1   An evaluation of biotic ligand models predicting acute copper toxicity to

2   *Daphnia magna* in wastewater effluent

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10

11   **Abstract -** The toxicity of copper to *Daphnia magna* was investigated in a series of 48 hour
12   immobilisation assays in effluents from four wastewater treatment works. The assay results
13   were compared with EC50 forecasts produced by the HydroQual BLM, the refined *D. magna*
14   BLM, and a modified BLM that was constructed by integrating the refined *D. magna* biotic ligand
15   characterisation with the WHAM VI geochemical speciation model, which also accommodated
16   additional effluent characteristics as model inputs. The results demonstrated that all the BLMs
17   were capable of predicting toxicity by within a factor of two, and that the modified BLM produced
18   the most accurate toxicity forecasts. However, this was generally dependent on the inclusion of
19   effluent specific water chemistry characteristics in the speciation modelling as well as optimising
20   the dissolved organic carbon 'active' fraction. Only the refined *D. magna* BLM predicted all
21   EC50 values by within a factor of two using default model parameters and standard model
22   inputs. The results also suggested that the biotic ligand stability constant for sodium may be a
23   poor approximation of the mechanisms governing the influence of sodium where concentrations
24   exceed the range within which the biotic ligand stability constant value had been determined.
25   These findings support the use of BLMs for the establishment of site-specific water quality
26   standards in waters that contain a substantial amount of wastewater effluent, but re-enforces
27   the need for regulators to scrutinize the composition of models, their thermodynamic and biotic
28   ligand parameters, and the limitations of those parameters.

29

30   Keywords: biotic ligand, daphnia, copper, *ethylenediaminetetraacetic acid*, sodium

31

32   **INTRODUCTION**
33   Copper is a high-volume usage metal with multiple pathways into the aquatic
34   environment, within which elevated concentrations are of concern due to the potential
35   for adverse ecotoxicological effects. Numerous studies have, however, revealed neither
36   the total nor the dissolved concentration to be a good indicator of toxicity. Rather,
37   copper toxicity has been found to be dependent on metal speciation, and competition
38   for binding at biologically sensitive receptors, which are both influenced by water
39   chemistry characteristics such as pH, hardness and dissolved organic matter [1, 2]. The
40   importance of water chemistry in influencing copper toxicity is implicit in the two classic

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41 models which describe metal toxicity; the free ion activity model, which relates toxicity to
42 the free ion interaction with biologically sensitive cell surfaces [3, 4], and the gill surface
43 interaction model, which is similar to the free ion activity model, but takes into account
44 competition from other cations for binding at biologically sensitive receptors [5]. Most
45 recently, the biotic ligand model (BLM) approach has been developed, which uses the
46 accumulation of toxic metal species at a generic biologically sensitive receptor as a
47 basis for predicting toxicity [6]. This approach incorporates elements from both classic
48 models and predicts a toxicity endpoint expressed as a dissolved metal concentration.
49 The BLM approach has consideration for both metal speciation and competition for
50 binding at the biotic ligand, thereby providing a quantitative and mechanistic framework
51 for the evaluation of metal toxicity [7].
52 A number of acute and chronic toxicity BLMs have been developed for copper [8-11],
53 and recently the BLM approach received favourable commentary in a review of the
54 European Union risk assessment for copper and its compounds [12]. However, in
55 addition to generating interest within the research and risk assessment communities,
56 BLMs have begun to find appeal with regulatory authorities, and appear increasingly
57 likely to be applied as tools to determine site specific water quality standards for a
58 number of metals. In the United States the HydroQual BLM has already been approved
59 for use in determining water quality criteria for copper by the United States
60 Environmental Protection Agency [13].
61 The increasing prospect of regulatory application, however, has required some
62 consideration for the environmental context within which BLMs may be applied.
63 Whereas the accuracy of the aforementioned BLMs has been validated in a wide range
64 of natural waters, regions which receive sewage effluent inputs with little dilution from
65 receiving waters may effectively require the application of BLMs to waters that comprise
66 significantly of treated wastewater effluent, and for which the accuracy of BLM forecasts
67 is less certain. This is of particular relevance for regions such as the United Kingdom,
68 parts of northern and southern Europe, as well as parts of North America that are
69 known to have low effluent dilution capacity (≤1:10) [14] and which may seek to apply
70 the BLM approach for regulatory purposes. Effluents represent complex mixtures that
71 contain a number of potentially toxic metals that may influence metal toxicity in a
72 manner not accommodated by BLMs that have been developed for a single metal only.
73 The complex and uncertain nature of effluents suggests these might also contain low
74 molecular weight lipophilic metabolites which form metal complexes that may lead to
75 enhanced metal uptake and toxicity [4]. Alternatively, effluents have been shown to
76 contain a greater number of complexing ligands per milligram of dissolved organic
77 carbon (DOC) compared with the DOC from natural waters [15] so that models
78 calibrated on the basis of DOC from natural waters may understate copper
79 complexation and produce inaccurate forecasts. Similarly, effluents are also known to
80 contain synthetic chelating agents, such as *ethylenediaminetetraacetic acid* (EDTA),

81 which is used in detergents because of its capability of sequestering metal ions [16],
82 which may offer additional metal complexation capacity not accommodated by existing
83 BLMs. In combination, and alongside the intrinsic uncertainties associated with
84 wastewater effluents, these factors may influence the accuracy of BLM forecasts, with
85 implications for regulatory regimes based on the approach. Consequently, an
86 assessment of the ability of BLMs to predict toxicity in a wastewater effluent medium is
87 useful alongside the efforts aimed at improving the accuracy of metal speciation
88 modelling and biotic ligand characterisation.
89 The aim of the present study was to evaluate the predictive accuracy of existing
90 BLMs in a selection of wastewater effluents, as well as to determine whether the
91 inclusion of additional effluent water chemistry characteristics in speciation calculations
92 could produce forecasts with improved accuracy.
93
94 **MATERIALS AND METHODS**
95 *Experimental approach*
96 Acute toxicity assays, with *D. magna* as the test organism, were conducted in
97 effluents from four wastewater treatment works. The assessment of BLM performance
98 evaluated forecasts produced by the HydroQual BLM [17], and the refined BLM
99 proposed by De Schamphelaere et al. [9, 10]. In order to examine the effect of
100 incorporating the most recent model of copper-DOC complexation, and of including
101 additional effluent specific water chemistry characteristics in the speciation calculation,
102 a modified BLM was constructed by integrating the refined *D. magna* biotic ligand
103 characterisation with the WHAM VI geochemical speciation model [18, 19]. This
104 modified BLM was used to produce two forecasts; one which used the same inputs as
105 the HydroQual BLM, and another where the concentrations of synthetic chelating agent,
106 zinc and iron were considered as additional model inputs. The potentially relevant
107 synthetic chelating agents were restricted to the aminopolycarboxylic acids, which
108 represent the highest volume category of organic synthetic chelating agent [20], and
109 which were expected to occur in at least two of the selected effluents at potentially
110 relevant concentrations. Iron was considered in the speciation calculation due to its
111 affinity for aminopolycarboxylate synthetic chelating agents and its ability to occupy
112 binding sites in organic matter [21, 22], which might reduce copper complexation
113 capacity. Zinc was included in the speciation calculation due to its relative abundance in
114 wastewater effluents, and affinity for aminopolycarboxylate chelating agents (e.g. Zn-
115 EDTA: log K 18.3) [23]. Zinc was not included in the calculation for binding at the biotic
116 ligand, or assumed to contribute towards toxicity since the concentrations of zinc in the
117 effluents were expected to be below the *D. magna* chronic toxicity threshold, and well
118 below the acute toxicity threshold [24].
119
120 *Description of models*

