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Toxicity of selected metals and their binary combinations to the freshwater snail *Physella acuta*

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Interim Report (Commercial in confidence)

Interim report in fulfilment of project milestones 3.2 and 3.3 of project 298/0586 for:

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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.

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.

The influence of low pH on the mobilisation of metals in surface freshwater environments is well known. However, the potential impacts of metal mixtures in freshwater molluscs has not been well studied. This report presents an analysis of the toxicity of six dissolved metals, alone and in binary combination, to the freshwater snail *Physella acuta*. Although it is an introduced species, *P. acuta* is well established in the Coorong, Lower Lakes and Murray Mouth (CLLMM) region and is therefore suitable for ecotoxicological studies relevant to the region.

In the present study

- six metals – Al, Cu, Co, Mn, Ni and Zn – were selected based on their occurrence in waters of the lower Murray and Lower Lakes affected by localised acidification due to ASS oxidation and re-wetting
- the toxicity of and their binary combinations to the freshwater snail *Physella acuta* at pH 7 and pH 5 was determined in acute 48 h immobilisation bioassays
- a comparison of experimental data with predicted toxicity – based on a mixture toxicity modelling approach – revealed that a number of binary combinations exhibited greater-than-additive toxicity
- while Al-containing mixtures at pH 5 exhibited markedly synergistic toxicity, a confounding influence of lower-than-expected pH on the concentration-response profiles cannot be ruled out and further study is warranted
- the combinations of copper and zinc at pH 7, and copper and manganese at pH 5, also exhibited significantly synergistic toxicity

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 Introduction

1.1 Potential impacts of acid sulfate soils on surface freshwater ecosystem health

The re-wetting of oxidised acid sulfate soils (ASS) can result in the acidification of estuarine and inland surface waters and promote the mobilisation and bioavailability of metals (Baldwin and Fraser, 2009; Burton et al., 2008; Campbell and Stokes, 1985; Chapman et al., 1998; Förstner, 1995). The impact of acid sulfate-related surface freshwater acidification on aquatic organisms has not been well studied but is crucial in understanding the ecological significance of the risks posed by ASS.

The Lower Lakes region in South Australia experienced extensive declines in water levels between 2005 and 2010 due to high levels of abstraction in combination with an extended period of low rainfall in upstream catchments. This culminated in the widespread drying of sediments and exposure of ASS to aerobic conditions. ASS within the Lower Murray Reclaimed Irrigation Area (LMRIA) between Mannum and Wellington were also subjected to prolonged drying during this period. LMRIA is a network of approximately 5,200 hectares of former flood plains protected by a levee bank and irrigated via controlled release of gravity-fed water from the lower Murray. Low flow during the drought period prevented regular irrigation and resulted in extensive desiccation of ASS in the LMRIA.

After significant rainfall in the Murray-Darling catchment from March 2010, oxidised ASS in exposed Lower Lakes sediments were re-wetted, resulting in localised acidification and the mobilisation of soil-associated metals and nutrients into surface waters (Fitzpatrick et al., 2011). Dissolved trace metal concentrations for Ag, Cd, Co, Cu, Mn and Zn in river water sampled from several sites in the lower Murray exceeded ANZECC/ARMCANZ (2000) water quality guidelines (Simpson et al., 2010). A follow-up study on LMRIA drainage waters affected by re-wetted acidified soils revealed high dissolved concentrations of Al (55 mg/L), Fe (205 mg/L), Mn (24.7 mg/L), Co (1.25 mg/L), Ni (1.3 mg/L) and Zn (1.1 mg/L).

Based on these and detailed 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)

The bioavailability of metals, and hence their toxicity to aquatic organisms, varies considerably with pH and other physicochemical parameters due to factors such as changes in speciation, aqueous solubility, and complexation with dissolved organic matter (DOM). Much of the ecotoxicological research to date in this area has focused on fish and microcrustaceans, with comparatively little work done on molluscs. Although some species were rarely found during the drought of 2005-2010, molluscs now contribute significantly to benthic macroinvertebrate biodiversity in the CLLMM (Dittmann et al., 2011; Dittmann et al., 2014). Molluscs occurring in the Lower Lakes include bilvalves such as *Soletellina alba* and *Arthritica helmsi*, and gastropods such as *Salinator fragilis*, *Coxiella striata* and various unidentified species of Hydrobiidae (Dittmann et al., 2014). *P. acuta* is an invasive species described in 2009 as the most abundant freshwater snail in the lower Murray (Zukowski and Walker, 2009) and was recently identified at several sites in the Lower Lakes a recent macroinvertebrate survey, albeit in small numbers (Dittmann et al., 2014),

While there is some data available on the toxicity of metals to molluscs, most is concerned with the toxicity of individual metals rather than mixtures. Since metals in the aquatic environment almost always occur as mixtures of varying complexity, understanding metal mixture toxicity is fundamental to gaining a better understanding of the potential effects of ASS-related acidification on metal toxicity in the 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. 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. 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 (Altenburger et al., 2004; Faust et al., 1993; Hewlett, 1969). CA also forms the basis of the Toxic Unit concept that describes the individual contributions of a mixture to the overall toxicity (Spehar and Fiandt, 1986). IA, on the other hand, 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'.

