$ in darkness to prevent
10 photodegradation. Copper stock solution (10 mg l^{-1}) was prepared by pipetting
11 appropriate amount of standard copper solution into ASW. Seventy five sets of copper
12 solution, i.e. a combination of 5 fulvic acid concentrations ranging from 0 to 64 mg l^{-1} x
13 15 copper concentrations ranging from 16 to $256 \mu\text{g l}^{-1}$, were prepared by mixing
14 appropriate amounts of fulvic acid and copper stocks. The highest copper concentration
15 used in this study was approximately 4 times the 48-h EC_{50} , as determined previously
16 under identical conditions (Wong, Wong, Shiu, & Qiu, 2006). The range of fulvic acid
17 concentrations used covered that encountered by coastal animals (Buffle, 1984). The
18 solutions were prepared in 50-ml volumetric flasks and kept in darkness for at least 24 h

1 to allow the complexation reaction to reach equilibrium. Copper concentrations were
2 measured by DPASV. Labile copper was determined in the non-acidified sample,
3 whereas total copper was determined after sample acidification with HNO₃ and UV
4 irradiation (Florence, 1986). Measurements were taken with a hanging mercury drop
5 electrode, an Ag/AgCl reference electrode and an auxiliary platinum electrode connected
6 to a Metrohm 694 VA stand coupled with a Metrohm 693 VA processor. Samples were
7 stirred at 3000 rpm and degassed by nitrogen for 5 min before measurement. The
8 scanning was performed from -500mV to 0 mV at a sweep rate of 6.67 mV s⁻¹ and an
9 increment step of 2 mV. The measured potential was -170 mV ± 35 mV and the
10 deposition time was 60 s. Three polarograms were recorded for each solution and the
11 mean peak height was used to calculate labile copper concentration.

12 Scatchard plot (Motulsky & Christopoulos, 2003) was used to visualize the binding
13 characteristics. The X axis is bound copper concentration and the Y axis is bound
14 copper concentration divided by labile copper concentration. The plot will be linear in
15 the presence of one class of binding sites, otherwise it will be curved (Van Ginneken et al.,
16 2001). Depending on the shape of the plot, the data of copper binding by FA were
17 described by a complexation model which assumes either one (Buffle, 1984; Lund,
18 Helbak, & Seip, 1990) or two (Ma et al., 1999) classes of ligand and a reaction

1 stoichiometry of 1:1:

$$2 \quad [\text{CuL}] = \sum_1^n \frac{[\text{L}_i]_T \times K_i' \times [\text{Cu}']}{1 + K_i' \times [\text{Cu}']}, \quad (1)$$

3 where [CuL] is bound copper concentration and [Cu'] is labile copper concentration.

4 The conditional stability constant(s) K_i' and ligand concentration(s) $[\text{L}_i]_T$ can be

5 calculated through nonlinear regression (Motulsky & Christopoulos, 2003).

6 2.3. Bioassays

7 Adult *H. elegans* were collected between August and November 2005 and in May 2006
8 from the underside of styrofoam floats previously placed into the water of a fish farm in
9 Sai Kung, Hong Kong (22°19' N, 114°16' E). They were brought to the laboratory at
10 Hong Kong Baptist University (HKBU) where they were reared in aerated seawater
11 aquaria. The procedures for obtaining gametes and performing artificial fertilization
12 had been described in Qiu & Qian (1997) and Pechenik & Qian (1998). Fertilized eggs
13 were incubated for 24 h to allow for development into trochophore larvae. Larvae were
14 concentrated using a sieve of 35 μm pore size, and then transferred into the exposure
15 solution using a micropipette.

16 Two separate bioassays were performed to determine the toxicity of fulvic acid and

17 copper to *H. elegans* larvae. The preparation of exposure solutions was identical to that

1 in section 2.2. The bioassays were performed in 6-well Iwaki™ polystyrene culture plates.
2 In the first bioassay, the toxicity of FA was examined by exposing the larvae to a series of
3 FA concentrations (0, 8, 16, 32, 64, 128, 256 mg l⁻¹) prepared by mixing FA stock
4 solution and ASW. In the second bioassay, the toxicity of Cu-FA solution was
5 determined in a combination of 4 fulvic acid concentrations (0, 5, 10, 20 mg l⁻¹) x 12
6 copper concentrations (0 to 362 µg l⁻¹). There were two replicates in the first bioassay
7 and four replicates in the second bioassay. Each replicate consisted of 20 to 30 larvae in
8 a 10 ml solution. The data were used to calculate overall percentage of abnormal larvae
9 in each treatment.

10 The relationship between percentage of abnormal larvae (Y) and total or labile copper
11 concentration (X) was expressed using a logistic model (Motulsky & Christopoulos,
12 2003):

$$13 \quad Y = B + \frac{A - B}{1 + 10^{C(\log EC_{50} - X)}} \quad (2)$$

14 where A is the maximum response, B the baseline response and C the slope. The
15 difference in log EC₅₀ between the FA treatments and the no-FA control was determined
16 using *F* test, which compares the goodness-of-fit of the two models with adjustment for
17 differences in degrees of freedom (Motulsky & Christopoulos, 2003).