121   BLMs combine a chemical equilibrium sub-model alongside a toxicity sub-model
122   to predict toxicity [6]. The chemical equilibrium sub-model determines the distribution of
123   metal species, with the affinity between solution components represented by stability
124   constants, and where the biotic ligand (BL) is included within this framework as an
125   additional ligand, with its own set of stability constants. The chemical equilibrium sub-
126   model determines ion binding with DOC, inorganic aquatic components, and the BL.
127   The toxicity sub-model predicts toxicity endpoints on the basis the amount of dissolved
128   metal required to achieve critical concentrations of toxic metal-BL species, where the
129   critical concentrations are predetermined constants that exist within the model
130   database. Whereas this basic framework is common to the models that have been
131   applied in the present study, some significant differences exist.

132   The HydroQual model incorporates a chemical description of copper-DOC
133   complexation from the Humic Ion Binding Model V [25], alongside a version of the
134   Chemical Equilibria in Soils and Solutions (CHESS) model [26], which determines
135   inorganic speciation. These are applied, in combination, as the chemical equilibrium
136   model. The HydroQual model characterisation of the *D. magna* biotic ligand is based on
137   that determined for the fathead minnow [6] (i.e. it applies the gill binding constants
138   determined for the fathead minnow), alongside a modified critical concentration value
139   that had been determined by calibration with *D. magna* toxicity data. The HydroQual
140   model considers Cu^{2+} and $CuOH^+$ as the toxic metal species and is expected to predict
141   48-h EC50 values by within a factor of two. A full description of this model is available
142   [8].

143   The refined *D. magna* BLM, developed by De Schamphelaere et al. [9, 10]
144   utilised a similar version of the HydroQual model described previously for speciation
145   calculations, however, with certain notable differences. The refined BLM utilised biotic
146   ligand stability constants that were determined experimentally from toxicity data, which
147   differed from the values applied in HydroQual model. The refined BLM also required a
148   greater proportion of BL binding sites to be occupied by toxic metal species in order for
149   toxicity to occur. In addition to Cu^{2+} and $CuOH^+$, the refined model also regarded $CuCO_3$
150   as a toxic metal species. Stability constants for inorganic complexes were also adjusted
151   to NIST recommended values. Whereas both models applied the Humic Ion Binding
152   Model V to model copper-DOC complexation, there were significant differences in the
153   parameterisation of this model. The Humic Ion Binding Model V relates the binding of
154   metals to DOC with proton binding characteristics through a single model parameter; a
155   metal-proton exchange constant (pK_{MHA}), which has been well described elsewhere [8,
156   21]. In the development of the refined BLM [9, 10] it was noted that the default pK_{MHA}
157   value (1.5) resulted in a significant overestimation of copper-DOC complexation. De
158   Schamphelaere et al. consequently determined a best fit pK_{MHA} value (1.9) which was
159   applied in speciation modelling. This modification implied a reduced affinity between
160   copper and DOC. The refined BLM is expected to predict 48-h EC50 values by within a

161 factor of two. A full description of the refined BLM and its development is also available
162 [2, 9, 10].

163   The modified BLM was constructed by integrating the refined *D. magna* biotic
164 ligand characterisation with the WHAM VI geochemical speciation model. WHAM VI
165 incorporates an inorganic speciation model with similar functionality to that of the
166 CHESS model, alongside Humic Ion Binding Model VI, which has been demonstrated to
167 provide a more accurate description of copper-DOC complexation than Model V [15,
168 21]. Since the differences between Model V and Model VI have been well described
169 elsewhere [18, 25], only a brief description is provided. The Humic Ion Binding Model
170 V, as applied within the HydroQual model framework (and the refined BLM), relates the
171 binding of metals to DOC with proton binding characteristics through a metal-proton
172 exchange constant (pK_{MHA}). Humic Ion Binding Model VI relaxes the relationship
173 between metal and proton binding and expresses metal-DOC binding affinity in terms of
174 a central log K_{MA} value, with a distribution of binding constants determined on the basis
175 of a primary 'spread' factor (ΔLK_1), and a secondary 'spread' factor ($\Delta LK2$). The
176 secondary 'spread' factor enables the creation of high affinity binding sites for which a
177 range of metal ions compete for binding, and to which the improved accuracy of the
178 model is attributed, in particular, at low copper concentrations ($<1\mu M$). The high affinity
179 binding sites, however, represent only a very small fraction of the total number of
180 binding sites.

181   For the modified BLM WHAM VI inorganic binding constants were adjusted to
182 values applied in the refined BLM, notably, the binding constant for $CuHCO_3^+$, which
183 was adjusted from 14.63 to 12.13, which was also in accordance with the
184 recommendation by Bryan et al. [21]. The modified BLM was applied to produce two
185 forecasts; one for which the identical inputs as the HydroQual and refined BLMs were
186 applied, and another where effluent characteristics (synthetic chelating agents, zinc and
187 iron) were included in the speciation calculation. Stability constants for
188 aminopolycarboxylates and the relevant metal cations (and their hydroxide products)
189 were obtained from Stumm and Morgan [23] (supplemental data **Table S1)**. The biotic
190 ligand parameters and inorganic thermodynamic constants for each model are
191 summarised in **Table 1**.

192

193 *Model inputs*

194   The models required pH, DOC, inorganic carbon (or alkalinity), calcium,
195 magnesium, sodium, sulphate, and chloride as standard inputs, as well as iron, zinc and
196 the relevant aminopolycarboxylate synthetic chelating agent for the modified BLM. The
197 total concentration of aminpolycarboxylate synthetic chelating agent was used for model
198 input, regardless of initial speciation. For modified BLM forecasts, the model input was
199 Cu^{2+} free ion activity. WHAM VI was able to accept Cu^{2+} free ion activity as a model
200 input (i.e. effectively the concentration remaining as Cu^{2+} free ion after complexation),

201 so that the speciation calculation determined the requisite dissolved Cu^{2+} concentration,
202 and the associated speciation. The Cu^{2+} free ion activity was calculated using the BLM
203 equation developed for the refined BLM [9, 10]. The equation is given below, with
204 values in square brackets indicating ion activities.
205

$$\text{EC50}_{Cu^{2+}} = \frac{f_{CuBL}^{50\%} \cdot \{1 + K_{CaBL} \cdot [Mg^{2+}] + K_{NaBL} \cdot [Na^+] + K_{HBL} \cdot [H^+]\}}{(1 - f_{CuBL}^{50\%}) \cdot \{K_{CuBL} + K_{CuOHBL} \cdot K_{CuOH} \cdot [OH^-] + K_{CuCO_3BL} \cdot K_{CuCO_3} \cdot [CO_3^{2-}]\}}$$

