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Oxidation of acid-volatile sulfide in surface sediments increases the release and toxicity of copper to the benthic amphipod *Melita plumulosa*

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Abstract

Acid-volatile sulfides (AVS) are an important metal-binding phase in sediments. For sediments that contain an excess of AVS over simultaneously extracted metal (SEM) concentrations, acute or chronic effects should not result from the metals Cd, Cu, Ni, Pb and Zn. While AVS phases may exist in surface sediments, the exposure to dissolved oxygen may oxidize the AVS and release metals to more bioavailable forms. We investigated the role of oxidation of AVS, and specifically copper sulfide phases, in surface sediments, in the toxicity to juveniles of the epibenthic amphipod, *Melita plumulosa*. Sediments containing known amounts of copper sulfide were prepared either in situ by reacting dissolved copper with AVS that had formed in field sediments or created in sediments within the laboratory, or by addition of synthesised CuS to sediments. Regardless of the form of the copper sulfide, considerable oxidation of AVS occurred during the 10-d tests. Sediments that had a molar excess of AVS compared to SEM at the start of the tests, did not always have an excess at the end of the tests. Consistent with the AVS–SEM model, no toxicity was observed for sediments with an excess of AVS throughout the tests. However, the study highlights the need to carefully consider the changes in AVS concentrations during tests, and that measurements of AVS and SEM concentrations should carefully target the materials to which the organisms are being exposed throughout tests, which in the case of juvenile *M. plumulosa* is the top few mm of the sediments.

Keywords

surface, sediments, increases, oxidation, release, acid, toxicity, copper, benthic, amphipod, melita, plumulosa, volatile, sulfide

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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10 Oxidation of acid-volatile sulfide in surface sediments increases the release and
11 toxicity of copper to the benthic amphipod *Melita plumulosa*

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22 **Abstract**

23 Acid-volatile sulfides (AVS) are an important metal-binding phase in sediments. For sediments that contain an
24 excess of AVS over simultaneously extracted metal (SEM) concentrations, acute or chronic effects should not
25 result from the metals Cd, Cu, Ni, Pb and Zn. While AVS phases may exist in surface sediments, the exposure to
26 dissolved oxygen may oxidise the AVS and release metals to more bioavailable forms. We investigated the role of
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30 within the laboratory, or by addition of synthesised CuS to sediments. Regardless of the form of the copper sulfide,
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32 compared to SEM at the start of the tests, did not always have an excess at the end of the tests. Consistent with the
33 AVS-SEM model, no toxicity was observed for sediments with an excess of AVS throughout the tests. However,
34 the study highlights the need to carefully consider the changes in AVS concentrations during tests, and that
35 measurements of AVS and SEM concentrations should carefully target the materials to which the organisms are
36 being exposed throughout tests, which in the case of juvenile *M. plumulosa* is the top few mm of the sediments.

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39 *Keywords: Acid-volatile sulfide, oxidation, copper, toxicity, amphipod, sediment*

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42 1. Introduction

43 It is widely recognised that for sediments containing a molar excess of acid-volatile sulfide (AVS) over
44 simultaneously-extracted metals ($SEM = \sum Ag, Cd, Cu, Ni, Pb, Zn$) the porewater concentrations of these metals will
45 be negligible and acute or chronic effects should not result from these metals (Ankley et al., 1993; Berry et al.,
46 1996; USEPA, 2005; Simpson and Spadaro, 2011). The metal concentrations in excess of the binding capacity
47 attributed to AVS may be bound by other solid phases, including particulate organic carbon (POC) and iron and
48 manganese oxyhydroxide phases, or partition to the pore waters (Di Toro et al., 2005; Simpson and Batley, 2007).
49 Sediment quality guidelines (SQGs) that vary in proportion with AVS, POC and silt contents have been shown to
50 offer a significant improvement on single value SQGs (Di Toro et al., 2005; Strom et al., 2011).

51 The redox conditions in sediments are usually stratified and zones are frequently referred to as oxic, sub-
52 oxic, and anoxic (Jørgensen and Revsbech, 1985; Kristensen, 2000). In the present study, we refer to oxic
53 sediments as surface sediments penetrated by dissolved oxygen, sub-oxic sediments as those contain mixtures of
54 dissolved Mn(II) and Fe(II) in equilibrium with iron(III) and manganese(IV) oxyhydroxide phases but containing
55 negligible dissolved oxygen or sulfide, and anoxic sediments as those in which dissolved sulfide and AVS are
56 dominant. At the sediment-water interface of surface sediments and at burrow walls, dissolved oxygen generally
57 only penetrates a few millimeters (Jørgensen and Revsbech, 1985; Kristensen, 2000; Gallon et al., 2008). While
58 AVS phases may persist in surface sediments, having been formed under anoxic conditions or brought to the
59 surface through bioturbation, the ongoing exposure to dissolved oxygen may result in oxidation of AVS and
60 transfer of metals to more bioavailable forms). When exposed to dissolved oxygen, copper sulfide phases are slow
61 to oxidise compared to iron and manganese sulfide phases (Simpson et al., 1998; Caetano et al., 2003). Copper that
62 is released during the oxidation of copper sulfide phases is expected to become rapidly adsorbed to particulate
63 organic carbon or iron and manganese oxyhydroxide phases (Chapman, et al., 1998; Simpson and Batley, 2003;
64 Eggleton and Thomas, 2004). Through the combination of the slow metal-sulfide oxidation processes, adsorption
65 to sediment particles and dilution in surrounding water, dissolved metal concentrations in surface pore water and
66 overlying water are commonly in the low $\mu g L^{-1}$ range and are generally not an environmental concern (Eggleton
67 and Thomas, 2004; Simpson and Batley, 2007).

68 Most organisms live within oxic surface sediments or in oxygenated microenvironments created within sub-
69 oxic and anoxic sediments (e.g. by irrigation of burrows with oxygen-containing waters). In these environments
70 the binding of metals by AVS may vary considerably with time (Foster, 1996, Motelica-Heiono et al., 2003; Naylor
71 et al., 2006; Gallon et al., 2008). Benthic invertebrates may also ingest significant quantities of sediment during
72 their foraging for food, and dietary exposure to metals may elicit toxicity effects to some species (Simpson, 2005;
73 Goulet et al., 2007; Wang et al., 2007; Mann et al., 2009; Casado-Martinez et al., 2010). The oxidation of AVS in
74 surface sediments has the potential to increase the bioavailability of both dissolved and particulate metals (Peterson
75 et al., 1996; Eriksson-Wiklund and Sundelin, 2002; De Lange et al., 2008).