18 **3. Results**

1 *3.1. Cu-FA binding characteristics and Cu-FA binding model*

2 Fig. 1A shows the relationship among FA, total copper concentration and labile copper
3 concentration in the exposure solution. It is clear that, in each total copper
4 concentration treatment, labile copper concentration declined with increasing FA
5 concentration. However, the magnitude of decline depended on FA and total copper
6 concentration. At the lowest FA concentration tested (8 mg l^{-1}), $[\text{Cu}']$ ranged from 82%
7 of total copper at $256 \text{ } \mu\text{g Cu l}^{-1}$ to 39% of total copper at $16 \text{ } \mu\text{g Cu l}^{-1}$. At 64 mg l^{-1} FA,
8 $[\text{Cu}']$ ranged from up to 21% of total copper at $256 \text{ } \mu\text{g Cu l}^{-1}$ to as low as 5% of total
9 copper at $16 \text{ } \mu\text{g Cu l}^{-1}$. Fig. 1B is the Scatchard plot showing the characteristics of the
10 Cu-FA binding. For each of the four FA treatments, the relationship between $[\text{CuL}]$ and
11 $[\text{CuL}]/[\text{Cu}']$ appeared linear, indicating there was one class of dominant binding sites
12 under the experimental conditions.

13 Hence, we fitted the data to a one-site binding model, i.e. $n = 1$ in Eq (1). The
14 conditional stability constants and ligand concentrations are shown in Table 1. The
15 fitting parameters differed slightly among the FA treatments, with an increase in $\log K'$
16 and decrease in $[\text{L}]$ with increasing FA concentration. The 95% confidence interval
17 (C.I.) for each fitting parameter was very small and the coefficient of determination (r^2)
18 was high, indicating the model was adequate in describing the binding reaction.

1 Moreover, when data for all FA treatments were pooled and the non-linear procedure
2 applied, the 95% C.I. for each fitting parameter was still quite small, and the r^2 was
3 reasonably high (Table 1), indicating the model could be used to describe the Cu-FA
4 binding reaction within the wide range of FA concentrations used. Based on the model
5 prediction, a curve describing the relationship between [Cu'] and [CuL] was plotted in
6 Fig. 2. Deviation of the predicted curve from the measured values was minimal.
7 Furthermore, deviation from the model prediction was checked by plotting measured vs.
8 predicted [Cu'] (Fig. 3). The two sets of data showed a good linear correlation, with an
9 r^2 of 0.97.

10 3.2. Bioassays

11 In the first bioassay that tested the toxicity of FA to the polychaete larvae, the
12 percentage of abnormal larvae at the end of the 48-h exposure ranged from 3 to 8, and
13 was independent of the FA concentration within the test range (0 to 256 mg l⁻¹). Thus,
14 FA itself was not toxic to the larvae under environmental realistic concentrations, and the
15 toxicity of the Cu-FA exposure solution in the second bioassay should not be caused by
16 FA. Without FA in the exposure solution, copper was very toxic to the larvae (Fig. 4).
17 At 23 µg l⁻¹ and higher copper concentrations, the percentage of abnormal larvae (19.5)
18 was significantly higher than that of the control (2.5) ($P < 0.05$). When the dosage data

1 (X) were transformed by $\log(X + 1)$, the dose-response curve displayed a sigmoid shape.
2 The data were fitted to Eq. (2). Because no larvae survived through the end of the
3 bioassay at the two highest copper concentrations, the maximum response of the model
4 was constrained to 100. The minimum response was constrained to > 0 , instead of 0, to
5 account for the presence of a small percentage of abnormal larvae in the copper-free
6 control. The fitting parameters are shown in Table 2 and the calculated EC_{50} was $55.6 \mu\text{g}$
7 l^{-1} .

8 With FA in the exposure solution, the toxicity curve was displaced to the right, and the
9 displacement increased with increasing FA concentration (Fig. 4A). At each FA
10 concentration, the fitting between percentage of abnormal larvae and total copper
11 concentration was quite satisfactory, with an r^2 higher than 0.98 (Table 2). The EC_{50}
12 was $65.0 \mu\text{g l}^{-1}$ at 2.5 mg l^{-1} FA, $75.3 \mu\text{g l}^{-1}$ at 5 mg l^{-1} FA, $103.0 \mu\text{g l}^{-1}$ at 10 mg l^{-1} FA and
13 $137.4 \mu\text{g l}^{-1}$ at 20 mg l^{-1} FA. A comparison of the $\log EC_{50}$ of the control and individual
14 FA treatments using F test showed significant differences (Table 2). However, when
15 data from all FA treatments were combined and plotted against labile copper
16 concentration (Fig. 4B), the data could be fitted to Eq (2), with an r^2 of 0.986 and an EC_{50}
17 of $58.9 \mu\text{g l}^{-1}$. A comparison between the $\log EC_{50}$ for all FA treatments and that for the
18 no-FA control by F test showed no significant difference (Table 2).

1 4. Discussion

2 In view of the experimental evidence accumulated in the 1960s and 70s that metal
3 toxicity is often related to the free ion concentration or inorganic complexes of the metal,
4 Morel (1983) formulated the free ion activity model (FIAM), which uses the free ion
5 activity as a predictor of metal toxicity in the presence of inorganic and organic
6 chemicals. The FIAM recognizes that many metals form complexes with inorganic and
7 organic chemicals both in solution and particulate forms in natural water and that such
8 complexation affects metal bioavailability and toxicity to aquatic organisms. Thus, it
9 emphasizes the importance of water chemistry, without taking into account of the
10 interaction among abiotic factors and between these factors and aquatic organisms.

