Toxicity of binary mixtures of selected metals to larvae of the midge *Chironomus tepperi*

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Department of Environment, Water and Natural Resources, as part of the Coorong, Lower Lakes and Murray Mouth Recovery Program

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This interim report satisfies milestone 3.2 and 3.3 of SA DEWNR project 298/0586 -The research of ecotoxicological effects of acidification on key aquatic organisms in the Lower Lakes. This is an interim report and part of a three year investigation. A final synthesis report will be written which will include all investigations of the Lower Lakes Ecotoxicology project.

Australian Government

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Executive summary

The present report forms part of a series investigating the potential ecosystem-level effects of increased dissolved metal concentrations in aquatic environments affected by the re-wetting of acid sulfate soils after an extended period of desiccation, as occurred in 2010 in wetlands of the lower Murray River and regions of the Lower Lakes. Inundation of oxidised sulfidic material can result in the dissolution of soil-associated minerals, resulting in the mobilisation of multiple metals that are known to be toxic to aquatic biota. Since metals associated with soil minerals rarely occur singly and multiple metal ions can occur at high concentrations in acidified surface waters, it is vital to gain a better understanding of the behaviour and toxicity of multiple dissolved metals.

Chironomids, or non-biting midges, are an important component of aquatic ecosystems and often dominate invertebrate communities in heavily polluted water bodies. In the present study, the toxicity of Al, Cu, Co, Mn, Ni and Zn and their binary combinations to 2nd instar larvae of *Chironomus tepperi* was determined in water-only exposures at pH 7 and pH 5. Comparison of experimentally-derived concentration-response curves with those predicted using an additive model revealed that numerous binary combinations tested in the present study exhibited antagonistic interactions, or less-than-additive toxicity.

The main findings of the study were:

- The toxicity of individual metals to midge larvae at pH 5 was less than that observed at pH 7
- At neutral pH, most binary combinations tested exhibited significantly less-than-additive or antagonistic toxicity
- At pH 5, with the exception of Al-containing mixtures, most combinations were additive
- All Al-containing combinations at pH 5 exhibited significantly less-than-additive toxicity
- The binary combinations that displayed greater-than-additive or syngergistic toxicity were:
  - at the median effect level (50% immobilisation):
    - Co + Ni at pH 7
    - Co + Zn at pH 5
  - at 10% immobilisation:
    - Co + Zn at pH 5
    - Zn + Ni at pH 5

The present study provides a large amount of mixture toxicity data that will inform modelling approaches for aquatic invertebrates and contribute to the development of improved management practices for acid-sulfate impacted water bodies. These findings will help to predict the possible risks to freshwater invertebrates of mixtures of metals in the aquatic environment, particularly those metals that are known to be mobilised from soils and sediments in the region after oxidation and re-wetting of acid-sulfate material.

1 Background

1.1 Chironomid midges as ecotoxicology test organisms and indicators of metal contamination

Midge larvae of the family Chironomidae (order Diptera) are relatively tolerant to waterborne metals in comparison with other aquatic organisms. A *Chironomus* species common in the norther hemisphere, *C. riparius*, was ranked the least sensitive of 55 species in a comprehensive literature review of cadmium toxicity in a wide range of aquatic biota (US_EPA, 2001). Consequently, in streams heavily contaminated by metals, midge larvae have been reported to dominate invertebrate communities, comprising more than 75% of all aquatic insects collected from impacted sites (Winner *et al.*, 1980; Canfield *et al.*, 1994; Marques *et al.*, 1999; De Bisthoven *et al.*, 2005; Carew *et al.*, 2007). This observation has seen the increasing use of midge larvae as bioindicators of metal contamination in surface freshwaters. There is evidence to suggest that chironomids can adapt to high levels of metal contamination (Wentsel *et al.*, 1978) and that this process of phenotypic adaptation involves metallothionein-like proteins capable of binding metals taken up into the organism (Yamamura *et al.*, 1983). Because metallothioneins are selective, sensitivity to different metals can vary. Differences in the status of these adaptive processes likely contribute to observed differences in the sensitivity of chironomids to heavy metals in experimental exposure scenarios, with some studies reporting high sensitivity in comparison with other invertebrates (Clements *et al.*, 1988; Milani *et al.*, 2003). Since ecosystem recovery following pulse events such as acid-sulfate soil inundation is dependent at least in part on the local survival of organisms at multiple trophic levels, it is important to better understand the threat posed by the presence of multiple dissolved metals to endemic invertebrates such as midge larvae.

Chironomus tepperi (Skuse) is widely distributed in Australia, reproduces readily in the laboratory (Martin & Porter, 1978; Stevens, 1993b), and has been used extensively in aquatic toxicology studies relevant to environmental issues in the Australasian region (Stevens & Warren, 1992; Stevens, 1993a; 1998; Wilson *et al.*, 2000; Stevens *et al.*, 2004; Phyu *et al.*, 2005; Deng *et al.*, 2014; Hale *et al.*, 2014; Jeppe *et al.*, 2014; Kellar *et al.*, 2014). Most studies on metal toxicity in *C. tepperi* have focused on spiked sediments (Deng *et al.*, 2014; Hale *et al.*, 2014; Kellar *et al.*, 2014), and the few reports published on waterborne exposures in this species have investigated metals in isolation (Colombo *et al.*, 2014; Jeppe *et al.*, 2014; Long *et al.*, 2015).

The acidification of inland surface waters following inundation of acid sulfate soils (ASS) can result in the dissolution of metals from soil minerals into the water column (Campbell & Stokes, 1985; Förstner, 1995; Chapman *et al.*, 1998; Burton *et al.*, 2008; Baldwin & Fraser, 2009). The ecotoxicological impacts of ASS re-wetting in the aquatic environment have not been well characterised, and a greater understanding is needed of the potential effects of increased metal ion concentrations combined with low pH. The bioavailability of metals – which determines their toxicity to aquatic organisms - varies considerably with pH and other physicochemical parameters due to factors such as changes in chemical speciation, aqueous solubility, and complexation with dissolved ortant to arganic matter (DOM). Since metals in soil minerals typically occur in combination, it is impossess whether toxicity is affected by possible interactions between metal ions in solution.

Based on monitoring of selected sites in the Lower Lakes conducted by SA DEWNR and CSIRO, the following six metals were selected for mixture toxicity assessments under laboratory conditions.

- Aluminium (Al)
- Cobalt (Co)
- Zinc (Zn)
- Copper (Cu)
- Nickel (Ni)
- Manganese (Mn)

To assess metal interactions with respect to acute toxicity in midge larvae, 2nd instar midge larvae were exposed to waterborne binary combinations of the above metals. The resulting concentration-response relationships were compared with those predicted using modelling approaches based on the toxicity of individual metals. In comparison to later larval stages, 2nd instar chironomid larvae are considerably more sensitive to metal toxicity (Nebeker *et al.*, 1984; Williams *et al.*, 1986; Ebau *et al.*, 2012) and hence may provide a more indicative measure of critical concentration ranges of dissolved metals in the aquatic environment.

1.2 Predictive modelling of mixture toxicity

In the present study, we used two conceptual models to predict the toxicity of binary combinations of metals, namely concentration addition (CA) and independent action (IA). CA (Equation 1), where ECx is a given EC value and p_i is the molar proportion of the *i*th component), was introduced by Loewe and Muischnek (1926) and has been used extensively to predict the effects of mixtures of similarly-acting substituents. CA assumes that components of the mixture act in the same way and as such the molar concentrations can therefore be simply added together to predict the resulting toxicity (Hewlett, 1969; Faust *et al.*, 1993; Altenburger *et al.*, 2004). CA also forms the basis of the Toxic Unit concept that describes the individual contributions of a mixture to the overall toxicity (Spehar & Fiandt, 1986).