76 In this study, we investigated the role of oxidation of AVS, and specifically copper sulfide phases, in surface
77 sediments on toxicity to juvenile *Melita plumulosa*. Previous studies have demonstrated that copper exposure from

78 the dissolved phase and through ingestion of sediment particles contribute to toxic effects for this epibenthic
79 deposit-feeding amphipod (Simpson and King, 2005; King et al., 2006; Spadaro et al., 2008; Mann et al., 2009;
80 Strom et al., 2011). This study aimed to demonstrate that AVS reduces the toxicity of copper, and to assess the
81 degree of oxidation of copper sulfide phases in surface sediments, and provide further insight into the role of
82 dietary exposure in any observed toxicity.

83 **2. Experimental procedures**

84 *2.1. General analytical*

85 New plasticware was used for all chemical analyses. All chemicals were analytical reagent grade or
86 equivalent analytical purity. Deoxygenated waters were prepared by bubbling solutions with high purity oxygen-
87 free nitrogen gas for >8 h to give dissolved oxygen concentrations <0.1 mg L⁻¹. Measurements of pH, salinity,
88 temperature and dissolved oxygen were made in accordance with the instrument manufacturers' instructions.
89 Methods for measurements of pH, particle size fractions (wet sieving and gravimetry), total organic carbon (TOC,
90 high temperature analyser), and particulate metals (2:1 concentrated HCl:HNO₃, heated) were made as described
91 previously (Simpson et al., 2000a; Simpson et al., 2007). Sediment pore water was extracted by centrifugation (5
92 min, 1700 g, 18 to 22 °C) under a nitrogen atmosphere (Simpson et al., 2000a). Pore water and overlying water
93 samples were rapidly filtered through acid-washed 0.45 µm membrane filters (Minisart, Sartorius) immediately
94 following collection and acidified to 2% HNO₃ (v/v) with concentrated HNO₃ (Tracepur, Merck). Acid-volatile
95 sulfide and simultaneously extracted metals (SEM) were analysed according to Simpson (2001). Any samples for
96 AVS analyses were stored frozen in container with no head space or a nitrogen atmosphere immediately after
97 sampling, and all subsequent handling, including thawing, was undertaken in a nitrogen gas atmosphere (De Lange
98 et al., 2008). Dissolved metal concentrations in water samples and digested sediments were determined by
99 inductively coupled plasma-atomic emission spectrometry (ICP-AES, Spectroflame EOP, Spectro Analytical
100 Instruments) calibrated with matrix-matched standards. Analyses of filter and digest blanks, replicates for 20% of
101 samples, analyte sample-spikes and the certified reference material (PACS-2, National Research Council Canada)
102 were made as part of the quality assurance and recoveries were within 85–110% of expected values. The limits of
103 reporting for the various methods were less than one tenth of the lowest reported values. All sediment related
104 concentrations are reported on a dry mass basis.

105 *2.2. Test media*

106 Clean seawater was collected from Port Hacking, Sydney, Australia, membrane filtered (0.45 µm), and
107 acclimated to room temperature of (21±1°C). Where necessary, the salinity of the filtered seawater was adjusted to
108 the test salinity of 30 PSU using deionised water (18 MΩ cm⁻¹ or 0.055 µS cm⁻¹, Milli-Q® Millipore). Silty
109 sediments with low to moderate metal contamination and negligible concentrations of organic contaminants were
110 collected from the mangrove forest lined Bonnet Bay, Woronora River, Australia, as described previously
111 (Simpson et al., 2004). At this site, sediments containing both low AVS (<0.5 to 2 µmol g⁻¹) and high AVS (28 to
112 32 µmol g⁻¹) were collected. The high AVS material was typically located in layer at 2–4 cm depth in sediments

113 within the mangroves, whereas the low AVS material was the dominant material on the bay side of the mangroves.
114 Both the low and high AVS sediments were hydrous (70% water), silty (95% of particles <63 μm), contained 4 %
115 TOC and had porewater pH values of 7.1 to 7.8. Particulate metal concentrations were similar for both the low and
116 high AVS materials: 2.1–2.6% Fe, and Cd, Cu, Mn, Ni, Pb and Zn of 1–3, 30–50, 50–80, 3–10, 40–70, and 150–
117 250 mg kg^{-1} , respectively (Simpson et al., 2004; Strom et al., 2011).

118 2.3. Preparation of sediments containing copper sulfide.

119 The preparation, manipulation and equilibration of the copper-spiked sediments were carried out in a
120 nitrogen gas-filled glove box at room temperature following the procedures described in Simpson et al. (2004).
121 The sediments were thoroughly homogenised by mixing with a plastic spoon and then on a bottle roller for 2 h at
122 least twice per week. At higher copper-spike concentrations, the pH decreased, so the pH of the sediments was
123 adjusted to pH 7.5 with 1 M NaOH one day after copper-spiking and maintained at this pH by small additions of
124 NaOH throughout the one-month equilibration period. Changes in pH, redox potential, and dissolved metals in the
125 pore water were monitored during this period.

126 Three methods were used to create substrates containing copper sulfide. The high-AVS sediment was
127 diluted with low-AVS sediment and equilibrated for 5 days to create sediments with AVS concentrations of 11, 18
128 and 30 $\mu\text{mol g}^{-1}$. Each of these three sediments was spiking with the required amounts of copper sulfate dissolved
129 in deoxygenated water to achieve a series of 5 to 6 concentrations up to 28 $\mu\text{mol Cu g}^{-1}$ and allow copper sulfide to
130 form *in situ* after 20 days equilibration (named the AVS-Cu series).

131 The second approach differed in that AVS-containing sediment was first prepared by reacting the low-AVS
132 sediment with sulfide to create a sediment with the desired AVS concentration (the FeS-Cu series). The FeS-Cu
133 sediments were also prepared in two stages, by first spiking dissolved Na_2S into sub-oxic sediments, which was
134 allowed to react for five days to form 30 $\mu\text{mol Fe g}^{-1}$ (as quantified by AVS). The FeS-Cu concentration series was
135 then prepared by reacting the 30 $\mu\text{mol Fe g}^{-1}$ sediment with different amounts of CuSO_4 dissolved in deoxygenated
136 water to form copper sulfide *in situ* (six concentrations up to 20 $\mu\text{mol Cu g}^{-1}$). At the end of the equilibration
137 period, the AVS and SEM concentrations were determined for each sediment to provide information on the
138 stoichiometry of the copper: sulfide reaction. Previous studies showed that copper spiked into sulfidic sediments is
139 reduced and forms Cu_2S , rather than CuS (Simpson et al., 2000a).