206

207   In addition, whereas the HydroQual and refined BLMs required DOC as an input,
208 WHAM VI required dissolved organic matter as a model input rather than DOC.
209 Therefore, for WHAM VI the input values were two times the DOC concentration. The
210 factor of two converts DOC to DOM by assuming carbon to comprise 50% of organic
211 matter by weight [27]. Additional attention is, however, required with regard to DOC and
212 iron inputs.
213 *Dissolved organic carbon.* For the purposes of modelling, DOC has traditionally been
214 considered to comprise of an 'active' fraction, to which metal ions bind, and another
215 fraction which is inert with regard to metal binding. The proportion of DOC considered
216 'active' therefore has a significant effect on modelled speciation. The 'active' fraction
217 has, however, been found to be variable. In copper titration experiments Dwane and
218 Tipping [28] found that measured and calculated free copper ion activity provided the
219 best agreement when 40-80% of DOC was considered 'active fulvic acid'. In the
220 development of the refined BLM De Schamphelaere et al. [9, 10], assumed 50% of
221 DOC to be 'active fulvic acid', and an evaluation by Bryan et al. [21] suggested that, on
222 average, 68% of DOC may be regarded to behave as 'active fulvic acid'. The extent by
223 which, for example, the 50% value applied by De Schamphelaere et al. differs from the
224 68% value determined by Bryan et al. is not, however, apparent since the effect of the
225 applied 'active' fraction on modelled speciation was also dependent on other differences
226 between models. Consequently, it was not sensible to apply a uniform 'active' fraction
227 across all models. Therefore, for the purposes of model comparison, the 'active' DOC
228 fraction was standardised in accordance with recommended values or those determined
229 in other studies so that, for the HydroQual BLM the DOC value applied for model input
230 was 100% of the DOC concentration, for the refined BLM the value was 50% of the
231 DOC concentration, and for the modified BLM the value was 68% of the DOC
232 concentration. However, since Sarathy and Allen [15] demonstrated that DOC derived
233 from wastewaters contained a greater proportion of copper complexing ligands
234 compared with DOC from natural sources, which might be indicative of a more
235 significant 'active' fraction compared to the DOC assessed in other studies, the DOC
236 input value was also treated as an adjustable parameter so that, for each sample and
237 model combination, the 'active' fraction was optimised to provide perfect agreement
238 between measured and forecast toxicity (EC50). The optimised 'active' fraction was

239 determined by linear interpolation using model outputs from a range of 'active' fraction
240 values and verified by model application. An additional assessment of forecast accuracy
241 was subsequently conducted using a best fit 'active' fraction, which was determined for
242 each model as that which minimised the sum of squared forecast residuals. For
243 consistency, and to enable comparison with results from other studies [2, 9, 10, 21],
244 DOC was considered to be fulvic acid only.

245   *Iron.* It was desirable to include iron, as Fe^{3+} in particular, in speciation modelling
246 due to the strong affinity between Fe^{3+} and aminopolycarboxylate chelating agents as
247 well as DOC. Iron speciation is, however, complex. Ordinarily, in the typical pH range
248 (6-9), the concentration of iron existing as Fe^{3+} or as an Fe^{3+}-DOC complex would be
249 expected to be extremely low due to hydrolysis and precipitation as colloidal hydroxides
250 that occurs in this pH range; however, the presence of aminopolycarboxylate chelating
251 agents may alter this significantly. Fe^{3+} was modelled as follows: WHAM VI was able to
252 accept Fe^{3+} free ion activity as a model input (i.e. effectively the concentration
253 remaining as Fe^{3+} free ion after speciation), so that the speciation calculation performed
254 determined the requisite dissolved Fe^{3+} concentration, and the associated speciation
255 (i.e. complexation with DOC and synthetic chelating agents etc.). Consequently, Fe^{3+}
256 ion activity was used as the model input, rather than the measured iron concentration.
257 Fe^{3+} ion activity was determined using the method of Lofts et al. [29]. The method takes
258 account of the effects of both temperature and pH solubility and is given by the equation
259 below:

$$\log \alpha_{Fe^{3+}} = 2.93 - 2.70\text{pH} + \frac{\Delta H^{\circ}}{2.3R}\left(\frac{1}{283} - \frac{1}{T}\right)$$

260 Where T represents temperature, $\Delta H°$ the standard enthalpy change for solubility
261 equilibrium with $Fe(OH)_3$ (-24.37kcal/mol), and *R* the log solubility product of $Fe(OH)_3$
262 (for these calculations a value of 2 was applied as *R*). The default WHAM VI
263 thermodynamic parameters for Fe^{3+} were applied in speciation modelling, except those
264 controlling reactions with aminopolycarboxylates, which were taken from Stumm and
265 Morgan [23]. The difference between measured dissolved iron and calculated dissolved
266 Fe^{3+} (as determined above) was assumed to be iron as either Fe^{2+} or colloidal iron
267 species.

268

269 *Sampling of effluents*

270   Two effluent samples were collected from each of four wastewater treatment
271 works (designated A, B, C and D), at least one week apart (e.g. A1 and A2 represent
272 separate samples from treatment works A), between November 2008 and February
273 2009. All treatment works utilised the activated sludge process and received mostly
274 domestic wastewater inputs. The activated sludge process treatment type produces a
275 relatively high quality effluent that is likely to contain relatively low ammonia and
276 biochemical oxygen demand which might otherwise contribute towards toxicity. Two

277 treatment works (A and B) received inputs from dairies and were expected to contain
278 relatively high concentrations of aminopolycarboxylate synthetic chelating agent, EDTA
279 in particular. Effluents from the other works (C and D) were expected to contain
280 concentrations resulting from domestic inputs only. At two of the wastewater treatment
281 works (A and D), iron dosing ($FeCl_3$) was being undertaken to remove phosphate to
282 meet discharge permits. Effluent samples were collected in acid washed 20L
283 polyethylene vessels. Upon return to the laboratory, samples were filtered through a
284 0.45μm cellulose nitrate membrane (Whatman, UK) and stored in darkness at 4°C. Prior
285 to testing, all solutions were equilibrated to 20°C (±1°C).

286

287 *Toxicity tests*

288 *D. Magna* acute toxicity tests were performed in accordance with OECD
289 guidelines [30] with juvenile daphnids less than 24 hours old at the start of each test
290 (neonates). The test organisms were IRCHA clone type 5 (clone type A in Baird et al.
291 [31, 32]) and originated from the culture maintained by the School of Biological
292 Sciences at the University of Reading, UK. In each assay, five concentrations and a
293 control group were assessed. For each concentration and control, four groups of five
294 neonates were used so that each assay required a total of 120 neonates. Each group
295 comprised of 40ml of filtered undiluted effluent in a 50ml borosilicate glass beaker. A
296 glass cap was placed over each beaker to minimise evaporation and the risk of
297 contamination. The exposure concentrations were arranged in a geometric series with a
298 fixed separation factor of 1.7 so that the test concentrations were between 80μg/L and
299 664μg/L. A broad test concentration range was selected to accommodate the
300 uncertainties associated with toxicity tests conducted in an effluent medium. Spiked
301 solutions were equilibrated for one hour before the addition of the neonates.
302 Immobilisation was recorded at 24 and 48 hours. EC50 concentrations were determined
303 using the Trimmed Spearman-Karber method [33].