IA (Equation 2), where E is a given effect, e.g. 0.5 for 50% survival, and *c* is concentration), was first described in the scientific literature by Bliss (1939) and is considered suitable for predicting the combined toxicity of agents with dissimilar modes of action. In contrast to CA, IA describes the probability that a given effect will occur based on the likelihood that each component will cause that effect, and is sometimes referred as ‘effect multiplication’.

$$ECx_{mixture} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1} \tag{1}$$

$$E(c_{mixture}) = 1 - \prod_{i=1}^{n}(1 - E(c_i)) \tag{2}$$

A straightforward approach for comparing predicted mixture toxicity with experimental data from toxicity tests is the model deviation ratio (MDR) (Belden *et al.*, 2007), which is the ratio of a predicted EC value to the corresponding EC derived from a mixture toxicity test (Equation 3).

$$MDR = \frac{predicted\ ECx}{observed\ ECx} \tag{3}$$

In the case of CA, and when the goal is to classify the interaction of the components of a mixture as additive, synergistic or antagonistic, the magnitude of deviation from the MDR must be considered. Additive toxicity is generally assumed if the experimental data falls within 30% of an MDR of 1 with respect to CA, with values 30% higher or lower than the model (above 1.3 or below 0.7) considered to represent synergistic or antagonistic mixture interactions, respectively (Belden *et al.*, 2007; Phyu *et al.*, 2011; Arora & Kumar, 2015), provided that testing for statistical differences is also conducted. Concordance of experimental data at a given EC with predicted ED values derived from the IA model, on the other hand, could indicate independent action, although this kind of assumption is not generally considered as hard evidence of differential modes of action of the mixture components.

1.3 Aims and scope

This report is one part of a larger study investigating the toxicity of metal mixtures to snails and midge larvae under varying conditions of pH and salinity. In the present study, the aims were to:

1. Determine the survival of the chironomid midge *Chironomus tepperi* in the acidic pH range
2. Determine the toxicity of six metals to the freshwater snail *C. tepperi* in 48 h acute toxicity tests conducted at pH 7 and pH 5
3. Based on individual metal toxicity, use modelling approaches to predict the toxicity of binary mixtures of metals at pH 7 and pH 5
4. Determine the toxicity of binary combinations of metals and ascertain whether mixtures were additive, synergistic or antagonistic by comparison with the toxicity predicted using an additive model

2 Methods

2.1 Midge 48 h immobilisation bioassay – waterborne metal exposures

To determine concentration-response relationships for acute toxicity of individual metals (Cu, Co, Mn, Zn, Ni and Al), 48 hour exposures were carried out using 2nd instar *C. tepperi* larvae in laboratory-prepared water at pH 7 and pH 5. Experimental conditions for the exposures are shown in Table 1. In order to maintain pH at 5, 2-(*N*-morpholino) ethanesulfonic acid (MES) was used as a buffering agent.

Range-finding tests were carried out prior to performing definitive exposures over a narrow concentration range comprising eight two-fold dilutions per test. At the conclusion of the tests, the survival of the test organisms was determined and pH, electrical conductivity, and dissolved oxygen (DO) of the water measured. The concentration of metal ions was analysed at 0 h and 48 h time points to determine whether concentrations had deviated significantly from nominal values.

Table 1. Summary of test conditions for the 2nd instar midge larva, *Chironomus tepperi* immobilisation bioassay

TEST PARAMETER	TEST CONDITION
Test type	Static, non-renewal
Test duration	48 h
Temperature	21 ± 1°C
Light quality	cool-white fluorescent tube lighting
Light intensity	800 ± 160 Lux
Photoperiod	16 h light : 8 h dark
Test chamber size	50 mL vial
Test solution volume	25 mL
Age of test organisms	2nd instar larvae
No. of organisms per replicate	5
No. of replicates per treatment	4
No. of organisms per treatment	20
Feeding regime	None
Dilution water	MHW
Test concentrations	8
Control treatments	Moderately hard water (MHW)
Endpoint	Immobilisation observed after 48h
Test acceptability criterion	≥ 90% survival in controls.

2.2 Binary combinations experimental design

All 15 possible binary combinations of the selected metals (Table 2) were tested in 48 h acute *C. tepperi* toxicity bioassays. The concentration ranges applied in mixture toxicity tests were based on EC50 values derived from tests conducted with individual metals. An approach which facilitated the use of predictive mixture toxicity models was employed. That was, for the binary mixtures, metals were combined in equitoxic concentrations with respect to the EC50 value, and a dilution series centred on the EC50 was established using a 50% dilution increment in a fixed-ratio mixture design (the ratio of concentrations of the two components of the mixtures remained constant throughout the dilution series). Mixture toxicity tests were conducted at pH 7 and pH 5.

Table 2. Binary mixture combinations

	Al	Co	Cu	Zn	Ni	Mn
Al		Al + Co	Al + Cu	Al + Zn	Al + Ni	Al + Mn
Co			Co + Cu	Co + Zn	Co + Ni	Co + Mn
Cu				Cu + Zn	Cu + Ni	Cu + Mn
Zn					Zn + Ni	Zn + Mn
Ni						Ni + Mn

2.3 Mixture toxicity modelling and comparison to experimental data

In the present study, mixture toxicity modelling using CA and IA models was undertaken with the aid of Prism 6 (GraphPad, La Jolla, CA, USA) and Microsoft Excel. Logistic concentration-response models were fitted to toxicity data comprising 4 replicate treatments of 5 midge larvae each using least-squares non-linear regression. The resulting concentration-response curves were used to derive EC values representing 10%, 25%, 50%, 75% and 90% mortality, which were implemented in CA modelling (equation 1) to predict the molar EC values of each binary mixture for the same mortality rates. The 95% confidence intervals (CI) for the EC values derived from the CA model were calculated by substituting the lower and upper 95% CI for each individual EC. IA modelling was conducted using the EC50 and slope of concentration-response curves from individual metals - multiplying the predicted effects of each component of the binary mixtures according to their proportion in the total mixture (equation 2). The EC values for binary combinations predicted using the IA model were derived by fitting a logistic model to the predicted effect for each point in the dilution series and interpolating EC10, EC25 and EC50 values from the resulting curve. The 95% CIs for EC values derived from the IA model were determined by substituting the the 95% CI of the EC50 for individual metals in each binary combination.

The MDRs were determined for EC50, EC25 and EC10 values at pH 7 and pH 5 using Equation 3.

2.4 Statistical significance testing of model deviation ratios

Since the experimental EC values for metal mixtures were derived by fitting a logistic concentration-response model to survival data, each EC value had an associated error that must be taken into account when determining significance of MDRs. In the present study, we deemed that MDRs could only considered significant in cases where the 95% CI of the EC predicted by mixture model and the 95% CI of the EC derived from experimental data did not overlap. This is effectively the same as comparing two means using a two-tailed t-test at a significance level of 0.05.

3 Results and Discussion

3.1 Toxicity of individual metals in 48 h midge immobilisation bioassays

To determine EC50 values, logistic concentration-response curves were fitted to metal toxicity data from acute toxicity tests conducted at pH 7 and pH 5 (Figure 1 and

). Concentrations used for non-linear regression were the nominal dissolved ionic concentrations. For the midge toxicity tests, aluminium was poorly soluble at pH 7, with the formation of visible precipitates indicating that the soluble concentration was likely to deviate markedly from the nominal concentration. Accordingly, Al toxicity data for individual exposures and binary mixtures at pH 7 were not considered for the present report.

For copper, 48 h acute EC50 values for 2nd instar *C. tepperi* determined at neutral pH in the present study were somewhat lower than some published EC50 values for later larval stages. For example, the acute EC50 for Cu exposure to 3rd instar *C. tepperi* via water for 96 h was 4.51 mg/L (Jeppe *et al.*, 2014), while the EC50 for Cu in the present study was 0.09 mg/L. On the other hand, the Cu EC50 for first instar *C. riparius* exposed in a water-only regime for 96 h was 0.043 mg/L (Milani *et al.*, 2003), which is lower than our study - perhaps due to the earlier life stage used and longer exposure time. The acute Zn EC50 for 3rd instar *C. tepperi* was 186 mg/L (Long *et al.*, 2015), which is close to the value presented here, 187 mg/L.