140 The third approach involved spiking the low-AVS sediments with synthesised copper sulfide that had been
141 prepared by reacting $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Univar, Ajax Finechem) dissolved in deoxygenated water with dissolved
142 $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (Sigma) for 6 h. The stoichiometry of the copper sulfide phases that formed was not determined, and
143 may have been CuS , Cu_2S or a mixture of the two. This series contained six concentrations up to 20 $\mu\text{mol Cu g}^{-1}$
144 was prepared by mixing with the low-AVS sediment, and was named the CuS series. The CuS series control was
145 created by spiking the sediment with 20 $\mu\text{mol g}^{-1}$ Na_2S . These sediments allowed comparison of the oxidation of
146 copper sulfide in sub-oxic sediments (CuS series) with that of anoxic sediments (FeS-Cu series). In addition to
147 these copper sulfide containing sediments, the low-AVS sediment was also spiked with dissolved copper using the
148 methods described previously (Simpson et al., 2004; Hutchinson et al., 2007) (the Oxic-Cu series).

2.4. General toxicity test procedures

Melita plumulosa is an epibenthic deposit-feeding amphipod commonly found in estuarine tidal mudflats ranging from silty to sandy sediments in freshwater, estuarine and marine environments throughout south-eastern Australia (Hyne et al., 2005). *M. plumulosa*, were obtained from laboratory cultures that were maintained as described previously (King et al., 2006). Juvenile *M. plumulosa* were harvested from 14-day old cultures of gravid females using a 210 µm sieve and the largest juveniles (11±3 days old, 0.5 to 1 mm body length) were selected for tests (Spadaro et al., 2008).

The 10-day water-only and whole-sediment toxicity tests with *M. plumulosa* were conducted in accordance with standard protocols (Spadaro et al., 2008; Strom et al., 2011). In brief, all toxicity tests were performed at 21 ± 2°C in a constant environmental chamber (Labec Refrigerated Cycling Incubator) on a 12-h light/12-h dark cycle (light intensity = 3.5 µmol photons s⁻¹ m⁻²) for the test duration. Glass beakers and acrylic beaker-lids used for toxicity tests were cleaned in a dishwasher (Gallay Scientific Pty Ltd) programmed for a phosphate-free detergent wash (Clean A, Gallay Scientific Pty), a dilute acid wash (1% HNO₃), followed by thorough rinsing with Milli-Q water. The physicochemical parameters of dissolved oxygen (>85%), pH (7.5 to 8.2), salinity (30 ± 1 PSU) and temperature (21 ± 2°C) were monitored and maintained throughout the test period according to Spadaro et al. (2008).

The water-only tests comprised four replicate 250 mL beakers containing 220 mL seawater and 20 juvenile *M. plumulosa* per treatment. Nominal copper concentrations were achieved by spiking seawater with 100 g L⁻¹ of CuSO₄·5H₂O and the beakers were conditioned with the test water for one day prior to test commencement. Food was added during water-only tests after each water change at a rate of 1 mg fish powder per organism (Sera micron, Sera Fishtamins®). The whole-sediment tests comprised four replicate containing 15 juvenile *M. plumulosa* per treatment, with the sediment and water volumes depending on test purpose (described below). As previous tests had demonstrated that food was generally not required to achieve adequate survival in controls (Spadaro et al., 2008), no food was added during sediment tests except in those specified below.

The test water or overlying water in sediment tests was changed before adding the amphipods and exchanged with new test water on days 3, 5 and 7. Water subsamples were taken (<0.45 mm filtered) for analyses at the start and finish of all tests, and before and after water renewals. The frequency of water changes required during the sediment bioassays was determined in preliminary tests run without organisms to ensure that dissolved copper concentrations were maintained below the 10-day water-only no observed effect concentration (NOEC) of 36 µg L⁻¹ for juvenile *M. plumulosa* (Spadaro et al., 2008; Strom et al., 2011). This involved measuring the dissolved copper to overlying waters for all sediments preparations over a 24-h period under the same conditions used in toxicity tests, but without amphipods. The reported concentration of dissolved Cu in each treatment was the time-averaged concentration (Angel et al., 2010).

Survival was determined at the completion of tests, and was indicated by active movement, confirmed under a dissection microscope. Sediment tests were terminated by gently sieving the sediment tests through a 180-mm stainless-steel sieve, and then the contents were transferred to large amphipod counting trays. Live amphipods were

185 counted and removed, and the sieved sediment was then transferred into 120-ml polycarbonate vials with
186 approximately 100 mL of seawater, fixed with 4 mL 10% v/v neutral phosphate-buffered formalin, and stained with
187 5 ml Rose Bengal solution (0.1 g Rose Bengal salt/100 mL Milli-Q water). The sediment was then left for 72 h to
188 enable any surviving amphipods, missed in the initial count, to take up the stain, and then these amphipods were
189 counted. Tests were considered acceptable when the physicochemical parameters remained within acceptable limits
190 throughout the test, and if survival of organisms was on average >80% in the controls.

191 2.5. Influence of AVS concentration, sulfide oxidation and added food on toxicity to *M. plumulosa*

192 To demonstrate the effects of AVS on the toxicity of copper to *M. plumulosa*, a comparison was made
193 between the toxicity of the Oxic-Cu (AVS <1 $\mu\text{mol/g}$) and the three AVS-Cu concentration series (AVS of 11, 18,
194 30 $\mu\text{mol/g}$). These tests were conducted in 250-mL beakers containing 20 g of sediment (approximately 40 mL)
195 and 200 mL of seawater. The homogenized sediments were added 24 h before the test was started, and seawater
196 was added slowly to minimize sediment resuspension. This created a sediment depth of 4–5 mm, whereas the
197 juvenile amphipods are epibenthic and were observed to burrow within the top 1–2 mm of the sediment.

198 The tests of the FeS-Cu and CuS concentration series were undertaken in 100-mL beakers containing 10 g
199 (approximately 20 mL) of test material and 90 mL seawater. The sulfide phases were added just an hour before the
200 amphipods were added and tests started. Seawater was added slowly to minimize sediment resuspension. The
201 materials formed a 1 to 2 mm layer and this resulted in there being no pore water. The survival of *M. plumulosa* in
202 FeS-Cu and CuS controls (no copper) was >80%, indicating adequate nutrition. As there was negligible pore water
203 in the FeS-Cu and CuS tests, and the dissolved copper concentrations in the overlying water were generally well
204 below the 10-day NOEC of 36 $\mu\text{g L}^{-1}$, any toxicity observed was likely to have a significant contribution from the
205 dietary exposure route. To determine whether added food influenced the observed toxicity, the FeS-Cu and CuS
206 series were tested both with and without feeding (1 mg fish powder per organism added after each water change).

207 For all of these experiments, the test waters were renewed on days 3, 5 and 7 by replacing 80% of the
208 overlying water with clean seawater. Sub-samples of overlying water were taken (<0.45 μm filtered) for analyses
209 at the start and finish of all tests, and before and after water renewals. Analyses of AVS and SEM-Cu were made at
210 the start and finish of all tests to provide information on the oxidation of AVS and copper sulfide phases.

211 2.6. Statistical analyses.