11 Although many studies have lent support to the FIAM, a number of exceptions have
12 been found (review by Campbell, 1995). One exception involves tests of freshwater
13 organisms performed under varying pH or water hardness, where cations compete for the
14 metal binding site and reduce metal bioavailability (Pärt, Svanberg, & Kiessling, 1985;
15 Meyer, Santore, Bobbitt, DeBrey, Boese, Paquin et al., 1999; Qiu, Xie, & Wang, 2005).
16 Another exception occurs where, in the presence of DOM, metal bioavailability is higher
17 than the value predicted by the FIAM (Table 8 in Campbell, 1995; Voets et al., 2004),
18 being consistent with the observation that some DOM-metal complexes can be taken up

1 by aquatic organisms (Tao, Liang, Liu, & Xu, 1999; Roditi, Fisher, & Sanudo-Wilhelmy,
2 2000; Pan & Wang, 2004). The biotic ligand model (BLM) (Di Toro et al., 2001), which
3 predicts metal toxicity based on the amount of metal accumulated at presumptive binding
4 sites of aquatic organisms, assumes that metal ions in the water bind to a biotic ligand,
5 such as fish gill, and that the toxic effect occurs when the concentration of the
6 metal-biotic ligand reaches a threshold. Cations (i.e. Ca^{2+} , Mg^{2+} , Na^{+} , and H^{+}), anions
7 (i.e. SO_4^{2-} and Cl^{-}), DOM and suspended particulates in the water affect such binding,
8 thus changing metal bioavailability and toxicity. Thus, the BLM is an important
9 extension of the FIAM, which considers both water chemistry and biological responses
10 when predicting metal toxicity. This model has been successfully applied to predict
11 metal bioavailability to a number of freshwater organisms, such as cladocerans and fish
12 (Di Toro et al., 2001; Zhou et al., 2005), amphipods (Borgmann et al., 2005), earthworms
13 (Steenbergen et al., 2005) and algae (De Schamphelaere et al., 2005). However, its
14 application to coastal organisms has not been widely reported. A number of studies
15 appear to indicate water chemistry alone can be used to predict metal toxicity to
16 echinoderm larvae (Lorenzo et al., 2002; Rivera-Duarte et al., 2005), bivalve larvae
17 (Rivera-Duarte et al., 2005), and algae (Stauber et al., 2005), which support the
18 application of the FIAM in coastal waters. However, the situation is not straightforward,

1 as Lorenzo et al. (2005) showed that, in a marine mussel, water chemistry alone can be
2 used to predict copper accumulated in the gills, but cannot be used to predict copper
3 accumulated in the whole body. Of course, given the few numbers of marine organisms
4 studied, it is premature to generalize whether the FIAM or BLM is only applicable to
5 certain groups of organisms.

6 *H. elegans* is a serpulid polychaete species commonly found in tropical and subtropical
7 waters. Our recent studies showed that larval development of this species was sensitive
8 to copper stress (Xie et al., 2005); addition of algae into the exposure medium reduced
9 aqueous copper concentration, and thus copper toxicity (Wong et al., 2006). In this
10 study, we quantified the effects of FA, an important component of DOM in natural waters,
11 on the toxicity of copper to the larvae. The results showed that FA was not toxic to the
12 larvae up to the highest concentration tested (256 mg l^{-1}), indicating FA is not toxic to the
13 larvae under environmentally realistic concentrations. Copper and FA binding reduced
14 ASV labile copper concentration, and such reaction could be quantified using a simple
15 ligand binding model. Although visual observation of the relationship between $[\text{CuL}]$
16 and $[\text{CuL}]/[\text{Cu}']$ in the Scatchard plot appeared linear, which indicated the presence of
17 one class of dominant binding sites, we explored the use of both one- and two-site
18 binding models because humic substances contain many functional groups, such as

1 carboxylic and phenolic groups, the binding might be better described by a poly-site
2 model. In fact, two- to five-site models have been used by a number of authors (Hering
3 & Morel, 1988; Cabaniss & Shuman, 1988; Kim et al., 1999; Ma et al., 2002). Using a
4 three-site model to describe the binding between copper and humic acid, Hering & Morel
5 (1988) found two stronger but less abundant ligands with $\log K'$ exceeding 9 and a
6 weaker but more abundant ligand with a $\log K'$ of 6.6. The ligand found in the one-site
7 model in Lorenzo et al. (2002) may thus correspond to the weaker ligand in Hering &
8 Morel (1988). To evaluate whether a multi-site model was a better description of our
9 data, we also fitted the pooled data for all FA treatments to a two-site model, i.e. $n = 2$ in
10 Eq. (1). The result showed only a slightly improved r^2 (0.989) over the value for the
11 one-site model (0.979). The fitting parameters for the weaker ligand were: $\log K_1' =$
12 5.95, with a 95% C.I. of 5.75 to 6.08, and $[L_1] = 0.19$, with a 95% C.I. of 0.18 to 0.21.
13 These values were very close to those predicted by the one site model (Table 1). For the
14 stronger ligand, $[L_2]$ was 0.025, with a 95% C.I. of 0.005 to 0.045, and K_2' was $2.55 \times$
15 10^7 , with a 95% C.I. of 0 to 2.70×10^7 . K_2' had a very large confidence interval
16 indicating the estimated value was not reliable; therefore the two-site model was
17 inappropriate for our data.