The rank order of toxicity of the metals in midge acute toxicity bioassays at pH 7 was Cu >> Mn > Co > Ni > Zn, and at pH 5 was Cu >> Al > Mn > Ni > Co > Zn. As expected, Cu toxicity was approximately three orders of magnitude greater than the other metals tested. Interestingly, the toxicity of individual metals at pH 5 was considerably lower than that observed at pH 7 – on average, acute toxicity to midge larvae was diminished by approximately four-fold at pH 5 compared to pH 7 (as the mean ratio of EC50 values was 4.2 ± 1). It should be noted that in the snail (*P. acuta*) toxicity experiments conducted as part of the present study, acute toxicity of the selected metals at pH 5 was similar or only marginally less than that observed at pH 7. The reasons for large pH-dependent differences in EC50s in the midge bioassay are currently unclear - and are somewhat surprising given that lower pH generally increases the proportion of total dissolved metal ions or hydroxides (Elder, 1988).

Since low pH conditions can represent an additional stress to the organism, it was important to establish the pH range at which midge larvae survival was not severely affected in the absence of other stressors. Survival of midge larvae was found to be at least 75% between pH 4-7 (Figure 2), with no difference in survival (90%) under the conditions used for metal toxicity testing (pH 5 and pH 7). This was a greater range than that tolerated by snails in the present study, which exhibited very low survival below pH 5.

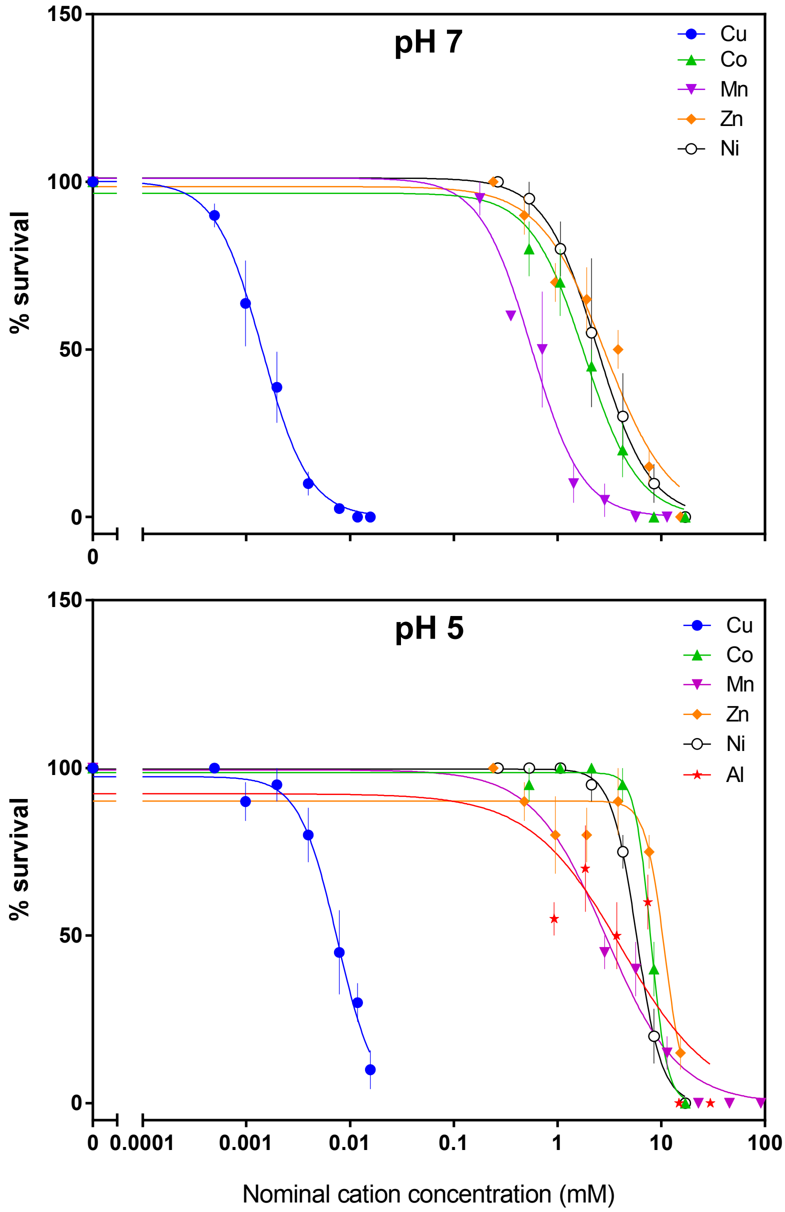


Figure 1. Concentration-response relationships for individual metals in 48 h acute toxicity tests conducted using the chironomid midge, *C. tepperi*. Data points represent means (±SEM) from four replicate treatments per concentration. Note that aluminium toxicity could not be accurately determined at pH 7 due to poor solubility.

Table 3. Median effective concentrations (EC50) for individual metals in 48 h acute *Chironomus tepperi* toxicity tests conducted at pH 7 (top) and pH 5 (bottom). NA indicates not applicable owing to poor solubility

		Cu	Al	Co	Mn	Zn	Ni
pH 7	EC50 (mg/L)	0.0901	NA	108	30.7	187	141
	EC50 (mM)	0.00142	NA	1.83	0.559	2.86	2.41
	95% CI (mM)	0.00113 to 0.00179	NA	1.38 to 2.41	0.415 to 0.752	2.14 to 3.8	1.67 to 3.47
	Hill slope	-1.92		-1.67	-1.77	-1.38	-1.71
	R^2	0.927	NA	0.9146	0.8954	0.9046	0.8328
pH 5	EC50 (mg/L)	0.479	112	467	161	706	339
	EC50 (mM)	0.00754	4.15	7.92	2.93	10.8	5.78
	95% CI (mM)	0.00627 to 0.00908	1.928 to 8.94	7.32 to 8.57	2.14 to 4.01	8.93 to 13.1	5.30 to 6.30
	Hill slope	-2.35	-0.99	-5.61	-1.24	-4.63	-3.59
	R^2	0.8818	0.6857	0.9624	0.9363	0.7574	0.9698

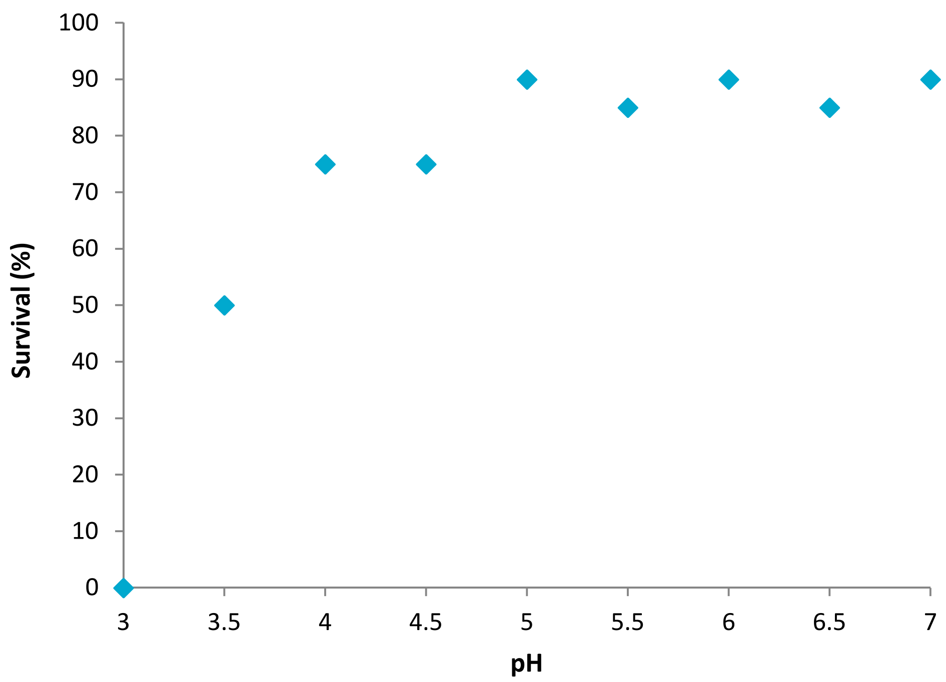


Figure 2. Average survival (from 4 replicate exposures) of midge larvae observed at pH 3-7