212 Results of the toxicity tests were reported as percentage survival in test sediments relative to controls.
213 Methods for statistical treatment of the data have been described previously (Spadaro et al., 2008). In brief, these
214 data were arcsine transformed and tested for normality of distribution (Shapiro-Wilks test) and homogeneity of
215 variance (Bartlett's test) prior to hypothesis testing. Dunnett's test (parametric) was then used if assumptions of
216 normality and homogeneity of variances were met, while Steel's Many-One Rank test (non-parametric) was used
217 when variances were heterogeneous or the distribution unequal. Maximum likelihood regression using probit
218 analysis with Abbott's correction or non-linear interpolation with bootstrapping (Icp) (if assumptions of the probit
219 analysis were not met) were also used to determine LC50, LC20, LC10 values and their 95% confidence limits

(CLs). Significance in all statistical tests was set at the $p < 0.05$ level and all statistical analyses were carried out using the software package Toxcalc (version 2.3) for Microsoft Excel (TidePool Scientific Software, California, USA).

3. Results and discussion

3.1. Properties of copper sulfide spiked sediments.

Copper sulfides are largely insoluble in 1 M HCl (Simpson et al., 1998; Cooper and Morse, 1998). Analyses of the synthesised copper sulfide (before addition to the sediments) found that $< 0.5 \mu\text{mol AVS g}^{-1}$ was extractable in 1 M HCl, whereas 1-h and 24-h extraction periods resulted in release of 11 ± 3 and $23 \pm 3\%$ (mean \pm SE, $n=3$) of the total copper, respectively. In the AVS analyses, iron(III) phases in sediments have been shown to catalyse the dissolution of copper sulfide phases in 1 M HCl, resulting in the liberation of copper and sulfur, rather than sulfide (Simpson et al., 1998; Simpson et al., 2000b). This is a key difference between the chemistry of copper sulfide to the other metals sulfides (e.g. FeS, MnS, ZnS, etc) and means that AVS and SEM analyses of sediments containing large portions of copper sulfide should also be interpreted differently. Firstly, increasing additions of copper should decrease the amount of AVS that is measureable in sediments, and this should be in proportion to the stoichiometry of the copper sulfide phases that form. Secondly, the varying solubilities in 1 M HCl of different iron (III) phases in sediments makes it difficult to predict the influence of iron on the extractability of copper from copper sulfide phases.

For the AVS-Cu and FeS-Cu series sediments, the copper sulfide was allowed to form in situ by reaction of copper with iron sulfide, FeS, which is the major AVS phase in most sediments. As AVS was liberated from the FeS phases, but not from copper sulfides phases, the changes in AVS measured were used to determine that the stoichiometry of the copper sulfide phases forming were Cu_2S , rather than CuS (Table S1 and Figure S1 of the Supplementary Information). Overall, the observations were consistent with previous studies that have demonstrated that Cu_2S forms in preference to CuS in sediments and that both CuS and Cu_2S are largely insoluble in 1 M HCl (Casas and Crecelius, 1994; Simpson et al., 1998; Cooper and Morse, 1998; Simpson et al., 2000a).

The concentrations of AVS and SEM-Cu measured for the three copper sulfide series at the start and at completion of the toxicity tests are shown in Figure 1 (Tables S1 of the Supplementary Information). All of the $30 \mu\text{mol g}^{-1}$ AVS-Cu series sediments contained a molar excess of AVS over added copper (i.e. positive values for AVS – Added Cu in Table S1) and the measured SEM-Cu was initially just 1–2% of the total copper (Figure 1d). The 18 and $11 \mu\text{mol g}^{-1}$ AVS-Cu series sediments were prepared by mixing the highly sulfidic and oxic base materials, and the copper-spiked sediments contained either no excess or a small excess of AVS over added copper. The SEM-Cu measured (before tests) represented 18–23% and 39–46%, respectively, of the added copper. Consequently, while the AVS analyses indicated that Cu_2S phases had formed, the SEM-Cu results indicated that a significant portion of the sulfidised copper was extractable due the presence of oxidised iron phases.

For the FeS-Cu series, the initial SEM-Cu concentrations ranged from 20–48% of total copper (Figures 1e, Table S1). While such high extractability of SEM-Cu was not expected due to the significant excess of AVS over

255 added copper in all sediments, this is likely to have been due to the high amounts of non-sulfidised iron(III) present
256 in the sediments. The sediments contained 140–180 $\mu\text{mol g}^{-1}$ SEM-Fe and further Fe(III) phases may have formed
257 by the oxidation of Fe(II) displaced from FeS during the reaction with added copper during the mixing and
258 equilibration of the sediments (Simpson et al., 2000b; Hutchins et al., 2008). For the CuS series, 83–100% of the
259 total copper was measureable as SEM-Cu, indicating that either much of the added CuS had been oxidised during
260 the mixing and equilibration period or the iron(III) phases present in these sediments were very effective at
261 oxidising CuS phases during the 1 M HCl extractions. Although negligible dissolved oxygen would have been
262 present in the sediments due to manipulation in a nitrogen atmosphere, sulfide is predicted to be
263 thermodynamically unstable in the presence of oxidised iron and manganese phases (Simpson et al., 2000b;
264 Millero, 2001) and the fine-sized synthetic copper sulfide may have been susceptible to oxidation through mixing
265 (Hutchins et al., 2008). Overall, with increasing spiked-copper concentrations the percentage of SEM-Cu extracted
266 decreased for the CuS series but increased for the FeS-Cu and three AVS-Cu series (Table S1). This is consistent
267 with the lower extractability of SEM-Cu in sediments with greater degrees of sulfidisation and lower proportions of
268 oxidised Fe phases (Simpson et al., 1998).

269 3.2. Oxidation of copper sulfide phases and copper release to overlying water.

270 The concentrations of AVS and SEM-Cu measured before and after the 10-day toxicity tests with the three
271 copper-spiked sediment preparations are shown in Figure 1 (Tables S1 of the Supplementary Information). Within
272 24 h in all tests, a brownish layer of oxidised sediment of approximately 1 mm thickness had formed over the
273 greyer sediments beneath. At the end of the AVS-Cu tests, the depth of the oxidised material had increased to 1–3
274 mm and the layering remained visible. In the FeS-Cu tests, no layering was visible after 48 h indicating that the
275 juvenile amphipods, with body lengths of 1 ± 0.1 mm (Spadaro et al., 2008), were causing significant disturbance of
276 the sediments.