304

305 *Water chemistry characteristics*

306 Copper was quantified by graphite furnace atomic absorption spectrophotometry
307 (GFAAS) using a Zeeman 4100ZL GFAAS (Perkin Elmer, Beaconsfield, UK). Zinc, iron,
308 calcium, magnesium, sodium, and potassium were quantified by flame atomic
309 absorption spectrometry using an AAnalyst 100 FAAS (Perkin Elmer, Beaconsfield,
310 UK). The instruments were calibrated using 1,000mg/L spectroscopic standards diluted
311 with deionised water (Millipore, Watford, UK). Chloride was quantified via UV
312 spectrometry (Series 200, Perkin Elmer) using an Aquanal®-plus chloride test kit
313 (Sigma-Aldrich 2009). Sample pH was determined using a SENTEK P11 pH probe
314 (Sentek, Braintree, UK). Sulphate was estimated from charge balance. Sulphate is
315 important only for determining charge balance and ionic strength, and does not directly
316 influence copper toxicity and consequently, an estimate was deemed acceptable [34].

317 DOC and inorganic carbon were quantified using a Model 700 TOC Analyser (OI
318 Corporation, Texas, USA).
319
320 *Synthetic chelating agents*
321   The aminopolycarboxylates *nitrilotriacetic acid* (NTA), EDTA and
322 *diethylenetriamine pentaacetic acid* (DTPA) were analysed by High Performance Liquid
323 Chromatography (HPLC) with UV detection using the method of Laine et al. [35]. HPLC
324 analysis was performed on a series 200 system (Perkin Elmer, Beaconsfield, UK) using
325 a 20µl loop. The analytical protocol required the addition of an excess of Fe^{3+} in order to
326 convert the aminopolycarboxylates into their Fe^{3+} forms, which display high UV
327 absorbance, and which forms the basis for quantification. Consequently, non-Fe^{3+}
328 aminopolycarboxylate was determined as the difference in the concentration detected in
329 Fe^{3+} spiked and non-Fe^{3+} spiked samples.
330
331 **RESULTS AND DISCUSSION**
332 *Water chemistry*
333   **Table 2** provides the water chemistry characteristics of the effluents, their
334 corresponding 48 hour EC50 dissolved copper values, and the concentrations of
335 synthetic chelating agent detected. Of the three synthetic chelating agents tested, only
336 EDTA was detected (41 to 547 µg/l). The EDTA concentrations were greatest for
337 treatment works which received inputs from dairies (A and B), and was found to be
338 predominately in Fe^{3+} form, in particular where treatment included iron dosing (>79%).
339 The concentration of EDTA detected was, in all cases, less than the PNEC of 2.2mg/l
340 determined by the EU Risk Assessment for EDTA [36]. The high concentrations of
341 sodium and chloride in effluents B1 and B2 were as a consequence of large natural salt
342 deposits that were characteristic of the regional geology. The calculated dissolved Fe^{3+}
343 concentrations were much lower than determined analytically, which suggested that the
344 dissolved iron existed predominately as either Fe^{2+} or colloidal species (>98%).
345
346 *Toxicity assays*
347   *Effluent assays*. In the effluent assays no immobilisation occurred in any of the
348 controls, indicating that the effluents were not acutely toxic to *D. magna*. The 48 hour
349 EC50 values for copper ranged between 288 and 401 µg/l, and displayed only a weak
350 linear relationship with each of the water chemistry characteristics presented in Table 3,
351 most notably, and in contrast with other studies [9, 10], with that of DOC ($n = 8$, $r^2 =$
352 0.227, $p = 0.23$). The weak linear relationship with DOC may, however, have arisen as a
353 consequence of the relative similarity in DOC concentration in the effluents (6.95 to 8.97
354 mg/l).
355   *Model Forecasts*. **Figure 1** shows the actual EC50 values in relation to each of
356 the model forecasts. None of the models predicted all EC50 values by within a factor of

357 two; however, the exceptions were not common to all models. The HydroQual model
358 overestimated toxicity for effluents A1 and A2, which were notable for their high EDTA
359 concentrations, whereas the refined and modified BLMs underestimated the toxicity of
360 effluent B2, which was notable for its high concentrations of sodium and chloride.
361 **Table 3** shows the percentage of 'active' DOC required for precise predictions of
362 toxicity for each of the models. The optimisation of DOC for the HydroQual BLM
363 suggested an 'active' range from 41% to 260%, with the highest values occurring for
364 effluents A1 and A2. An 'active' fraction of >100% does not present any conceptual
365 difficulty, since this may be indicative that the sample DOC incorporated a ligand
366 intensity greater than was assumed in the model description of DOC; however, as a
367 consequence of applying DOC as an adjustable parameter it was realistic that the
368 optimised value might also incorporate the effect of other unrecognised influences (e.g.
369 the high 'active' fraction values for effluents A1 and A2 might reflect the influence of
370 EDTA detected within these samples).
371 The optimisation of 'active' DOC for the refined BLM suggested an 'active'
372 fraction range of between -39% and 86%. A negative 'active' fraction value did not
373 present any conceptual difficulty since negative values might have been indicative of
374 some toxic influence that counterbalanced the influence of DOC. Whereas positive
375 optimised 'active' fraction values were estimated initially by linear interpolation, negative
376 values were estimated by extrapolation. The optimisation for the refined BLM indicated
377 that effluents B1 and B2 were likely to have contained some toxic influence that offset
378 the protective influence of the DOC. Similarly, the optimisation for the modified BLMs
379 also indicated that effluents B1 and B2 contained some toxic influence that offset the
380 influence of DOC. The apparent toxicity of effluents B1 and B2 as determined by the
381 optimisation for the refined and modified BLMs, however, deserved closer inspection
382 prior to evaluating the effect of the best fit 'active' fraction on forecast accuracy.
383
384 *The significance of model parameters*
385 Whereas the unusually high 'active' DOC fractions for effluents A1 and A2
386 indicated by the HydroQual BLM optimisation may have been attributable to the
387 influence of EDTA, the toxic nature of effluents B1 and B2 was uncertain. The lack of
388 mortality in the controls for effluents B1 and B2 suggested these may not be
389 significantly more toxic than the others and, indeed, the EC50 value for effluent B1
390 indicated this to be the least toxic of the tested effluents. The high concentrations of
391 sodium and chloride were, however, a notable characteristic of these effluents (sodium:
392 B1=1586mg/L, B2=1,452mg/L; chloride: B1=2,347, B2=2,173mg/L), far exceeding the
393 upper range threshold values suggested for use with the HydroQual BLM (236.9mg/L
394 and 279.7mg/L, respectively) [34] and the highest concentrations that were used in the
395 development of the refined *D. magna* biotic ligand characterisation (347mg/L and
396 553mg/L, respectively) [9, 10]. Consequently, the model parameters governing the

397   influence of sodium and chloride may have been inaccurate for the concentrations
398   detected in these effluents. Whereas chloride is included in speciation calculations only
399   for its contribution to ionic strength and is not expected to directly influence copper
400   toxicity [34], sodium has a significant influence on copper toxicity in that it is assumed to
401   compete with toxic metal species for binding at the biotic ligand.