3.2 Mixture toxicity modelling and comparison with experimental data

For the present study, the aims were to investigate whether the toxicity of combinations of metals to midge larvae exposed in water-only test conditions exceeded that predicted from additive toxicity of individual metals (i.e. synergistic mixture toxicity) or was less than predicted (i.e. antagonistic interactions) and to determine the effects of pH on the toxicity of metal mixtures. To this end, we undertook a laboratory-based approach, using controlled water chemistry, to determine the short-term acute toxicity of selected metals and their binary combinations. This approach assumes both negligible change in metal speciation over the pH range tested, and minimal complexation due to low dissolved organic matter (DOM) levels. The predicted toxicity of binary combinations was determined using two mixture toxicity models and compared to experimentally-derived concentration-response relationships for the mixtures. The statistical significance and magnitude of deviation from the two models was determined. After experimentally-derived concentration response relationships for metal mixtures were compared with those modelled from individual exposure data using an additive toxicity model (*concentration addition*, CA), conclusions were made regarding whether the observed toxicity of the mixtures differed from the additive model. The accuracy of the chosen models at EC values of interest (EC10, EC25 and EC50) was represented by the MDR, with values 30% higher or lower than the model (above 1.3 or below 0.7) considered to represent synergistic or antagonistic mixture interactions, respectively (Belden *et al.*, 2007; Phyu *et al.*, 2011; Arora & Kumar, 2015).

Concentration-response curves predicted by CA and IA models are shown in **Error! Reference source not found.** - superimposed with curves derived from experimental data for 10 binary combinations at pH 7 and 15 combinations at pH 5. From visual inspection of the concentration-response curves, the toxicity of most combinations was overestimated by both CA and IA. In other words, using either model to predict mixture toxicity based on the toxicity of metals applied individually resulted in a conservative estimate of toxicity of their mixture, with experimental data indicating that toxicity of metal mixtures to 2nd instar midge larvae was less than predicted by either CA or IA. Notable exceptions were the combinations of Co + Zn at pH 5 and Co + Ni at pH 7, which were more toxic than predicted by either model.

The combination of Cu + Mn at pH 5 did not elicit the expected concentration-response profile, with all treatments showing at least 70% survival. Consequently, non-linear regression for this combination was ambiguous, and the confidence intrervals of the resulting EC values were wide. MDRs for this combination were thus deemed non-significant. It is recommended that this experiment be repeated to confirm the result, since the same combination in the snail bioassay resulted a typical concentration-response and did not deviate from the additive model.

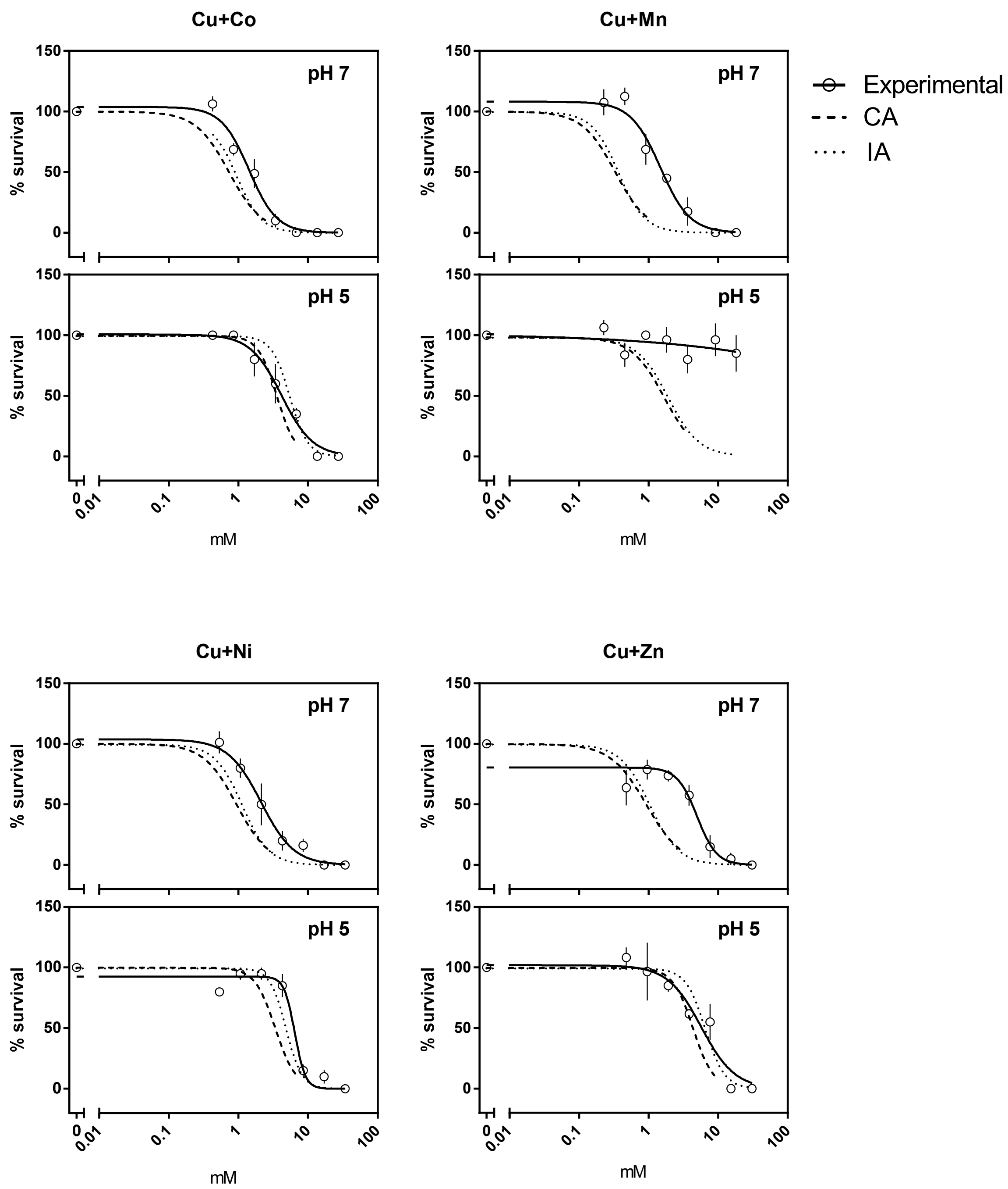
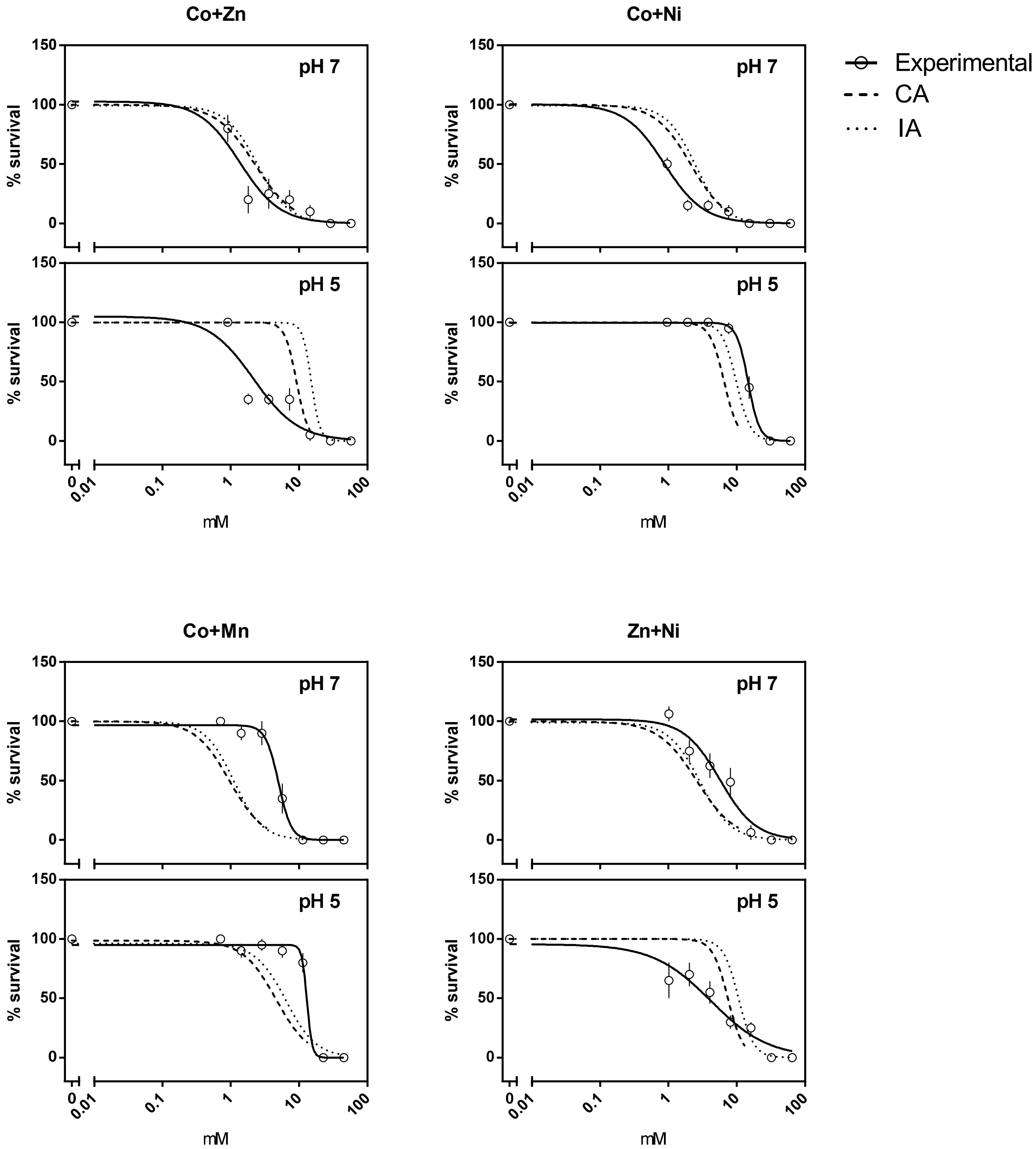