277 Comparison of AVS concentrations measured for the AVS-Cu and FeS-Cu series sediments at the start and
278 completion of the tests demonstrated that a significant percentage of AVS had oxidised during the 10-day test
279 period (Figures 1a and b). For sediments that initially had >1 $\mu\text{mol AVS g}^{-1}$, the AVS concentrations decreased by
280 32–67%, 76–97% and 82–97% in the 30, 18 and 11 $\mu\text{mol g}^{-1}$ AVS-Cu series, and by 46–88% for the FeS-Cu series.
281 Signification oxidation of AVS was expected to be detected due to the thin layers of sediments used in these tests.
282 Previously studies have also observed significant decreases in AVS concentrations during toxicity tests (Casas and
283 Crecelius, 1994; Besser et al., 1996; DeLange et al., 2008). The oxidation of AVS was not able to be accurately
284 assessed for the CuS series due to the low concentrations at the start of the tests (Figure 1c). The majority of
285 whole-sediment toxicity test procedures utilise test sediment depths of 2 cm or less (Greenstein et al., 2008), and it
286 is likely that oxidation of AVS in the surface sediments occurs during most test procedures. While the source of
287 oxygen was expected to have been predominantly from the overlying water, we expect that the rate of oxidation
288 will have been increased due to the burrowing activity of the amphipods (Peterson et al., 1996).

289 The degree of oxidation of the surface sediments was very evident from the changes in SEM-Cu. During the
290 10-day test period the copper extractable as SEM-Cu increased from 1–2% to 70–90%, 18–23% to 52–71%, and

291 39–46% to 72–92% for the 30, 18 and 11 $\mu\text{mol g}^{-1}$ AVS-Cu treatments, respectively (Figure 1d, Table S1). The
292 rate of oxidation of FeS and MnS by oxygen is considerably more rapid than that of CuS or Cu_2S (Simpson et al.,
293 1998; 2000) and the increased extractability of SEM-Cu at the end of the tests will be a result of increased amounts
294 of iron hydroxide phases and also the direct oxidation of the copper sulfide phases. The FeS-Cu series sediments
295 contained Cu_2S and FeS, whereas the CuS series sediments contained CuS and mostly iron oxyhydroxide phases.
296 Owing to the differences in iron chemistry and the influence of iron on the extraction of SEM-Cu, it was not
297 possible to determine from these experiments which of the Cu_2S or CuS phases oxidised more readily. It was also
298 not possible to determine the relative contribution of dissolved oxygen or iron and manganese oxyhydroxides in the
299 oxidation process. It was evident from the experiments that, over the 10-day period, the exposure to oxygenated
300 overlying water and the foraging of amphipods resulted in considerable oxidation of copper sulfide phases in
301 surface sediments, regardless of the form of the copper sulfide or iron chemistry.

302 For all of the copper-spiked sediment series, the porewater copper concentrations measured at the start of the
303 tests were less than $10 \mu\text{g L}^{-1}$. For the AVS-Cu series, the sediment depth of 4–5 mm allowed for a small amount
304 of porewater to exist, but the pore water was not reanalysed at the completion of the tests owing to the small
305 amount of material. For the FeS-Cu and CuS series the tests were conducted on 1 to 2 mm layers that contained
306 negligible porewater. Initial experiments found that, over a 24-h period, there was a significant release of dissolved
307 copper to the overlying water from each of the test materials. This was consistent with oxidation of the copper
308 sulfide phases, and was used to determine that renewing the overlying water every 2 to 3 days would prevent
309 dissolved copper concentrations increasing above the 10-day NOEC of $36 \mu\text{g L}^{-1}$ (Spadaro et al., 2008; Strom et al.,
310 2011). In both the pore waters (before tests) and overlying waters during the tests, the concentrations of dissolved
311 Cd, Ni and Pb were $<3 \mu\text{g L}^{-1}$, and Zn was $<15 \mu\text{g L}^{-1}$. Past studies have shown that copper is much more toxic
312 than each of these metals and that no lethality would occur to juvenile *M. plumulosa* at these concentrations (King
313 et al., 2006; Mann et al., 2010).

314 The time-averaged dissolved copper concentrations were determined from dissolved copper measurements
315 on days 1, 5 and 10 of the tests (Figures 2a and b). In general, the mean dissolved copper concentrations increased
316 linearly with increasing (spiked) sediment copper concentration. For the AVS-Cu series, the measured dissolved
317 copper concentrations were greater in the sediments that had the lower AVS concentrations (Figure 2a), which is
318 consistent with the greater binding of copper as copper sulfide phases in sediments with greater AVS
319 concentrations. The concentrations of dissolved and particulate copper were strongly correlated ($r>0.9$, $p<0.01$)
320 and indicated that desorption of copper from the sediments was the principal source of the dissolved copper in the
321 overlying water.

322 3.3. Copper toxicity and exposure pathways

323 From the 10-day water-only tests, LC50 (95% confidence limits), LC20 and LC10 values of 87 (70–104), 53
324 and $41 \mu\text{g/L}$, respectively, were determined for juvenile *M. plumulosa*. Previous studies had reported LC50 and
325 NOEC values of $76\pm 15 \mu\text{g/L}$ and $36 \mu\text{g L}^{-1}$ (Spadaro et al., 2008). The survival in the control sediments for each
326 series was $>80\%$, and any lethality observed for other sediments in that series was considered to be due to the

327 added copper. The sediments comprised >95% silt and 4% TOC and, consistent with past studies (Spadaro et al.,
328 2008), the adequate survival in the controls indicated that sediment nutrition was not significantly influencing the
329 results. In each copper sulfide series, the sediments became toxic to *M. plumulosa* at the higher copper
330 concentrations (Figures 2c and d). At the same total copper concentrations, the FeS-Cu series sediments caused
331 greater toxicity than the CuS series (Figure 2d), despite the very similar dissolved copper concentrations in the
332 overlying water (Figures 2b). Toxicity was observed for each series even though the mean dissolved copper
333 concentrations in the overlying waters were generally below the NOEC value of 36 $\mu\text{g L}^{-1}$ (Figures 2a and b).

334 *M. plumulosa* reside at the sediment-water interface (SWI) and the dissolved copper exposure will occur via
335 copper in the porewater or overlying water. Recent studies have shown that the flux of copper may be significantly
336 greater in the few mm below the SWI compared to both the deeper pore water and the overlying water (Tankere-
337 Muller et al., 2006). The use of thin layers of sediments meant that negligible pore water existed in the FeS-Cu and
338 CuS experiments, and the dissolved copper released at the SWI was expected to be rapidly diluted due to the
339 continual mixing of the overlying waters created by the gas bubbling. In past studies of copper with juvenile *M.*
340 *plumulosa*, Strom et al. (2011) calculated LC15 and LC50 values of 36 and 68 $\mu\text{g/L}$ in overlying water of tests
341 using copper-spiked sediments, by extrapolation to exposure conditions containing no particulate copper. The
342 closeness of those values to the values determined in the present study indicates that the dissolved copper
343 concentrations at the SWI and in the overlying waters were similar in the sediment bioassays. Based on the results
344 of the present study and the earlier studies, we do not believe that dissolved copper in the pore water, SWI or
345 overlying water were the only copper contributions to the observed toxicity (Figure 2).