402   In the development of the initial BLM for *D. magna*, De Schamphelaere et al. [2]
403   commented that the biotic ligand stability constant for sodium was high in comparison
404   with the values for calcium and magnesium considering their relative affinities for other
405   biogenic chelating agents and suggested that the protective influence offered by sodium
406   might be attributable to some other direct physiological effect, rather than competition
407   with Cu^{2+} alone. This is plausible since studies have shown environmental sodium to
408   assist sodium uptake in *D. magna*, and in juvenile *D. magna* in particular [37].
409   Consequently, the biotic ligand stability constant for sodium potentially incorporates the
410   protective influence of competition for binding, alongside the uptake 'assistance'
411   provided by environmental sodium. Following on from this, Bianchini and Woods [37]
412   identified a typical Michaelis-Menton type relationship between environmental sodium
413   and sodium uptake in juvenile *D. magna*, which implies that the sodium uptake
414   'assistance' is saturable. Consequently, where environmental sodium concentrations
415   exceed the organism's uptake ability, only the true competitive binding effect is likely to
416   exist, and for which a smaller biotic ligand binding constant is likely to be representative.
417   Therefore, in effluents B1 and B2, the concentrations of sodium appear to have
418   exceeded the organism's uptake ability so that the BL stability constant for sodium
419   overstated the influence of sodium, causing the BLMs to suggest these effluents to be
420   relatively toxic. Therefore, an additional scenario was considered whereby the model
421   input concentrations for sodium (and chloride) were limited to the upper threshold
422   values specified for the HydroQual BLM. Whereas these values are lower than the
423   maximum sodium concentration applied in the model developed by De Scamphelaere et
424   al. [9, 10], it was preferable to apply the HydroQual upper values in order to ensure that
425   the input values would be appropriate for the purposes of model comparison. More
426   elaborate schemes could have been tried, for example, by assuming a multiple-site
427   binding model for sodium, with differing binding affinities and saturation thresholds.
428   However, in the absence of experimental evidence upon which to base such a
429   characterisation, we considered the simplest approach to be most appropriate, that is,
430   to apply an upper threshold input value.

431   **Figure 2** shows the actual EC50 values in relation to model forecasts, using the
432   identical 'active' DOC fraction as applied in Figure 1, but where the upper threshold
433   concentration values for sodium and chloride had been applied as model inputs for
434   effluents B1 and B2. The DOC optimisation results are for the adjusted sodium and
435   chloride inputs are presented parenthetically in Table 3.

436   For the HydroQual BLM, the adjustment of sodium and chloride inputs did not
437   produce forecasts that were accurate by within a factor of two, in particular, since the
438   forecasts for effluents A1 and A2 were unaffected by the adjustment of sodium and
439   chloride inputs, however, the accuracy of forecasts for effluents B1 and B2 was
440   improved. The standard deviation of the average optimised 'active' fractions also
441   revealed the largest degree of variability (61%) in comparison with other model
442   forecasts, which suggested that the HydroQual model description of DOC complexation
443   was not consistent with the observed complexation. The large degree of variability
444   arose primarily from effluents A1 and A2, which also contained the highest
445   concentrations of EDTA.

446   For the refined BLM, the adjustment of sodium and chloride inputs improved
447   forecast accuracy in that all EC50 values were predicted by within a factor of 1.6. The
448   standard deviation of the average optimised 'active' fractions also revealed the smallest
449   degree of variability (16%), indicating the refined BLM parameterisation to provide the
450   most consistent representation of the complexation attributable to DOC.

451   For the modified BLM which did not include the additional effluent characteristics
452   the adjustment of sodium and chloride inputs improved forecast accuracy for effluents
453   B1 and B2 so that forecasts were accurate by within a factor of two, however, the
454   forecast for effluent A1, which was unaffected by the adjustment of sodium and chloride
455   inputs, was unchanged and accurate only within a factor of 2.2. The standard deviation
456   of the average optimised 'active' fraction revealed a degree of variability (29%) larger
457   than that of the refined BLM, although substantially smaller than that of the HydroQual
458   BLM. For the modified BLM which included the additional effluent characteristics the
459   adjustment of sodium and chloride inputs improved forecast accuracy in that all EC50
460   values were predicted by within a factor of 1.7. The standard deviation of the average
461   optimised 'active' fraction (22%) revealed a degree of variability similar to that of the
462   refined BLM, indicating a relatively consistent representation of complexation
463   attributable to DOC.

464   Where the adjusted concentrations of sodium and chloride were applied as
465   model inputs, the refined BLM provided the most accurate forecasts. The relative
466   accuracy of the forecasts suggested that the refined BLM biotic ligand characterisation
467   produced the most accurate forecasts, and that the Humic Ion Binding Model V, as
468   parameterised by De Schamphelaere et al. [9, 10], offered the most accurate
469   description of the complexation observed in these effluents. In comparison with the
470   results from where actual concentrations of sodium and chloride were applied, these
471   forecasts revealed that the HydroQual BLM parameterisation resulted in the greatest
472   sensitivity to departures from the model description of the influence of water chemistry,
473   but was relatively insensitive to inaccuracies related to the biotic ligand characterisation.
474   The modified BLM was relatively sensitive to departures from both the model
475   description of the influence of water chemistry as well as to inaccuracies relating to the

476 biotic ligand characterisation. However, this sensitivity, alongside the ability to
477 accommodate additional water chemistry inputs, enabled a more deterministic
478 description of the influence of water chemistry characteristics. The refined BLM, which
479 provided the most accurate predictions of copper toxicity, was also sensitive to
480 inaccuracies relating to the biotic ligand characterisation but was relatively insensitive to
481 departures from the model description of the influence of water chemistry.
482