18 An important result of Fig. 2 and Table 1 was that the fitting parameters for the

1 one-site model were quite close within the range of FA concentrations tested, as was the
2 case in another study of copper binding by Suwannee River FA (Lund et al., 1990). This
3 consistency indicates aggregation and other structural changes in the FA were minimal in
4 the exposure system, and that a single set of K' and $[L]$ could be used to predict labile
5 copper concentration across a relatively wide range of FA concentrations. One-site
6 binding model has been used in a number of studies of the binding between copper and
7 humic substances (Turner, Vaney, Whitfield, Manroua, & Riley, 1987; Lund et al., 1990;
8 Lorenzo et al., 2002). In Lorenzo et al. (2002), the model was successfully applied to
9 describe the binding between humic acid (HA) and copper (Lorenzo et al. 2002), with an
10 r^2 of 0.993. The $[L]$ in Lorenzo et al. (2002) was $0.23 \mu\text{mol site mg}^{-1} \text{HA}$, being higher
11 than the value obtained from this study ($0.19 \mu\text{mol site mg C}$, equivalent to $0.10 \mu\text{mol site}$
12 $\text{mg}^{-1} \text{FA}$). The $\log K'$ for HA (6.53) in Lorenzo et al. (2002) was also higher than the
13 value for FA from this study (6.19). A comparison of these binding parameters shows
14 that the binding of Cu-FA is weaker than that of Cu-HA, being consistent with previous
15 reports (Stevenson, 1982). However, under different experimental conditions, $\log K'$ for
16 copper complexation with natural water or fulvic acid can vary from 5.1 to 5.6 (Lund et
17 al., 1990) to 6.6 to 7.4 (Turner et al., 1987).

18 Although FA is not as strong as HA in terms of binding to copper, the modeling result

1 showed that FA still greatly affected labile copper concentration. Assuming that FA
2 content of seawater reaches a level of 2 mg l^{-1} and a complexation capacity of 0.19 mmol
3 Cu g C^{-1} or $0.10 \text{ mmol Cu g}^{-1}$ FA, the maximum concentration of cupric ion buffered by
4 the system can be estimated as follows:

5 $0.1 \text{ mmol Cu}/1000 \text{ mg FA} \times 2 \text{ mg FA l}^{-1} = 0.2 \text{ } \mu\text{mol Cu}$. $0.2 \text{ } \mu\text{mol Cu}$ is equivalent to
6 $12.7 \text{ } \mu\text{g l}^{-1}$ of Cu. This simple calculation shows that when coastal water is used as
7 dilution water in laboratory bioassay, metal toxicity could be underestimated substantially.
8 Thus, albeit a weak ligand, FA could lead to an appreciable reduction in copper toxicity.

9 ASV labile copper has been widely used to correlate copper toxicity in the presence of
10 pure FA, synthetic ligands or in natural water (review by Florence 1986; Deaver &
11 Rodgers, 1996; Wang et al. 2002; Rivera-Duarte et al., 2005; Stauber et al. 2005). As
12 noted by Florence (1986), when synthetic ligands such as nitrolotriactic acid,
13 8-hydroxyquinoline or ethyl xanthogenate were present, ASV copper did not show a clear
14 relationship with toxicity. In natural water or synthetic water containing FA or HA, the
15 pH at which ASV measurements are made greatly affect the data because at low pH more
16 metal ions are labile. Srna, Garrett, Miller, & Thum (1980) noted that ASV measured
17 ligands were about half of those that could result in the observed amelioration in copper
18 toxicity. They explained that this discrepancy might be due to the fact that the ASV

1 measurements were taken at a pH of 4.8, whereas the bioassays were conducted at a pH
2 of 8. Such differences in pH conditions should not be a problem in a number of recent
3 studies (Lorenzo et al., 2002; Rivera-Duarte et al., 2005; Stauber et al. 2005) conducted
4 in seawater as the ASV measurements as well as bioassays were performed at natural pH
5 for seawater, following the speciation scheme proposed by Florence (1986). In all these
6 studies, ASV concentration was an accurate predictor of the toxicity of copper in the
7 presence of various concentrations of humic substances. In this study, we found copper
8 toxicity to be a function of labile rather than total metal concentrations, and the curve
9 fitting for the pooled data from all of the FA treatments was not significantly different
10 from that for the no-FA treatment. The toxicity of seawater samples containing FA was
11 thus dependent on labile copper rather than total copper concentration.

12 From these data we can conclude that FA is reactive with copper, fulvic acid at
13 environmentally realistic concentrations can have a protective effect on copper toxicity to
14 *H. elegans* larvae, and such effect can be predicted based on ASV labile copper. This
15 study extends the application of the FIAM in predicting copper toxicity to polychaete
16 larvae in seawater containing FA. It should be noted that the bioassays in this study
17 were conducted under constant salinity and temperature conditions, and the conditional
18 constants for the binding model were determined after allowing the Cu-FA reaction to

1 reach equilibrium, whereas in natural seawater, environmental parameters fluctuate and
2 the levels of FA and HA, as well as particulate organic matter change spatially and
3 temporally. Further studies should thus test the applicability of ASV in the prediction of
4 metal toxicity to *H. elegans* larvae in natural seawater of different complexation
5 capacities.

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- 10

1

2 Table 1. Fitting parameters for the binding between copper and fulvic acid in artificial seawater as estimated by a one-site binding model.