346 Previous studies have demonstrated that *M. plumulosa* ingest sediments while foraging for food (King et al.,
347 2005; Simpson and King, 2005), and this may contribute to the observed toxicity (Simpson, 2005; Strom et al.,
348 2011). In the present study, adequate survival was observed in the controls with and without added food but
349 survival in the FeS-Cu and CuS series increased when additional food (dried, powdered fish) was added (Figure
350 2d). Unexpectedly, the dissolved copper concentrations were significantly greater (often double) in the tests with
351 no added food (Figure 2b) indicating that the higher dissolved copper concentrations were not increasing the
352 toxicity. Copper forms strong complexes with organic ligands present in sediment porewaters and overlying water
353 (Vink, 2009). It is possible that the added food also provided an additional source of copper complexing ligands
354 that increased the rate of release of copper from particles but decreased the bioavailability of copper in the
355 dissolved phase. This was discounted as the sole reason for the decrease in toxicity as the 10 g of sediment
356 (containing 4% TOC) was expected to contain a greater source of copper-complexing ligands than the 15 mg of
357 fish food was added at each water change (45 mg in total per test).

358 The increase in amphipod survival in the CuS and FeS-Cu series with food added was attributed to a
359 combination of factors, including increased robustness of the organisms, greater complexation of dissolved copper
360 in the overlying water, and ingestion of less of the copper-contaminated sediment. The observations add to the
361 growing body of evidence of toxic effects through diet for this amphipod species (Simpson and King, 2005; King et
362 al., 2006; Spadaro et al., 2008; Mann et al., 2009; Strom et al., 2011). While the added food will have adsorbed

363 some of the released copper and would act as an avenue of metal exposure, this will not have been a significant
364 source of copper to the amphipods.

365 Because some copper sulfide phases were oxidised during the tests (Figure 1), it was not possible to
366 distinguish between the possible effects of ingesting sulfide and non-sulfide forms of particulate copper. Previous
367 studies have demonstrated that benthic bivalves and polychaetes can accumulate metals from sediments that have a
368 molar excess of AVS compared to SEM (Lee et al., 2000; Otero et al., 2000; Griscom and Fisher, 2004; De Jonge
369 et al., 2009 and 2010). It is possible that the metal accumulation observed in those studies may have also occurred
370 following the partial oxidation of metal sulfide phases in the sediments, or through the existence
371 microenvironments that contained oxidised forms of the metals within the anoxic sediments.

372 3.4. AVS-SEM model predictions and copper effects thresholds

373 For sediments that contain an excess of AVS over SEM concentrations, the porewater concentrations of Cd,
374 Cu, Ni, Pb and Zn are predicted to be negligible and acute or chronic effects should not result from these metals
375 (USEPA, 2005). This forms the basis of the AVS-SEM model for predicting toxicity of these metals in sediments
376 (Di Toro et al., 2005). For all treatments, the SEM-Cd, Ni, Pb and Zn concentrations were 0.002, 0.044, 0.15 and
377 $2.1 \mu\text{mol g}^{-1}$, respectively. This $2.3 \mu\text{mol g}^{-1}$ of non-copper SEM was a minor contribution to the total SEM
378 (Figure 1, Table S1). For the three AVS-Cu series, the toxicity was less in the sediments that had the greater AVS
379 concentrations (Figure 2c), which is consistent with AVS reducing the bioavailability of copper.

380 Due to oxidation of AVS, sediments that had a molar excess of AVS compared to SEM-Cu at the start of the
381 tests, did not always have an excess at the end of the tests (Figure 1). Casas and Crecelius (1994) and Besser et al.
382 (1996) have previously observed the oxidation of copper and zinc sulfide phases during toxicity tests of both
383 marine and freshwater sediments, respectively. When the initially measured AVS and SEM concentrations were
384 used, the AVS-SEM model did not provide useful predictions of metal toxicity (Figure 3a). However, when the
385 AVS and SEM concentrations measured at the end of the tests were used the AVS-SEM model provides a suitable
386 prediction of the transition from non-toxic to potentially toxic metal concentrations (Figure 3b).

387 For two of the FeS-Cu series, toxicity was observed in the sediments having a small molar excess of AVS
388 over SEM-Cu at the completion of the tests. While this is considered to be within the experiment error of the SEM
389 - AVS analyses, it is also possible that the small amount of AVS that was present after 10 days was present inside
390 the particles and having little influence on the bioavailability of metals adsorbed to the oxidised outer layer. At the
391 same total copper concentrations, the FeS-Cu series sediments cause greater toxicity than the CuS series (Figure
392 2d), despite the CuS series having much higher SEM-Cu concentrations and similar dissolved copper
393 concentrations in the overlying water (Figure 2b). At the start of the tests, the FeS-Cu series had 20 to $30 \mu\text{mol}$
394 AVS g^{-1} , while the CuS series has $0.7 \mu\text{mol AVS g}^{-1}$. While the oxidation of excess AVS in the FeS-Cu series
395 (comprising mostly FeS) may have occurred quite rapidly in the surface layers, the CuS series was expected to
396 have a greater density of oxidised iron and manganese oxyhydroxide phases to which copper released during
397 oxidation of CuS could rapidly bind.

398 Overall, the results are consistent with past studies that have demonstrated that AVS reduces the
399 bioavailability of copper in sediments (Ankley et al., 1993; Berry et al., 1996) and the predictions of the AVS-SEM
400 model (Di Toro et al., 2005; USEPA, 2005; Simpson et al., 2011). Effects thresholds (LC50, LC20, and LC10)
401 based on total particulate copper concentrations were calculated for the AVS-Cu, FeS-Cu and CuS series sediments
402 (Table 1). Recently, Simpson et al. (2011) used acute effects data for 12 benthic organisms to calculate an acute no
403 effects threshold of 510 mg kg⁻¹ for copper-spiked oxic silty sediments from the same origin as those used in this
404 study. In that study, the LC50, LC20 and LC10 values for juvenile *M. plumulosa* in sediments equivalent to the
405 Cu-oxic series were 940, 790, and 720 mg kg⁻¹ (no added food), approximately 200 mg kg⁻¹ higher than the value
406 determined in the present study (730, 570, and 490 mg kg⁻¹, Table 1). While those differences may be attributed to
407 variations in the sensitivity of different batches of juveniles, the much lower LCx values of the FeS-Cu and CuS
408 series (140–690 mg kg⁻¹) indicate that differences in test design (e.g. depth of test material) and the copper
409 exposure were contributing to the greater toxicity of the FeS-Cu and CuS series.