Figure 3. Comparison of concentration addition (CA, dashed line) and independent action (IA, dotted line) predictive models with experimental data (circles and solid line) for all 15 possible binary combinations of Al, Cu, Co, Mn, Ni, and Zn at pH 7 and 5. (cont'd over next 2 pages)

Data points represent means (±SEM) from four replicate treatments per concentration. Note that aluminium toxicity could not be accurately determined at pH 7 due to poor solubility.



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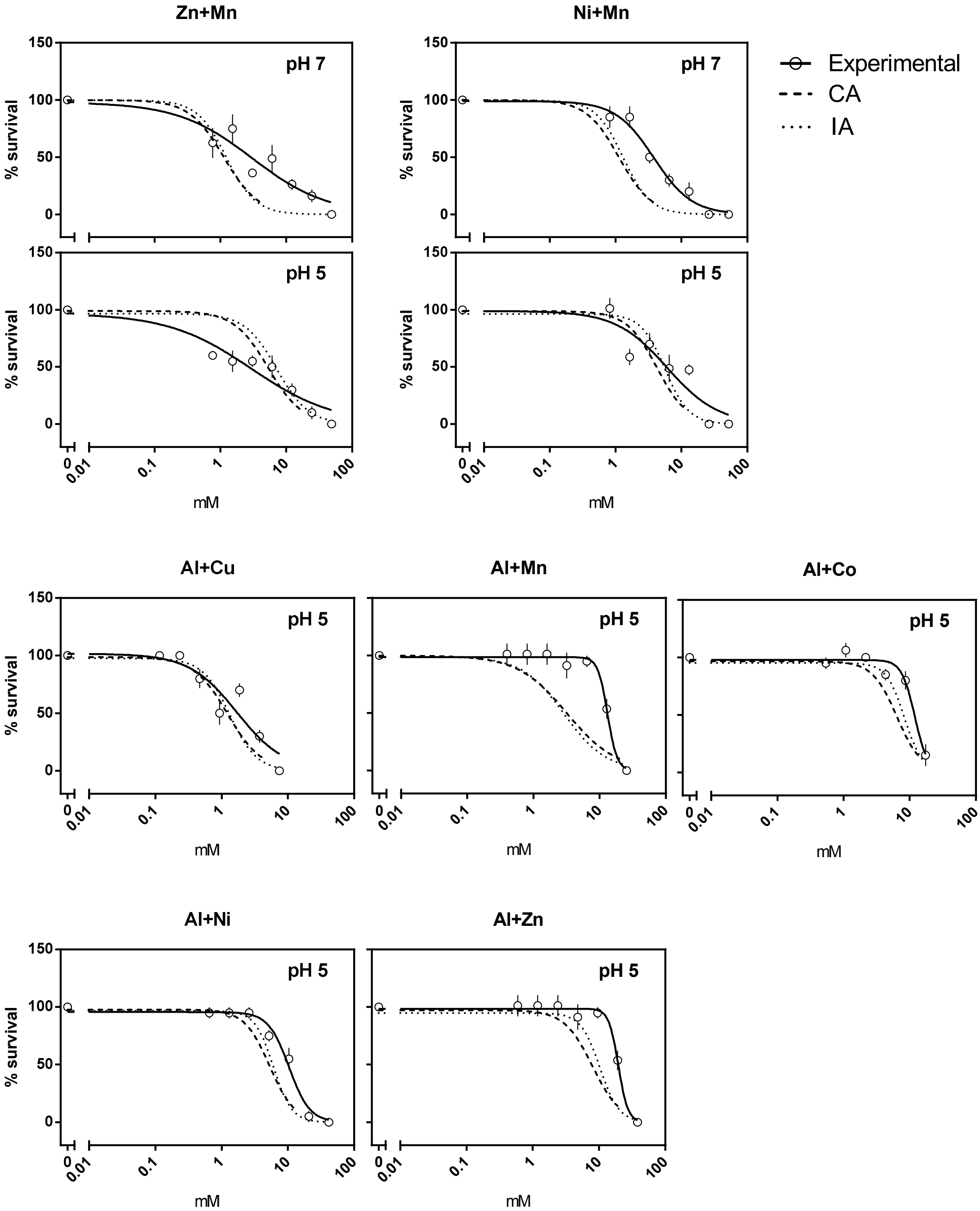
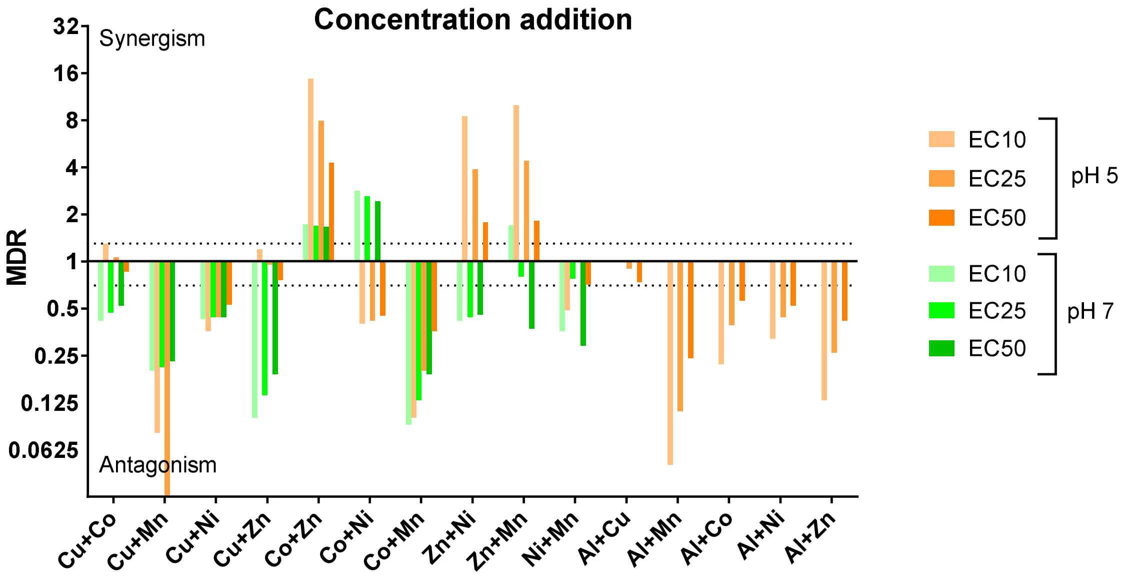