483 *Model input optimisation*
484   **Figure 3** shows the toxicity predictions with adjusted sodium and chloride
485 concentrations using the best fit 'active' DOC fraction given in Table 3. The best fit
486 'active' fraction values were all greater than those recommended or determined in other
487 studies, and were potentially indicative of the more significant complexation capacity of
488 effluent derived organic matter that had been observed by Sarathy and Allen [15].
489   For the HydroQual model the best fit 'active' fraction (134%) resulted in forecasts
490 that were accurate by within a factor of two for all effluents. This suggested that the
491 HydroQual BLM was capable of producing accurate forecasts for these effluents, but
492 that this was specifically dependent on optimising model inputs. For the refined BLM,
493 the best fit 'active' DOC fraction (57%) resulted in forecasts that were accurate by within
494 a factor of 1.5. This indicated that forecast accuracy was not dependant on optimising
495 the 'active' DOC fraction, or the inclusion of additional water chemistry characteristics in
496 speciation modelling. For the modified BLM which did not include the additional effluent
497 characteristics, the best fit 'active' DOC fraction (118%) resulted in forecasts that were
498 accurate by within a factor of 1.4. This indicated that although the modified BLM was
499 capable of predicting EC50 values with greater accuracy than the refined BLM, this was
500 also dependent on optimisation of model inputs. For the modified BLM which included
501 the additional effluent characteristics the best fit 'active' DOC fraction (102%) resulted in
502 the most accurate forecasts that, but still only by within a factor of 1.4. Although
503 inclusion of the selected effluent characteristics improved forecast accuracy, optimising
504 the 'active' DOC fraction, appeared relatively more important to forecast accuracy.
505   Whereas these experiments demonstrated that the modified BLM produced the
506 most accurate forecasts, and that all BLMs were capable of predicting acute copper
507 toxicity in these effluents by within a factor of two, this was generally dependent on
508 knowledge of additional water chemistry characteristics, and optimisation of the DOC
509 input in particular. The refined BLM [9, 10] was found to offer the most robust
510 assessment of acute toxicity, in that it predicted EC50 values by within a factor of two
511 using standard model inputs and input assumptions.
512

513 *Solution equilibration in toxicity assays*
514   An important consideration in the wider interpretation of the assessment results
515 is the equilibration period that was applied in the toxicity assays. The equilibration

516 period that was applied in the present study (1hr), may have been insufficient for the
517 test solutions to equilibrate prior to the addition of the neonates - potentially exposing
518 them to more toxic metal forms in the early stage of the toxicity assays than might
519 otherwise have occurred given a longer equilibration period [38, 39]. Indeed, the
520 relevance of this kinetic consideration was also recognised in the original BLM paper of
521 Santore et al. [6]. However, given the comparative nature of the objectives in this
522 assessment, the equilibration period will not have influenced the relative accuracies
523 reported, although for comparison with other reported studies, the actual EC50s may
524 have been higher (i.e. the effluents less toxic) with a longer equilibration period.
525

526 *The significance of D. magna genotype*
527 The clone type applied in the present study differed from that used in the original
528 determination of *D. magna* biotic ligand characteristics, and the compatibility of the
529 IRCHA clone with the existing *D. magna* biotic ligand characteristics was assessed by
530 applying the refined BLM to copper toxicity data reported in literature for IRCHA clone
531 type 5 (clone A). Toxicity data from two studies [31, 40] (EC50 range 21-41µg/L, mean
532 = 31µg/L) conducted in ASTM hard water [41], and another [42](EC50 = 32 µg/L)
533 conducted in APHA hard water were assessed. Since pH was not reported for the
534 studies in ASTM water, BLM predicted EC50 values were determined within the range
535 pH 7.4 and pH 8.5, with a nominal range of active DOC, between 101 and 175 µg/L (the
536 95% confidence interval range that De Schamphelaere et al. [2] determined to be
537 present in bioassay test media). These inputs produced EC50 forecasts ranging
538 between 30 µg/L and 50 µg/L (mean = 40µg/L). The EC50 forecast for the study
539 conducted in APHA water produced a forecast of 50µg/L, indicating that the biotic ligand
540 characterisation developed for clone type K6 was broadly applicable to the clone type
541 employed in the present study.
542

543 *The significance of EDTA*
544 Some recognition of the potential relevance of the kinetics of EDTA speciation,
545 as well as the implications of considering the total concentration of EDTA (as opposed
546 to Fe^{3+}-EDTA only) in speciation calculations is useful. Whereas a detailed
547 consideration of EDTA reaction kinetics is beyond the scope of this paper, the following
548 features of metal exchange are notable; the overall reaction rate is determined by two
549 independent processes, a *disjunctive* process which determines the rate at which a
550 metal complex dissociates and which is usually inversely proportional to the stability of
551 the complex, and an *adjunctive* process which determines the rate by which a new
552 complex is formed and which is dependent on the water exchange rate of the metal ion
553 (i.e. the rate at which water enters and leaves the coordination sphere of the ion) [43].
554 The high stability of the Fe^{3+}-EDTA complex (non-labile EDTA) suggests that, if initially
555 available in this form, the disjunctive process will be the rate determining step.

Consequently, non-labile EDTA, which for these effluents was a significant proportion of total EDTA, might be considered unlikely to dissociate and complex with the added copper within the time frame of the toxicity assays. Indeed, the slow rate of dissociation of non-labile EDTA has been noted in other studies which demonstrated that the dissociation of this complex may occur over a time scale of many days [44]. Since the present study speciation calculation suggests that, at the optimised 'active' DOC concentration, the majority of EDTA adsorption sites would be occupied by copper, it should be recognised that the effect of EDTA may have been overstated.

Conclusions

The BLMs assessed all predicted acute copper toxicity to *D. magna* in wastewater effluents by within a factor of two. However, with the exception of the refined BLM, this was dependent on the inclusion of additional water chemistry characteristics or through the optimisation of the DOC fraction that was 'active' with regard to metal binding. The optimisation of the DOC input was, however, relatively more important to forecast accuracy than the inclusion of additional water chemistry characteristics in speciation modelling. Whereas the modified BLM offered the most accurate assessment of copper toxicity, the refined BLM offered the most robust assessment, in that it was not reliant on the inclusion of effluent characteristics or optimisation of the 'active' DOC fraction to produce forecasts that were accurate by within a factor of two. This study has also demonstrated that the existing biotic ligand stability constant value for sodium may be an inaccurate approximation of the mechanisms that govern the influence of sodium, and where sodium concentrations exceed the concentration range within which the biotic ligand stability constant value had been determined, inaccurate forecasts may result.

These findings support the use of BLMs for the determination of water quality standards for waters impacted by wastewater effluents, but re-enforces the need for regulators to understand their limitations.

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695

696

Table 1. *D. magna* biotic ligand parameters and inorganic species stability constants

	Hydroqual BLM	Refined BLM	Modified BLM
log K_{CuBL}	7.4	8.02	8.02
log K_{CuOHBL}	6.22	7.32	7.32
log $K_{CuCO3BL}$		7.01	7.01
log K_{CaBL}	3.6	3.47	3.47
log K_{MgBL}	3.6	3.58	3.58
log K_{NaBL}	3.0	3.19	3.19
log K_{HBL}	5.4	5.4	5.4
$f_{CuBL}^{50\%}$ [a]	0.397%	47%	47%
BL binding sites (nmol/g dw)	30	30	n/a[b]
BL critical concentration (nmol Cu/g dw)	0.119	14.1	n/a[b]
log K_{CuOH^+}	6.48	6.48	6.48
log K_{CuOH2}	11.78	11.78	11.78
log K_{CuHCO3^+}	14.62	12.13	12.13
log K_{CuCO3}	6.75	6.77	6.77
log $K_{Cu(CO3)2}$	9.92	10.2	10.2
log K_{CuCL^-}	0.4	0.4	0.4
log K_{CuSO4}	2.36	2.36	2.36

[a] The proportion of biotic ligand binding sites that are required to be occupied by copper to induce a 50% acute toxicity effect.