3 [L] is ligand concentration in $\mu\text{mol site mg}^{-1} \text{C}$, and K' is conditional stability constant. Data in parentheses are 95% confidence

4 intervals.

5

Treatment	$\log K'$	[L]	df	r^2
8 mg FA l ⁻¹	6.154 (6.076 to 6.249)	0.203 (0.190 to 0.216)	14	0.989
16 mg FA l ⁻¹	6.172 (6.089 to 6.274)	0.197 (0.183 to 0.212)	14	0.988
32 mg FA l ⁻¹	6.363 (6.309 to 6.424)	0.162 (0.153 to 0.169)	14	0.996
64 mg FA l ⁻¹	6.663 (6.595 to 6.744)	0.112 (0.105 to 0.119)	14	0.992
All data	6.186 (6.137 to 6.240)	0.194 (0.185 to 0.203)	59	0.979

6

1

2 Table 2. Fitting parameters for Fig. 4. Percentage of abnormal larvae was expressed as a function of total (A) or labile (B) copper

3 concentration. Data in parentheses are 95% confidence intervals. Data in the *F* and *P* columns are results of *F* test that compares the4 log EC₅₀ of a FA treatment and the control. A *P* value of <0.05 indicates significant difference.

5

Figure	Treatment	Copper	log EC ₅₀	Hill slope	Baseline	<i>r</i> ²	<i>F</i>	<i>P</i>
4A	Control	Total	1.745 (1.694 to 1.795)	2.843 (2.058 to 3.628)	5.300 (0 to 11.680)	0.990		
4A	2.5 mg FA l ⁻¹	Total	1.813 (1.789 to 1.837)	2.900 (2.509 to 3.291)	2.380 (0 to 5.411)	0.997	7.32	0.0145
4A	5 mg FA l ⁻¹	Total	1.877 (1.818 to 1.935)	2.290 (1.717 to 2.873)	0.673 (0 to 7.343)	0.988	17.25	0.0006
4A	10 mg FA l ⁻¹	Total	2.013 (1.967 to 2.060)	2.634 (2.001 to 3.268)	5.597 (0.714 to 10.480)	0.990	71.59	<0.0001
4A	20 mg FA l ⁻¹	Total	2.138 (2.085 to 2.192)	3.124 (2.085 to 4.164)	3.591 (0 to 9.331)	0.980	133.80	<0.0001
4B	All FA data	Labile	1.770 (1.742 to 1.797)	2.189 (1.937 to 2.441)	2.839 (0.295 to 5.383)	0.986	0.80	0.374

6

7

1 **Figure captions**

2 Fig. 1. Binding between fulvic acid (FA) and copper. (A), For each total copper
3 concentration ranging from 16 to 256 $\mu\text{g l}^{-1}$, the line shows a decline in labile
4 copper concentration with increasing FA concentration. (B), Scatchard plot,
5 showing a linear relationship for each of the four FA treatments (64 mg FA l^{-1} :
6 $Y = 19.543 - 5.539X$, $r^2 = 0.966$; 32 mg FA l^{-1} : $Y = 7.084 - 2.778X$, $r^2 =$
7 0.961 ; 16 mg FA l^{-1} : $Y = 2.682 - 1.681X$, $r^2 = 0.914$; 8 mg FA l^{-1} : $Y =$
8 $1.494 - 1.889X$, $r^2 = 0.868$).

9 Fig. 2. Relationship between ASV labile copper and normalized bound copper on
10 mg carbon basis. The curve is the result of data fitting to a one-site binding
11 model.

12 Fig. 3. Relationship between predicted (X) and measured (Y) ASV labile copper
13 concentration ($Y = 1.053X - 0.021$, $r^2 = 0.969$, $n = 60$).

14 Fig. 4. Percentage of abnormal larvae as a function of total (A) and labile (B) copper
15 concentration. The bioassay data are mean \pm S.D. of four replicates. In
16 (A), regression was performed for individual FA treatments. In (B), the
17 solid line shows regression for the no-FA treatment, whereas the dash line
18 shows the regression for the pooled data from all FA treatments.