Zn+Mn
Ni+Mn
Experimental
CA
IA
pH 7
pH 5
% survival
150
100
50
0
0
0.01
0.1
1
10
100
mM
Al+Cu
Al+Mn
Al+Co
Al+Ni
Al+Zn

3.3 Significant deviations from the concentration addition model

Model deviation ratios are depicted graphically in Figure 4 and presented alongside EC values and 95% confidence intervals in Table 4 to Table 9. In general, most of the MDRs that deviated significantly from CA had values of less than 0.7, indicating an antagonistic or less-than-additive interaction. Considering MDRs at the EC50 level at pH 7, seven of ten combinations were significantly antagonistic, two were additive and one displayed a greater-than-additive or synergistic interaction (Co + Ni). At pH 5, 6 of 15 combinations exhibited significantly less-than-additive toxicity, 8 combinations were additive, and 1 combination was greater-than-additive (Co + Zn). At pH 7, all combinations were either additive or less-than-additive at both EC25 and EC10, while at pH 5, Co + Zn and Zn + Ni exhibited greater-than-additive toxicity at both EC25 and EC10.

Since metal ions in solution are considered to competitively interact with biological target sites in aquatic organisms such as fish gill surfaces (Di Toro *et al.*, 2001; Paquin *et al.*, 2002; Playle, 2004), CA could be expected to be a more appropriate model than IA for predicting the toxicity of dissolved metal ions. As was observed in the snail acute toxicity tests, this was also borne out in the midge bioassay results, with 10 of 10 combinations at pH 7 and 9 of 15 combinations at pH 5 deviating significantly from IA at the EC50 level.

A number of combinations exhibited strongly antagonistic interactions. In terms of EC50 at pH 7, MDRs for Cu + Mn, Cu + Zn and Co + Mn were less than 0.25, equivalent to four-fold less toxic than the values predicted by CA. At pH 5, interactions of all Al-containing combinations were significantly less than additive, with Al + Mn exhibiting an MDR of 0.24 and other combinations 0.42-0.56. These values are in contrast with the results of the snail bioassay, which showed a synergistic interaction of Al-containing binary metal mixtures at pH 5 and a synergistic interaction of Cu + Mn.



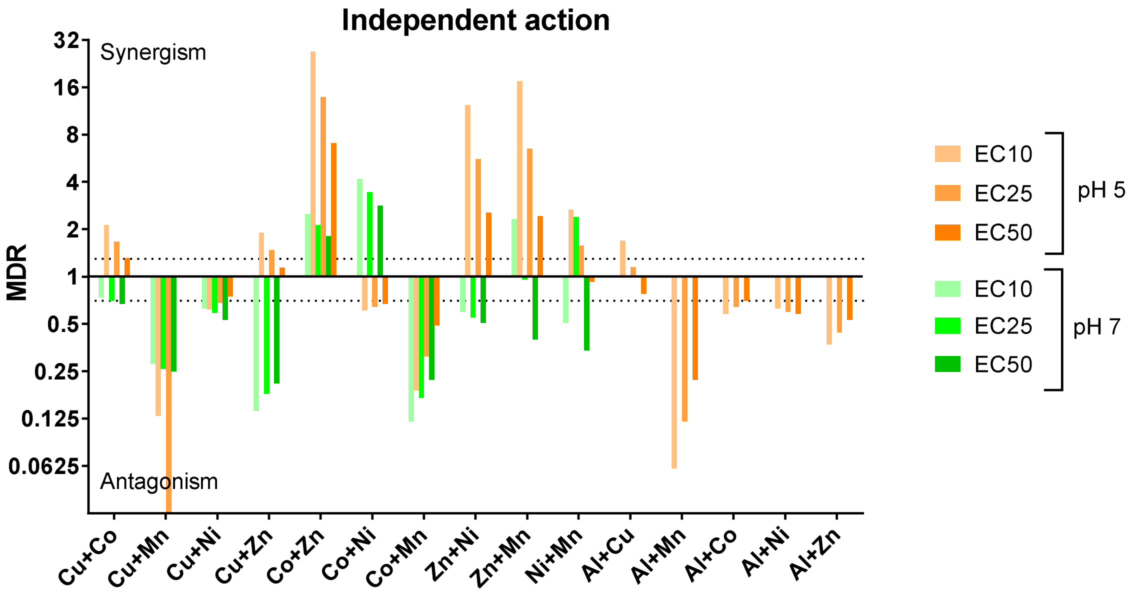


Figure 4. Model deviation ratios (MDR) for concentration addition (CA) or independent action (IA) models. MDRs for each binary combination are shown for 10%, 25% and 50% effect levels at pH 7 and pH 5 for CA (top) and IA (bottom) models. Dotted lines denote the range within which mixture toxicity can be considered as additive (0.7 to 1.3) regardless of statistical significance testing.

Table 4. Determination of model deviation ratios (MDR) at 50% effective concentration (EC50) for *Chironomus tepperi* 48 h acute toxicity experiments at pH 7. Green shading of the metal combination indicates less than additive toxicity and red shading indicates greater than additive toxicity (P < 0.05).

	Experimental data			Concentration addition model					Independent action model				
Mixture	EC50 (mM)	lower 95% CI	upper 95% CI	EC50 (mM)	lower 95% CI	upper 95% CI	MDR	P<0.05 CA?	EC50 (mM)	lower 95% CI	upper 95% CI	MDR	P<0.05 IA?
Cu+Co	0.733	0.571	0.941	1.42	1.15	1.74	0.52	Y	0.948	0.905	0.994	0.67	Y
Cu+Mn	0.333	0.254	0.436	1.47	1.13	1.92	0.23	Y	0.369	0.351	0.388	0.25	Y
Cu+Ni	0.939	0.705	1.24	2.12	1.55	2.89	0.44	Y	1.12	1.07	1.18	0.53	Y
Cu+Zn	0.931	0.725	1.19	4.98	3.74	6.62	0.19	Y	1.04	0.987	1.09	0.21	Y
Co+Zn	2.26	1.70	2.99	1.36	0.874	2.12	1.66	N	2.46	2.31	2.63	1.81	Y
Co+Ni	2.11	1.53	2.90	0.870	0.671	1.13	2.42	Y	2.46	2.32	2.61	2.83	Y
Co+Mn	0.956	0.715	1.28	5.00	4.37	5.72	0.19	Y	1.11	1.06	1.16	0.22	Y
Zn+Ni	2.60	1.87	3.62	5.66	4.05	7.92	0.46	Y	2.86	2.68	3.06	0.51	Y
Zn+Mn	1.13	0.840	1.51	3.05	1.46	6.35	0.37	N	1.23	1.18	1.27	0.40	Y
Ni+Mn	1.12	0.814	1.54	3.81	2.81	5.16	0.29	Y	1.30	1.24	1.36	0.34	Y

Table 5. Determination of model deviation ratios (MDR) at 50% effective concentration (EC50) for *Chironomus tepperi* 48 h acute toxicity experiments at pH 5. Green shading of the metal combination indicates less than additive toxicity and red shading indicates greater than additive toxicity (P < 0.05).