410 The present study highlights the importance of considering the temporal nature of AVS in sediments and
411 the need to monitor the presence of this phase and its influence on the bioavailability of metals in surface
412 sediments. The existence of an excess of AVS over SEM in a bulk sediment may not necessarily mean that the
413 microenvironment in which an organism lives, e.g. the burrows or surface sediments, contains the similar level of
414 metal binding by sulfide. As most whole-sediment toxicity test procedures utilise shallow sediment depths
415 (Greenstein et al., 2008), oxidation of significant amounts of AVS will occur during most test procedures (Ankley
416 et al., 1993; Peterson et al., 1996; Eriksson-Wiklund and Sundelin, 2002; De Lange et al., 2008).

417 While copper sulfide phases are relatively slow to oxidise, and released copper is readily adsorbed by a range
418 of other sediment phases, other AVS-bound metals may become much more bioavailable within oxidised surface
419 sediments. It is now well recognised that along with AVS, the bioavailability of metals in sediments is also
420 strongly influenced by POC and iron and manganese oxyhydroxide phases associated with silt (Besser et al., 2003;
421 Di Toro et al., 2005; Simpson and Batley, 2007; De Jonge et al., 2009; Strom et al., 2011). The absence of toxicity
422 in the AVS-Cu and CuS series until a 10 μmol g⁻¹ excess of SEM-Cu over AVS (greater than 600 mg Cu/kg) is
423 consistent with the presence of these copper-binding phases (Figure 4b). While methods for incorporating both
424 dissolved and dietary exposure routes into mechanistic based guidelines still requires further development
425 (Simpson, 2005), the use of guidelines that vary with changes in AVS, POC, and silty contents provide significant
426 improvement over single values guidelines that are frequently applied to all sediment types (Di Toro et al., 2005;
427 Strom et al., 2011; Simpson et al., 2011).

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431 **Supporting information available**

432 Chemical properties, including AVS and SEM-Cu concentrations measured before and after toxicity tests, of the
433 copper sulfide spiked sediments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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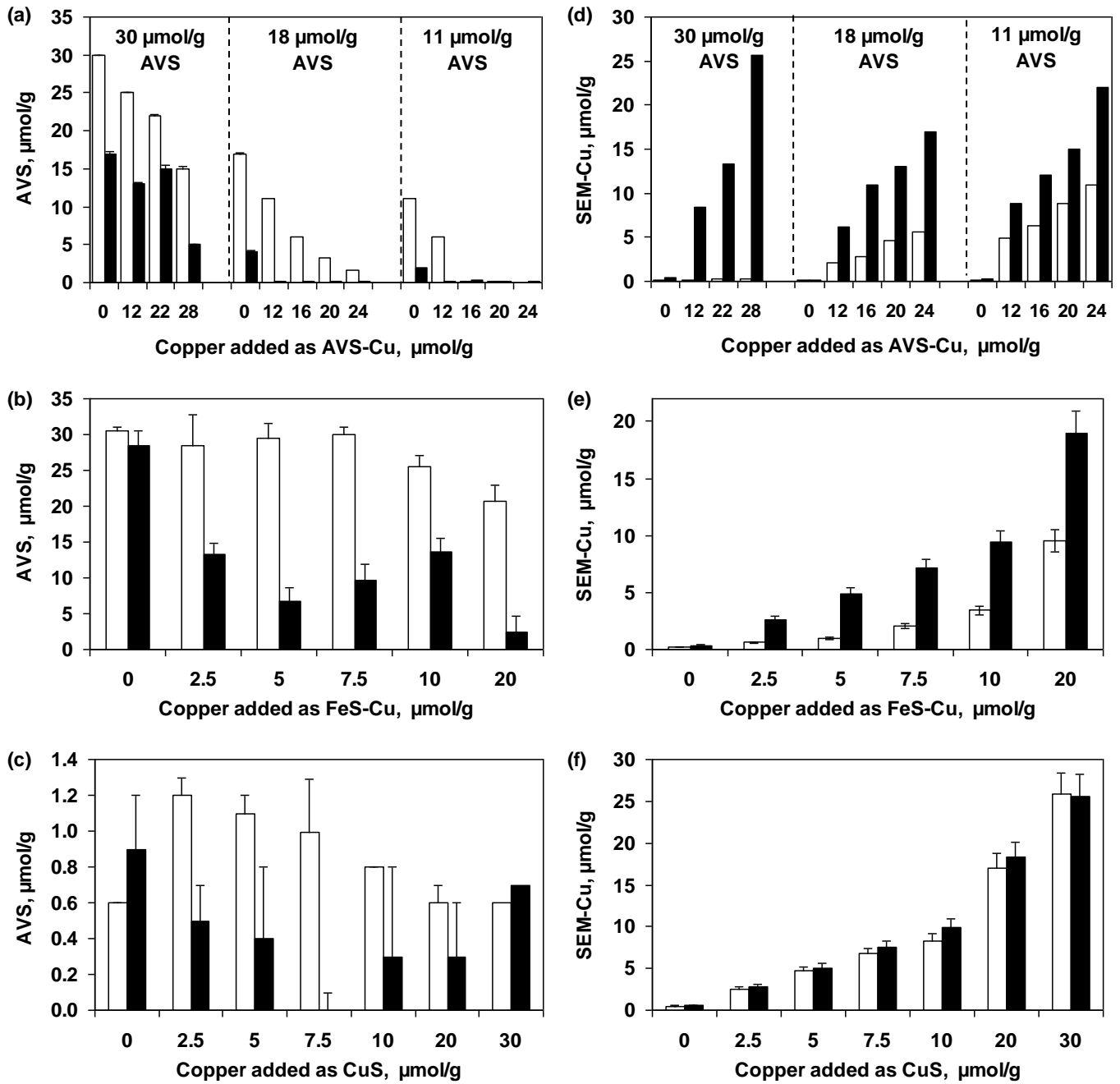
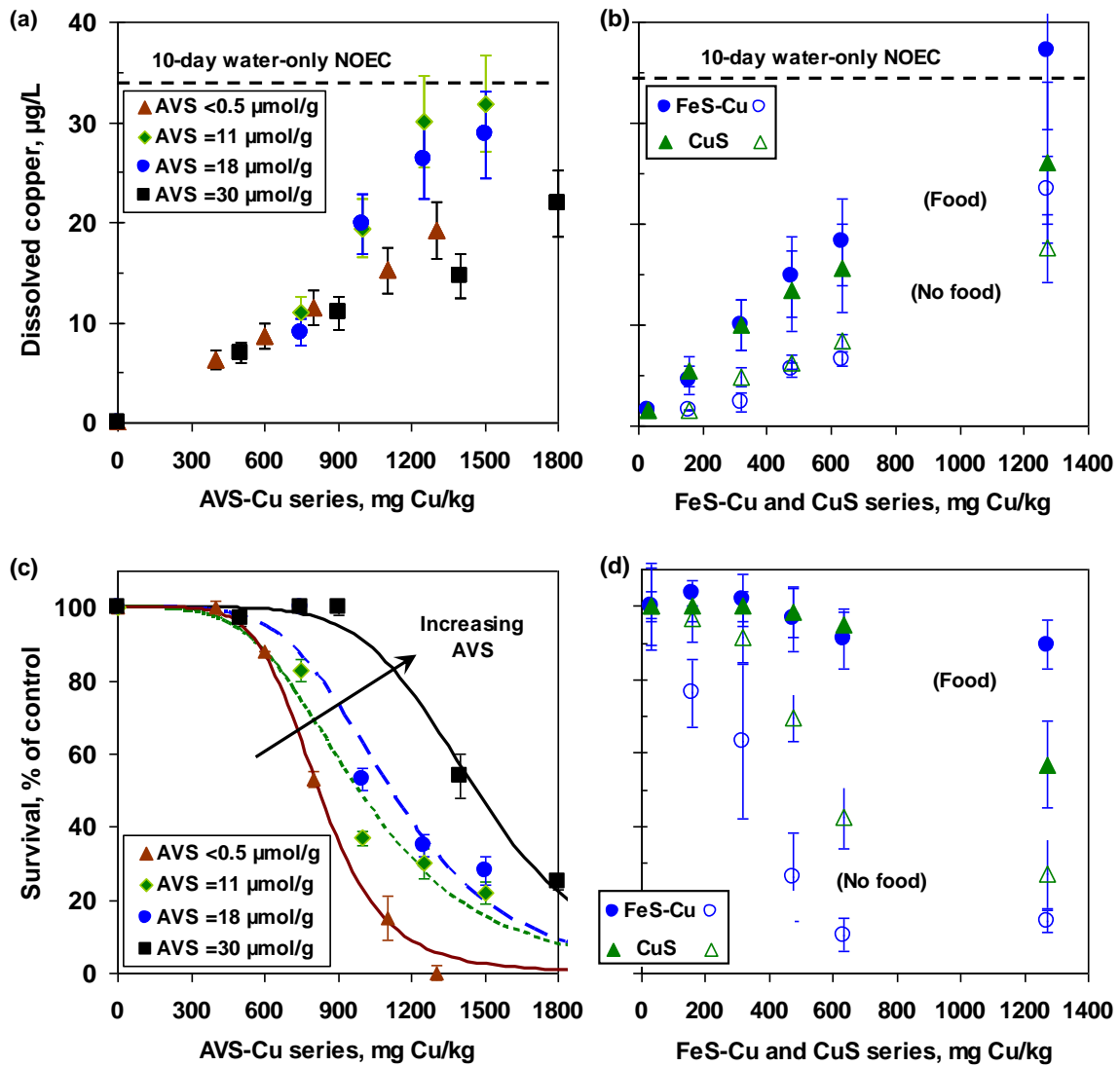