[b] Only the BLM equation [9, 10] was required. Refer to text.

Table 2. Water chemistry characteristics and results of acute *Daphnia magna* toxicity tests

Effluent	pH	DOC (mg/L)	IC[a] (mg/L)[b]	Concentration of major ions (mg/L)					Effluent characteristics (µg/L)			48-hr EC50 (µg/L)[b]
				Ca^{2+}	Mg^{2+}	Na^+	K^+	Cl^-	Zn^{2+}	Fe^c	$EDTA^d$	
A1	7.60	7.49	44	52	6	87	15	90	43	87 (0.51)	547 (0)	401 (356-452)
A2	7.50	6.95	40	54	6	95	15	95	34	98 (0.48)	393 (0)	309 (272-352)
B1	7.96	8.97	60	69	24	1,586	19	2,347	68	131 (0.47)	234 (120)	483 (402-581)
B2	8.14	8.22	63	71	26	1,452	19	2,173	60	109 (0.50)	164 (81)	301 (254-355)
C1	7.90	7.88	41	54	13	136	20	135	46	26 (0.44)	47 (8)	288 (245-337)
C2	7.91	7.92	38	52	11	118	16	121	87	69 (0.45)	41 (16)	331 (308-357)
D1	7.94	7.91	42	40	16	71	12	96	57	36 (0.47)	67 (18)	347 (307-392)
D2	7.89	8.29	50	44	20	87	16	100	34	53 (0.46)	124 (0)	305 (280-332)

[a] IC = inorganic carbon

[b] numbers between brackets indicate 95% confidence interval.

[c] numbers between brackets indicate WHAM VI calculated dissolved Fe^{3+} concentration

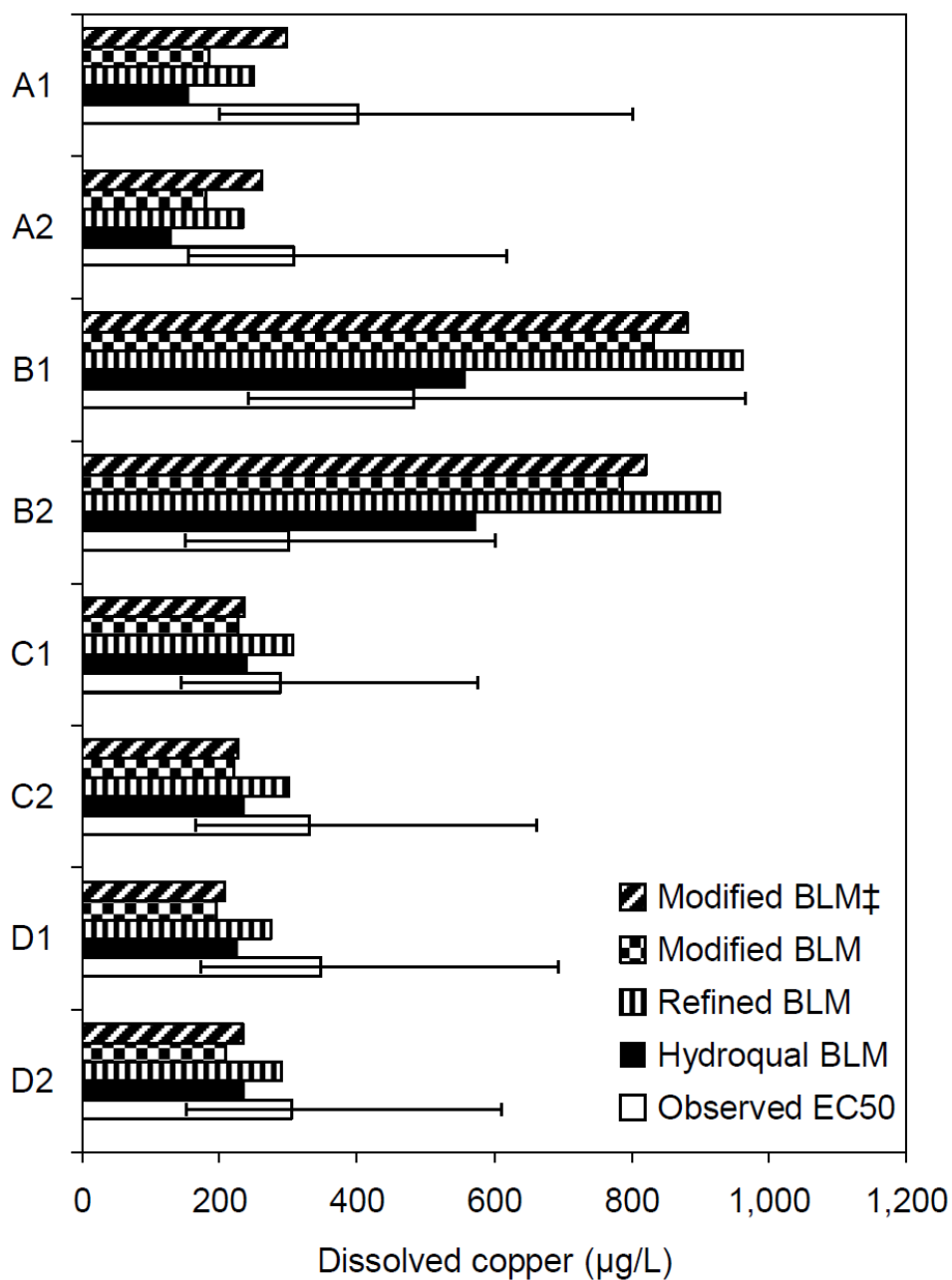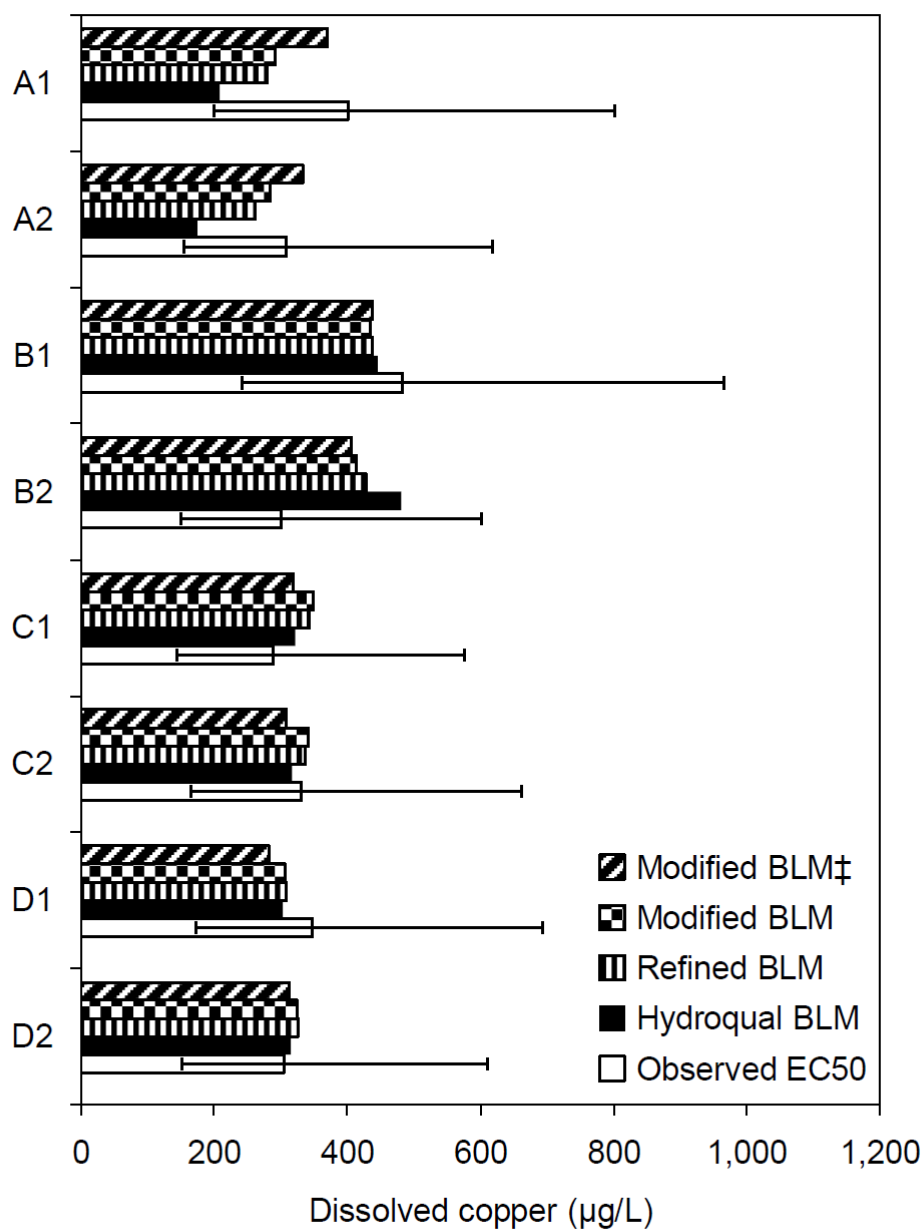[d] numbers between brackets indicate the labile EDTA concentration