	Experimental data			Concentration addition model					Independent action model				
Mixture	EC50 (mM)	lower 95% CI	upper 95% CI	EC50 (mM)	lower 95% CI	upper 95% CI	MDR	P<0.05 CA?	EC50 (mM)	lower 95% CI	upper 95% CI	MDR	P<0.05 IA?
Cu+Co	3.58	3.11	4.10	4.165	3.161	5.487	0.86	N	5.45	5.12	5.81	1.31	N
Cu+Mn	1.76	1.35	2.28	6011	3.21E-05	1.13E+12	0.00	N	1.90	1.74	2.08	0.00	N
Cu+Ni	3.39	2.98	3.84	6.443	5.503	7.543	0.53	Y	4.82	4.69	4.96	0.75	Y
Cu+Zn	4.37	3.63	5.27	5.722	3.827	8.554	0.76	N	6.52	6.04	7.03	1.14	N
Co+Zn	9.22	8.09	10.5	2.151	1.444	3.206	4.29	Y	15.2	15.0	15.4	7.06	Y
Co+Ni	6.56	6.04	7.14	14.7	13.81	15.65	0.45	Y	9.89	9.75	10.0	0.67	Y
Co+Mn	4.70	3.71	5.88	13.12	5.087	33.82	0.36	N	6.44	5.20	7.97	0.49	N
Zn+Ni	7.41	6.56	8.34	4.168	2.446	7.101	1.78	N	10.6	10.5	10.8	2.55	Y
Zn+Mn	5.39	4.09	7.09	2.948	1.562	5.565	1.83	N	7.16	5.74	8.92	2.43	Y
Ni+Mn	4.32	3.50	5.25	6.048	3.464	10.56	0.71	N	5.64	4.87	6.53	0.93	N
Al+Cu	1.25	0.839	1.73	1.698	1.124	2.565	0.74	N	1.32	1.22	1.43	0.78	N
Al+Mn	3.20	2.07	4.77	13.18	11.8	14.72	0.24	Y	2.89	2.69	3.11	0.22	Y
Al+Co	6.62	4.57	8.64	11.89	10.22	13.83	0.56	Y	8.32	6.89	10.0	0.70	Y
Al+Ni	5.40	4.04	6.65	10.4	8.905	12.14	0.52	Y	6.02	5.56	6.51	0.58	Y
Al+Zn	8.23	5.22	12.0	19.55	17.5	21.84	0.42	Y	10.3	8.73	12.2	0.53	Y

Table 6. Determination of model deviation ratios (MDR) at 25% effective concentration (EC25) for *Chironomus tepperi* 48 h acute toxicity experiments at pH 7. Green shading of the metal combination indicates less than additive toxicity and red shading indicates greater than additive toxicity (P < 0.05).

	Experimental data			Concentration addition model					Independent action model				
Mixture	EC25 (mM)	lower 95% CI	upper 95% CI	EC25 (mM)	lower 95% CI	upper 95% CI	MDR	P<0.05 CA?	EC25 (mM)	lower 95% CI	upper 95% CI	MDR	P<0.05 IA?
Cu+Co	0.400	0.274	0.584	0.858	0.626	1.18	0.47	Y	0.605	0.562	0.652	0.70	N
Cu+Mn	0.183	0.121	0.275	0.861	0.570	1.30	0.21	Y	0.227	0.210	0.245	0.26	Y
Cu+Ni	0.516	0.331	0.794	1.16	0.720	1.87	0.44	N	0.686	0.637	0.739	0.59	N
Cu+Zn	0.483	0.312	0.436	3.48	2.322	5.20	0.14	Y	0.610	0.566	0.658	0.18	Y
Co+Zn	1.09	0.669	1.77	0.646	0.315	1.32	1.69	N	1.37	1.25	1.51	2.13	N
Co+Ni	1.10	0.672	1.80	0.420	0.261	0.676	2.62	N	1.45	1.32	1.58	3.44	Y
Co+Mn	0.508	0.327	0.789	3.94	3.07	5.06	0.13	Y	0.652	0.608	0.699	0.17	Y
Zn+Ni	1.27	0.718	2.24	2.91	1.73	4.89	0.44	N	1.61	1.45	1.77	0.55	N
Zn+Mn	0.576	0.355	0.933	0.725	0.225	2.34	0.80	N	0.699	0.657	0.743	0.96	N
Ni+Mn	0.598	0.367	0.971	0.766	0.714	0.822	0.78	N	1.83	1.15	2.91	2.39	Y

Table 7. Determination of model deviation ratios (MDR) at 25% effective concentration (EC25) for *Chironomus tepperi* 48 h acute toxicity experiments at pH 5. Green shading of the metal combination indicates less than additive toxicity and red shading indicates greater than additive toxicity (P < 0.05).

	Experimental data			Concentration addition model					Independent action model				
Mixture	EC25 (mM)	lower 95% CI	upper 95% CI	EC25 (mM)	lower 95% CI	upper 95% CI	MDR	P<0.05 CA?	EC25 (mM)	lower 95% CI	upper 95% CI	MDR	P<0.05 IA?
Cu+Co	2.51	1.92	3.27	2.36	1.54	3.61	1.06	N	3.93	3.58	4.32	1.67	N
Cu+Mn	0.841	0.515	1.35	167	0.0244	1.15E+06	0.01	N	1.08	0.935	1.238	0.01	N
Cu+Ni	2.33	1.89	2.84	5.32	4.28	6.60	0.44	Y	3.62	3.48	3.76	0.68	Y
Cu+Zn	2.99	2.25	3.25	3.11	1.67	5.8	0.96	N	4.58	4.07	5.14	1.47	N
Co+Zn	7.44	5.99	9.23	0.94	0.508	1.72	7.95	Y	12.9	12.6	13.2	13.82	Y
Co+Ni	5.03	4.35	5.80	11.9	10.2	13.9	0.42	Y	7.60	7.47	7.73	0.64	Y
Co+Mn	2.36	1.44	3.78	11.9	8.57	16.6	0.20	Y	3.67	2.64	5.09	0.31	Y
Zn+Ni	5.58	4.79	6.48	1.43	0.634	3.24	3.90	Y	8.02	7.88	8.17	5.60	Y
Zn+Mn	2.62	1.58	4.27	0.59	0.211	1.68	4.41	N	3.87	2.75	5.45	6.51	Y
Ni+Mn	2.27	1.48	3.32	2.26	0.948	5.38	1.00	N	3.56	2.84	4.45	1.57	N
Al+Cu	0.616	0.274	1.14	0.68	0.350	1.34	0.90	N	0.785	0.692	0.891	1.15	N
Al+Mn	1.25	0.576	2.51	11.0	8.21	14.7	0.11	Y	1.30	1.16	1.45	0.12	Y
Al+Co	3.59	1.49	6.85	9.24	7.68	11.1	0.39	Y	5.88	4.37	7.91	0.64	N
Al+Ni	3.09	1.53	4.73	7.00	5.53	8.86	0.44	Y	4.23	3.77	4.74	0.60	Y
Al+Zn	4.21	1.67	8.50	16.3	12.2	21.7	0.26	Y	7.15	5.59	9.15	0.44	Y

Table 8. Determination of model deviation ratios (MDR) at 10% effective concentration (EC10) for *Chironomus tepperi* 48 h acute toxicity experiments at pH 7. Green shading of the metal combination indicates less than additive toxicity and red shading indicates greater than additive toxicity (P < 0.05).