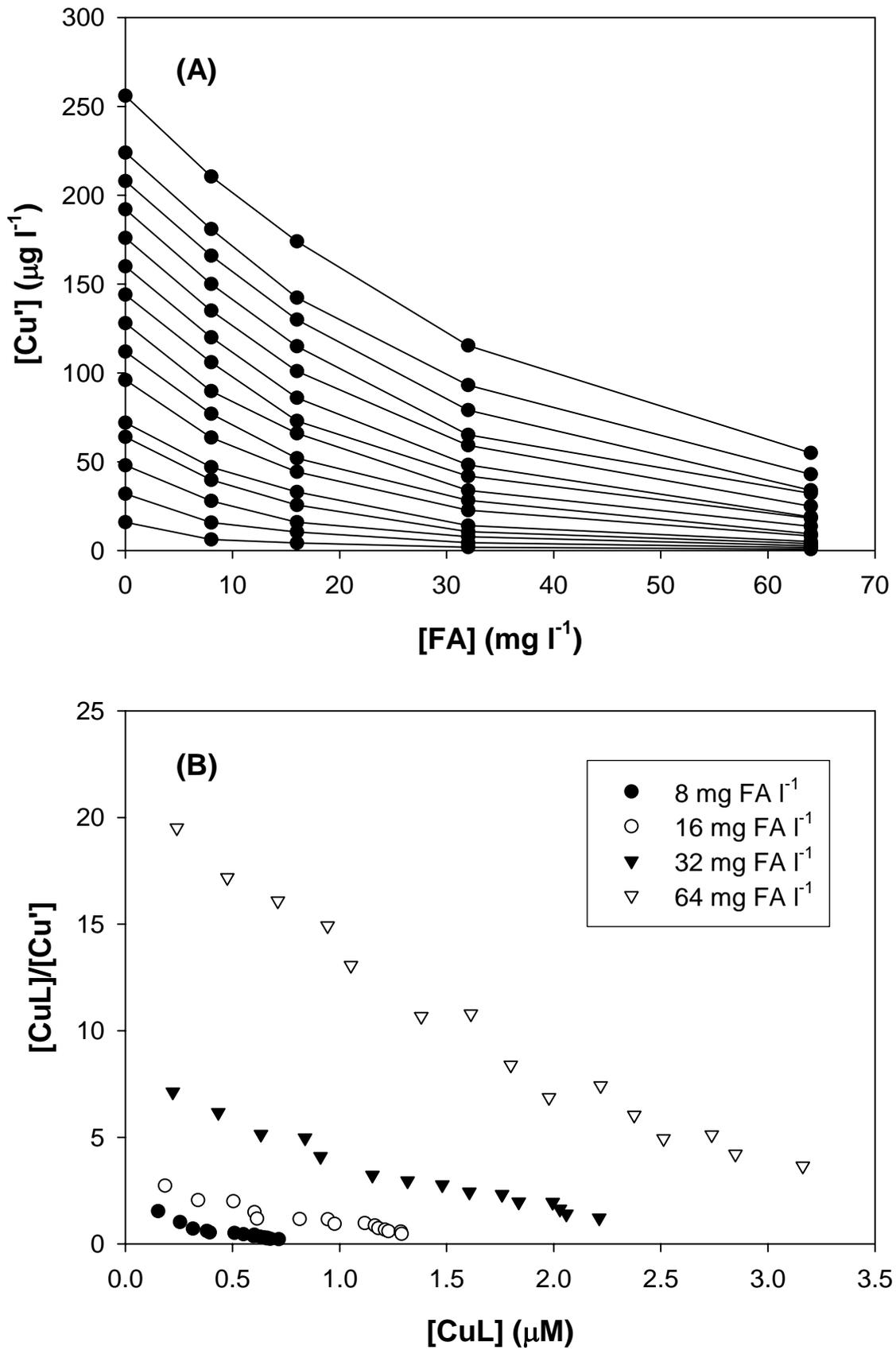


Fig. 1

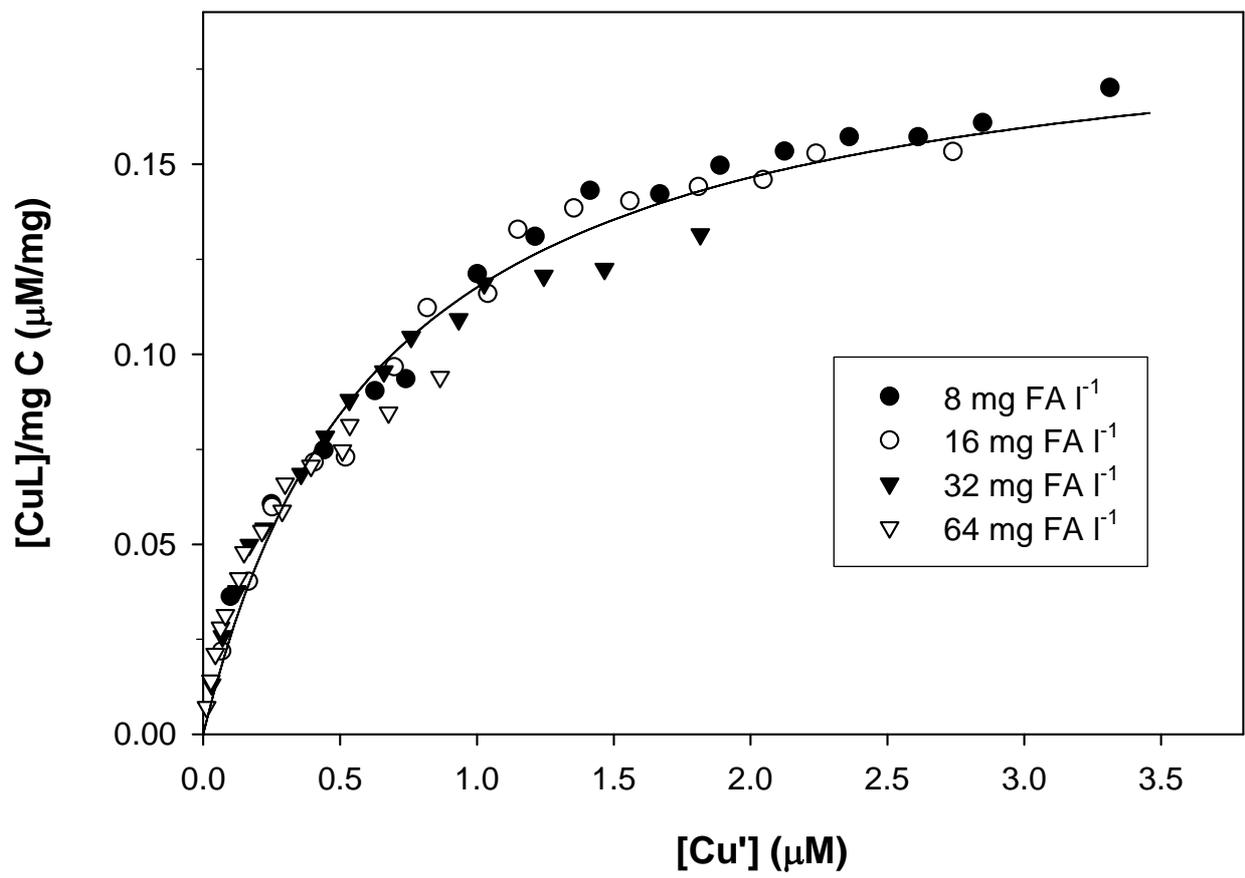