709
710 Fig.1. The observed EC50 in relation to model forecasts. Error bars represent the factor

711 of two range so that the upper bar is twice the observed EC50 value, and the lower bar

712 half the observed EC50 value.

713

714
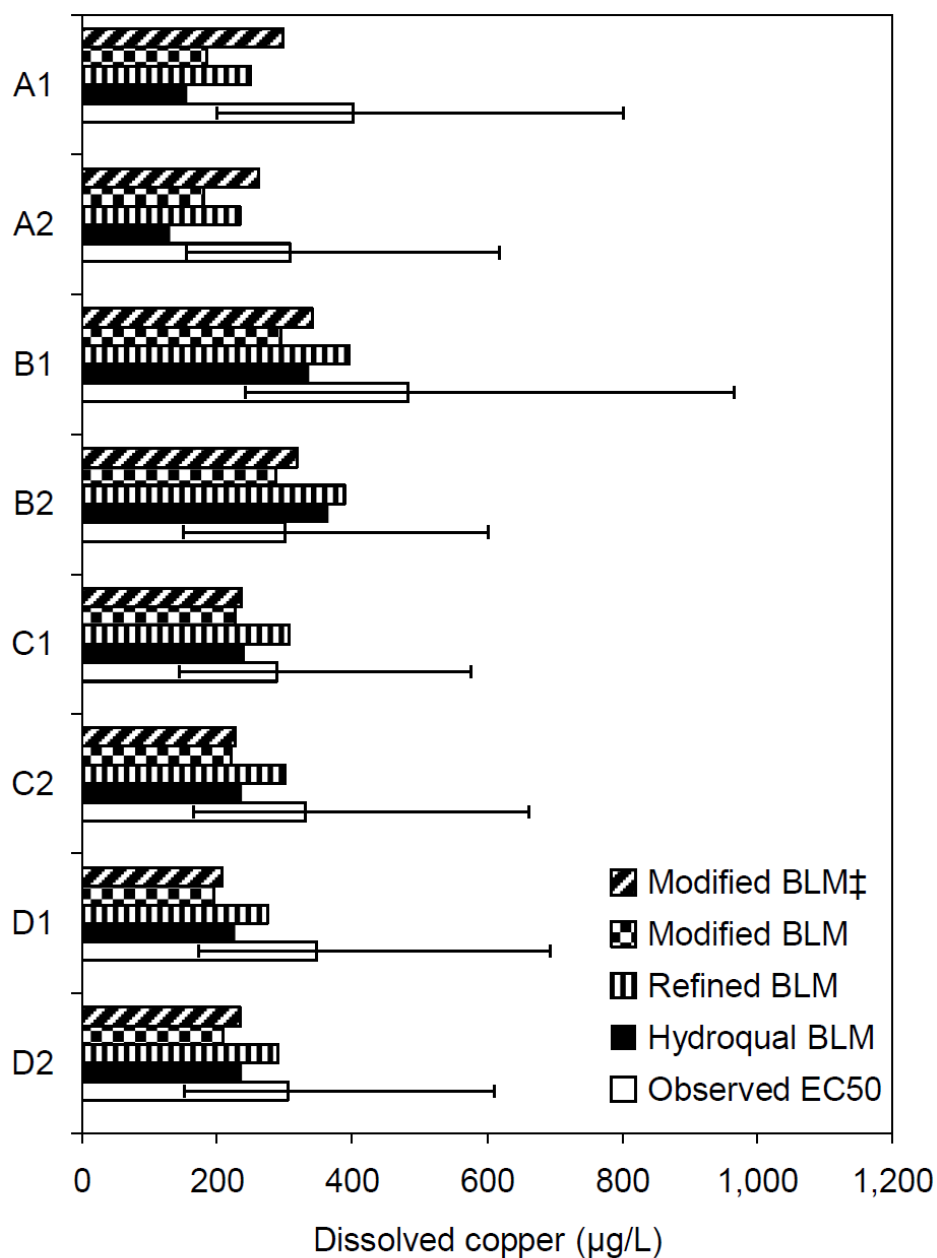
Fig.2. The observed EC50 in relation to model forecasts produced with adjusted sodium
and chloride concentrations and standard 'active' DOC. Error bars represent the factor
of two range so that the upper bar is twice the observed EC50 value, and the lower bar
half the observed EC50 value.

Fig.3. The observed EC50 in relation to model forecasts with adjusted sodium and chloride concentrations and best fit 'active' DOC fraction. Error bars represent the factor of two range so that the upper bar is twice the observed EC50 value, and the lower bar half the observed EC50 value.