	Experimental data			Concentration addition model					Independent action model				
Mixture	EC10 (mM)	lower 95% CI	upper 95% CI	EC10 (mM)	lower 95% CI	upper 95% CI	MDR	P<0.05 CA?	EC10 (mM)	lower 95% CI	upper 95% CI	MDR	P<0.05 IA?
Cu+Co	0.513	0.312	0.844	0.218	0.124	0.381	0.42	N	0.38	0.335	0.432	0.74	N
Cu+Mn	0.502	0.258	0.978	0.100	0.055	0.183	0.20	Y	0.138	0.122	0.157	0.28	Y
Cu+Ni	0.661	0.313	1.40	0.283	0.144	0.539	0.43	N	0.414	0.367	0.467	0.63	N
Cu+Zn	2.46	1.31	4.62	0.251	0.139	0.450	0.10	Y	0.354	0.315	0.398	0.14	Y
Co+Zn	0.307	0.103	0.912	0.534	0.278	1.025	1.74	N	0.765	0.662	0.885	2.49	N
Co+Ni	0.203	0.098	0.418	0.575	0.274	1.188	2.84	N	0.849	0.744	0.970	4.19	Y
Co+Mn	3.10	2.10	4.60	0.270	0.141	0.517	0.09	Y	0.384	0.346	0.427	0.12	Y
Zn+Ni	1.50	0.694	3.23	0.623	0.288	1.340	0.42	N	0.901	0.777	1.044	0.60	N
Zn+Mn	0.172	0.030	0.994	0.296	0.151	0.578	1.71	N	0.399	0.364	0.437	2.31	N
Ni+Mn	0.879	0.444	1.740	0.320	0.154	0.656	0.36	N	0.452	0.406	0.502	0.51	N

Table 9. Determination of model deviation ratios (MDR) at 10% effective concentration (EC10) for *Chironomus tepperi* 48 h acute toxicity experiments at pH 5. Green shading of the metal combination indicates less than additive toxicity and red shading indicates greater than additive toxicity (P < 0.05).

	Experimental data			Concentration addition model					Independent action model				
Mixture	**EC10 (mM)**	**lower 95% CI**	**upper 95% CI**	**EC10 (mM)**	**lower 95% CI**	**upper 95% CI**	**MDR**	**P<0.05 CA?**	**EC10 (mM)**	**lower 95% CI**	**upper 95% CI**	**MDR**	**P<0.05 IA?**
Cu+Co	1.73	1.13	2.65	1.34	0.703	2.55	1.30	N	2.84	2.46	3.27	2.12	N
Cu+Mn	0.388	0.179	0.823	4.66	0.00225	9652.000	0.08	N	0.609	0.492	0.755	0.13	N
Cu+Ni	1.59	1.14	2.16	4.39	3.27	5.89	0.36	Y	2.71	2.55	2.89	0.62	Y
Cu+Zn	2.02	1.32	3.09	1.69	0.655	4.35	1.20	N	3.21	2.69	3.84	1.90	N
Co+Zn	6.00	4.30	8.36	0.407	0.165	1.01	14.73	Y	11.0	10.5	11.6	27.06	Y
Co+Ni	3.84	3.07	4.78	9.64	7.35	12.6	0.40	Y	5.83	5.69	5.98	0.61	Y
Co+Mn	1.09	0.491	2.37	10.8	7.99	14.7	0.10	Y	2.09	1.27	3.43	0.19	Y
Zn+Ni	4.19	3.37	5.19	0.492	0.148	1.63	8.53	Y	6.05	5.89	6.21	12.30	Y
Zn+Mn	1.19	0.533	2.60	0.120	0.0250	0.575	9.95	N	2.09	1.25	3.51	17.46	Y
Ni+Mn	1.11	0.533	2.12	0.844	0.234	3.04	0.49	N	2.24	1.59	3.16	2.66	N
Al+Cu	0.274	0.063	0.79	0.27	0.099	0.764	1.00	N	0.467	0.385	0.566	1.70	N
Al+Mn	0.484	0.138	1.40	9.13	5.07	16.5	0.05	Y	0.582	0.494	0.687	0.06	Y
Al+Co	1.60	0.321	5.43	7.19	5.53	9.35	0.22	Y	4.16	2.60	6.66	0.58	N
Al+Ni	1.51	0.366	3.48	4.71	3.27	6.80	0.32	N	2.97	2.48	3.55	0.63	N
Al+Zn	1.80	0.356	6.32	13.6	7.52	24.4	0.13	Y	4.97	3.37	7.31	0.37	Y

The present study examined toxic interactions in 2nd instar midge larvae exposed to binary combinations of metals in short-term (48 h) water-only exposures. As is also highlighted in the snail mixture toxicity report that forms part of the present study, the exposure conditions comprised moderately hard water containing negligible dissolved organic matter (DOM); hardness, DOM and other physicochemical characteristics that vary widely in natural waters and can affect metal speciation, bioavailability, and therefore toxicity. Toxicity modelling that considers the bioavailable fraction of dissolved metal ions can be achieved using approaches such as the Windermere humic aqueous model with toxicity function (WHAM-F_{TOX}) (Stockdale *et al.*, 2010), which addresses the influence of DOM on metal bioavailability, or a modified biotic ligand model (BLM), which also takes into account competitive binding of various metals sites in or on the organism (Di Toro *et al.*, 2001; Paquin *et al.*, 2002).

Numerous derivatives of BLM and WHAM-F_{TOX} have been developed for metal mixtures in order to take into account parameters such as DOM and hardness (these are summarised in a recent evaluation of metal mixture modelling approaches (Meyer *et al.*, 2014; Farley & Meyer, 2015; Farley *et al.*, 2015)). These models should be considered when assessing the potential risks to aquatic biota of metal mixtures in real-world scenarios. BLM and WHAM-F_{TOX} approaches rely on the availability of species-specific toxicity data for the initial generation of model parameters. As such, there are different models available for different metals that are applicable to different species. When investigating metal toxicity in a new species or class of species, the establishment of laboratory-based toxicity data is essential. The extensive concentration-response data generated in the present study will therefore be valuable for the development and calibration of models suitable for predicting the toxicity of dissolved metal mixtures to midge larvae.

4 Conclusions

Utilising an additive model, it was clear in the present study that many mixtures at neutral pH exhibited antagonistic toxic interactions. That is, the experimentally determined toxicity of a large proportion of mixtures at neutral pH was less than that predicted by the additive model, suggesting that in general CA is a conservative model for mixture toxicity to midge larvae at neutral pH. The same was true for aluminium-containing combinations at pH 5. Conversely, the majority of mixtures not containing Al at pH 5 exhibited additive toxicity, indicating that disregarding Al, CA can accurately predict the toxicity of the metal mixtures in this pH range.

The combinations that exhibited significantly greater-than-additive toxicity and can be recommended for further study to elucidate the underlying mechanisms were:

- At the EC50:
    - Co + Ni at pH 7
    - Co + Zn at pH 5
- At the EC10:
    - Co + Zn at pH 5
    - Zn + Ni at pH 5

The abundance of Al in ASS-affected soils in the Lower Lakes region means that a better understanding of the potential toxicity of Al-containing mixtures is required. The additive model used in the present study, based on nominal concentrations of dissolved metal salts, is clearly inadequate for Al-containing combinations at pH 5, since antagonistic toxic interactions were observed in midge and synergistic toxic interactions were observed in aquatic snails. However, even if mixture toxicity under laboratory conditions can be accurately predicted, there are a number of additional caveats that must be considered before translating the approaches used here to real-world scenarios. In the laboratory, the following physicochemical parameters that are known to affect metal speciation and bioavailability may differ from the variable conditions occurring in the natural aquatic environment:

- Dissolved organic matter
- Particulate organic matter and sedimentation
- Hardness
- Temperature
- Salinity

The present study highlights (through identification of gaps in our understanding) the need for further research into the behaviour and toxicity of metals in combination at a range of conditions relevant to water bodies potentially impacted by acid-sulfate soils.

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