Fig. 2

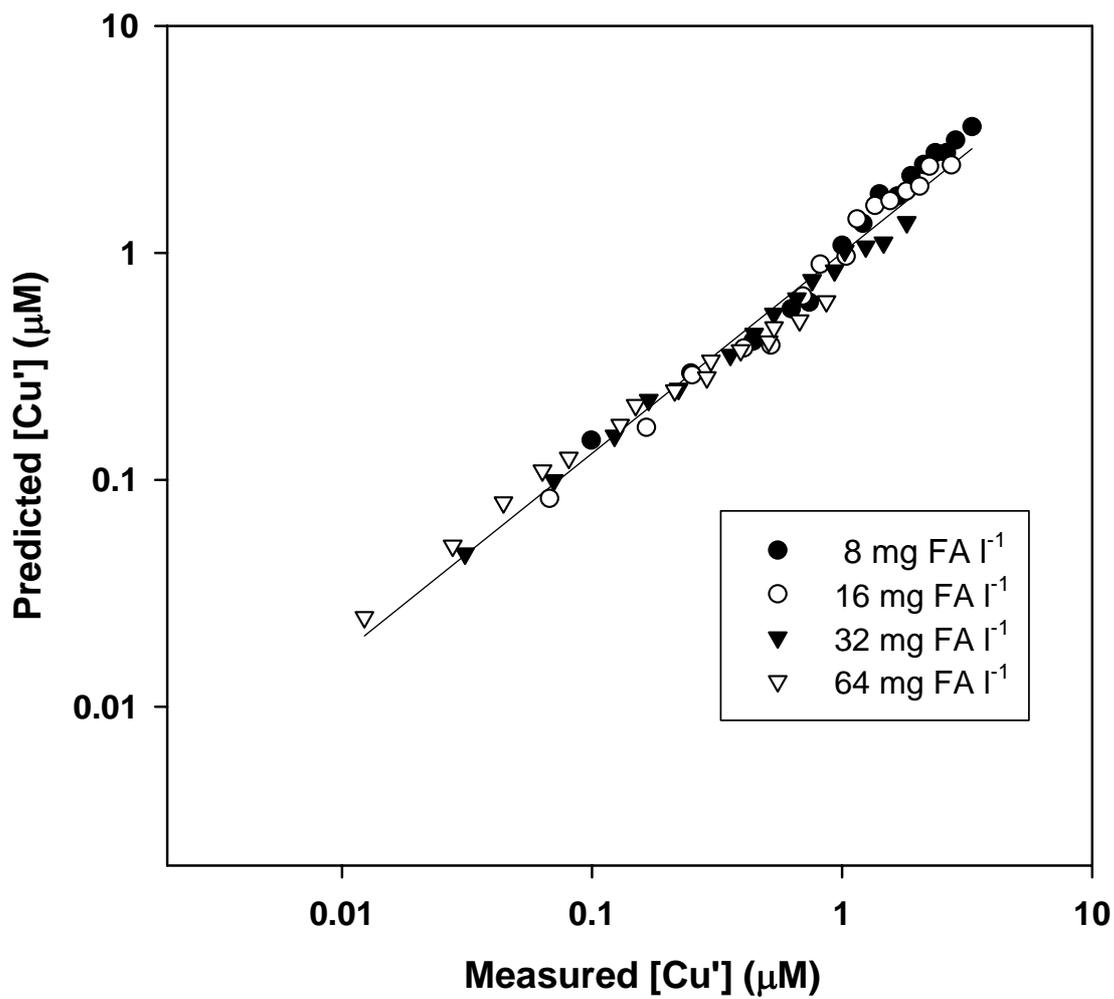


Fig. 3