Fig. 1. AVS and SEM-Cu concentrations (mean ± 1 SD, n = 3) before (■) and after (□) 10-day toxicity tests for the copper sulfide spiked sediments: (a, b) AVS-Cu series; (c, d) FeS-Cu series, and (e, f) CuS series.

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29 **Fig. 2.** Dissolved copper concentrations ($\mu\text{g/L}$) in the overlying water and 10-day survival of juvenile *M.*

30 *plumulosa* in 10-day whole-sediment toxicity tests of copper sulfide spiked sediments: (a, c) AVS-Cu series; (b, d)

31 FeS-Cu series and CuS series with (filled symbols) and without food added (open symbols). The data are means

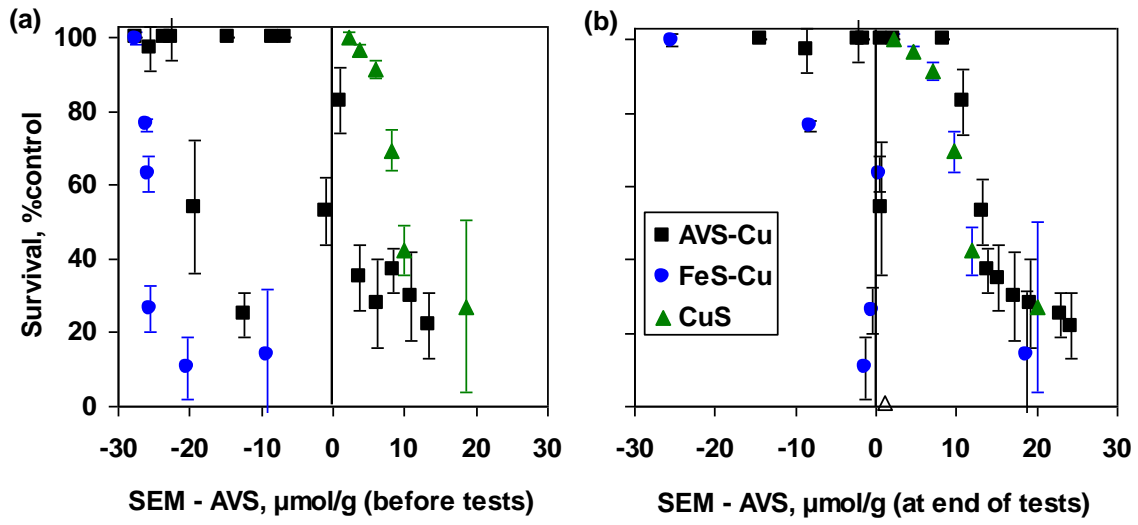
32 with error bars representing standard error for three replicates.

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43 **Fig. 3.** Survival of juvenile *M. plumulosa* in relation to SEM - AVS models using measurements from the start and
 44 end of 10-day tests. The data are means with error bars representing standard error for three replicates.

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Tables

Table 1

Effects concentrations for juvenile *M. plumulosa* exposure to copper-spiked sediments

Copper spiked sediment	Particulate copper, mg kg ⁻¹		
	LC50 (95% CL)	LC20 (95% CL)	LC10 (95% CL)
Oxic/sub-oxic Cu	730 (690-770)	570 (530-620)	490 (440-550)
AVS_11-Cu	970 (900-1050)	690 (600-780)	560 (600-780)
AVS_18-Cu	1100 (1030-1180)	810 (730-910)	680 (590-790)
AVS_30-Cu	1460 (1420-1500)	1150 (1090-1210)	1000 (930-1100)
CuS-oxic (no food)	690 (580-810)	330 (260-430)	220 (150-310)
CuS-oxic (food)	1800 (1500-2000)	850 (670-1100)	560 (390-790)
FeS-Cu (no food)	320 (220-400)	190 (92-410)	140 (50-390)
FeS-Cu (food)	No toxicity up to 1200 mg Cu kg ⁻¹		

95% CL = 95% Confidence Limit.