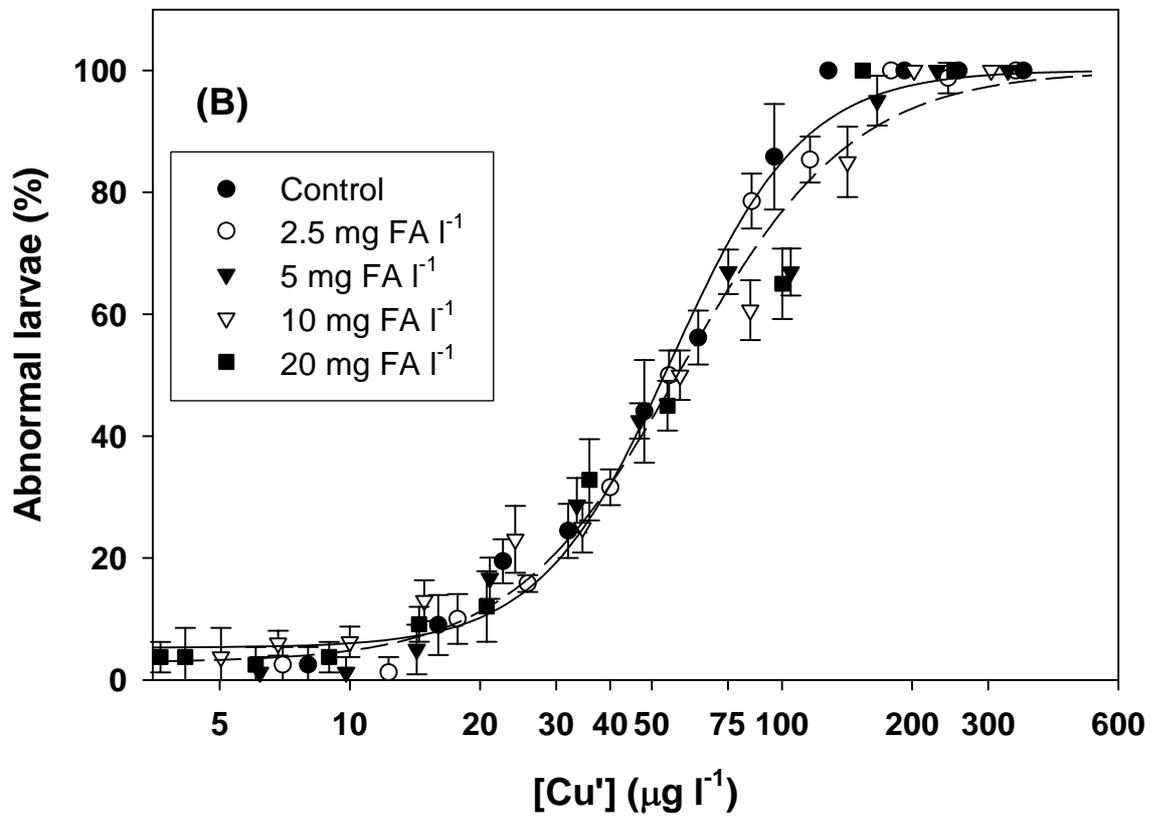
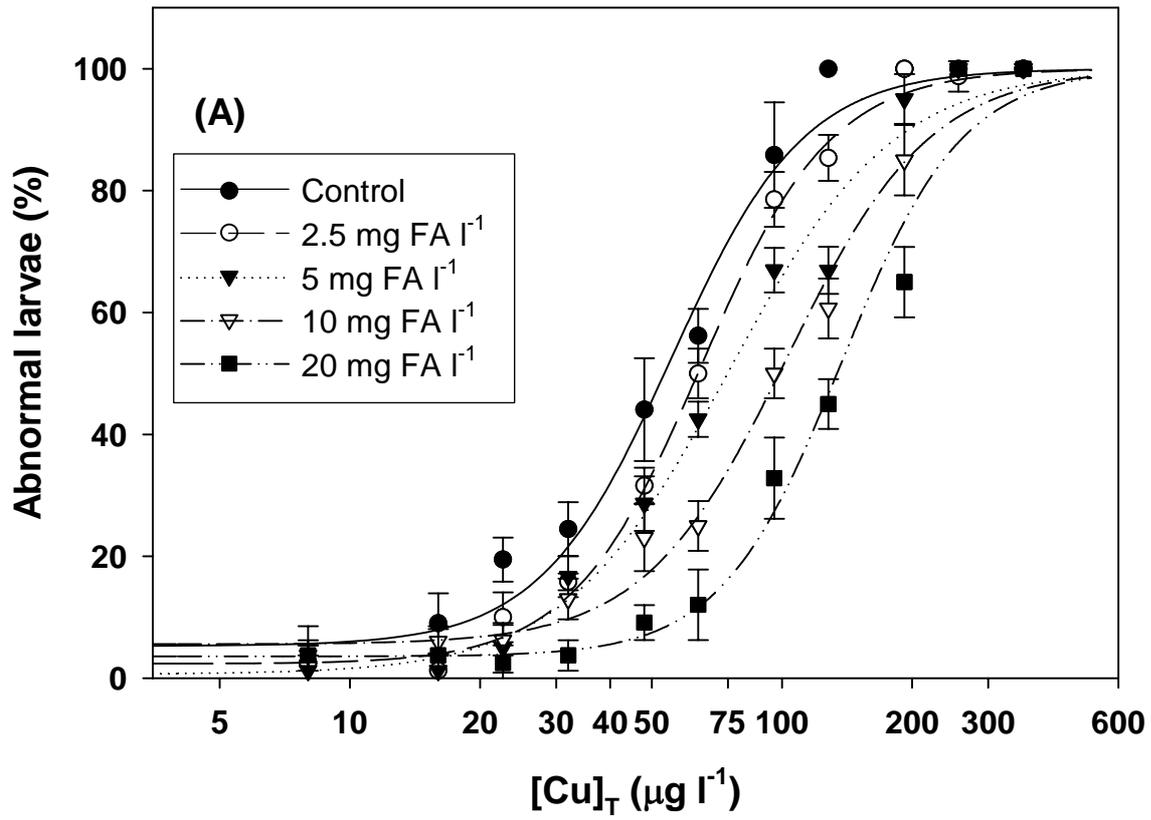


Fig. 4