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

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

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).

$$MDR = \frac{predicted\ ECx}{observed\ ECx} \quad (3)$$

In the case of CA, the magnitude of deviation from the MDR must be considered when the goal is to classify the interaction of the components of a mixture as additive, synergistic or antagonistic. 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 (Arora and Kumar, 2015; Belden et al., 2007; Phyu et al., 2011), 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 forms part of a larger study investigating the toxicity of metal mixtures to snails and midge larvae. In the present study, the aims were to:

1. Determine the survival of the freshwater snail *Physella acuta* in the acidic pH range
2. Determine the toxicity of six metals to the freshwater snail *P. acuta* in 48 h acute toxicity tests conducted at pH 7 and pH 5
3. Based on individual toxicity, use modelling approaches to predict the toxicity of binary mixtures at pH 7 and pH 5
4. Compare the predicted toxicity with experimental data – in order to determine whether mixtures were additive, synergistic or antagonistic with respect to acute toxicity

2 Methods

2.1 Snail 48 h immobilisation bioassay

To determine concentration-response relationships for individual metals (Cu, Co, Mn, Zn, Ni and Al), 48 hour acute toxicity tests were carried out using newly hatched (48 h post-hatch) *P. acuta* in laboratory-prepared water at pH 7 and pH 5. Experimental conditions are shown in Table 1. We observed a decrease in pH after dissolving some metals in aqueous solutions at pH 5. Thus, 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 (EC) and dissolved oxygen (DO) of the water measured. The concentration of metal ions was analysed at 0 h and 48 h time points to ensure that concentrations did not deviate significantly from nominal values.

Table 1. Summary of test conditions for the newly hatched freshwater snail (*Physella acuta*) 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	25 mL vial
Test solution volume	22 mL
Age of test organisms	48 h hatched snail
No. of organisms per replicate	5
No. of replicates per treatment	4
No. of organisms per treatment	20
Feeding regime	None
Dilution water	Modified FETAX Solution (MFS), 3000 µS/cm EC
Test concentrations	8 (7 + control)
Control treatments	MFS, 3000 µS/cm EC
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 *P. acuta* toxicity bioassays. The concentration ranges applied in mixture toxicity tests were based on EC50 values derived from tests conducted with individual metals. For the binary mixtures, metals were combined in equitoxic concentrations with respect to the EC50 value, and a dilution series centred around 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). This approach facilitated the use of predictive mixture toxicity models. 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 snails 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 and multiplying the predicted effects of each component of the binary mixtures according to their proportion in the total mixture (equation 2). 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 are derived by fitting a logistic concentration-response model to survival data, each EC value has 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 snail immobilisation bioassays

Logistic concentration-response curves were fitted to metal toxicity data from acute toxicity tests conducted at pH 7 and pH 5 (Figure 1), with EC50 values presented in

Table 3. Concentrations used for non-linear regression were nominal. For metals with extremely steep concentration-response curves (Hill slopes > -10), model parameters were deemed ambiguous and thus the reported EC values (EC10, EC25 and EC50) should be considered as approximate values. These cases are indicated with a preceding tilde (~) in

Table 3. Confidence intervals could not be calculated for these EC values.

The toxicity of individual metals was similar at pH 7 and pH 5, with the exception of Mn, which exhibited somewhat diminished toxicity at pH 5 compared to pH 7. The rank order of toxicity of the metals in snail acute toxicity bioassays was Cu > Zn ~ Ni > Al > Co > Mn. Interestingly, Al did not exhibit markedly different toxicity at pH 7 and pH 5 despite expected solubility issues at neutral pH.

The EC50 values obtained for individual metals were in a similar range to data from other freshwater snails reported in previous studies conducted under similar conditions using comparable exposure times. For example, EC50 values for 48 h acute toxicity tests conducted using the freshwater snail *Melanoisdes tuberculata* for Cu, Zn, Ni, Al and Mn were 0.39, 13.2, 36.4, 307 and 120 mg/L, respectively (Shuhaimi-Othman et al., 2012), which were somewhat higher values than we report in the present study, but it should be noted that the *M. tuberculata* were adult snails while in the present study we used 48 h post-hatch juveniles.

Table 3 Median effective concentrations (EC50) for individual metals in 48 h acute *Physella acuta* toxicity tests conducted at pH 7 (top) and pH 5 (bottom).

		Cu	Al	Co	Mn	Zn	Ni
pH 7	EC50 (mg/L)	0.130	~ 6.64	70.7	72.5	0.968	1.44
	EC50 (mM)	0.00204	~ 0.246	1.20	1.32	~ 0.0148	0.0245
	95% CI (mM)	0.00180 to 0.00231	(Very wide)	0.906 to 1.58	1.16 to 1.49	(Very wide)	0.0218 to 0.0274
	Hill slope	-3.31	~ -35.9	-2.45	-4.28	~ -27.2	-7.58
	R^2	0.9628	0.9945	0.9238	0.9497	0.9814	0.9846
pH 5	EC50 (mg/L)	0.120	9.45	92.2	316	1.56	1.37
	EC50 (mM)	0.00189	0.350	1.56	~ 5.76	0.0239	0.0233
	95% CI (mM)	0.00171 to 0.00208	0.311 to 0.395	1.27 to 1.92	(Very wide)	0.0227 to 0.0252	0.0211 to 0.0257
	Hill slope	-5.40	-7.81	-2.98	~ -32.8	-6.24	-6.49
	R^2	0.9589	0.9843	0.9293	0.978	0.9956	0.9826

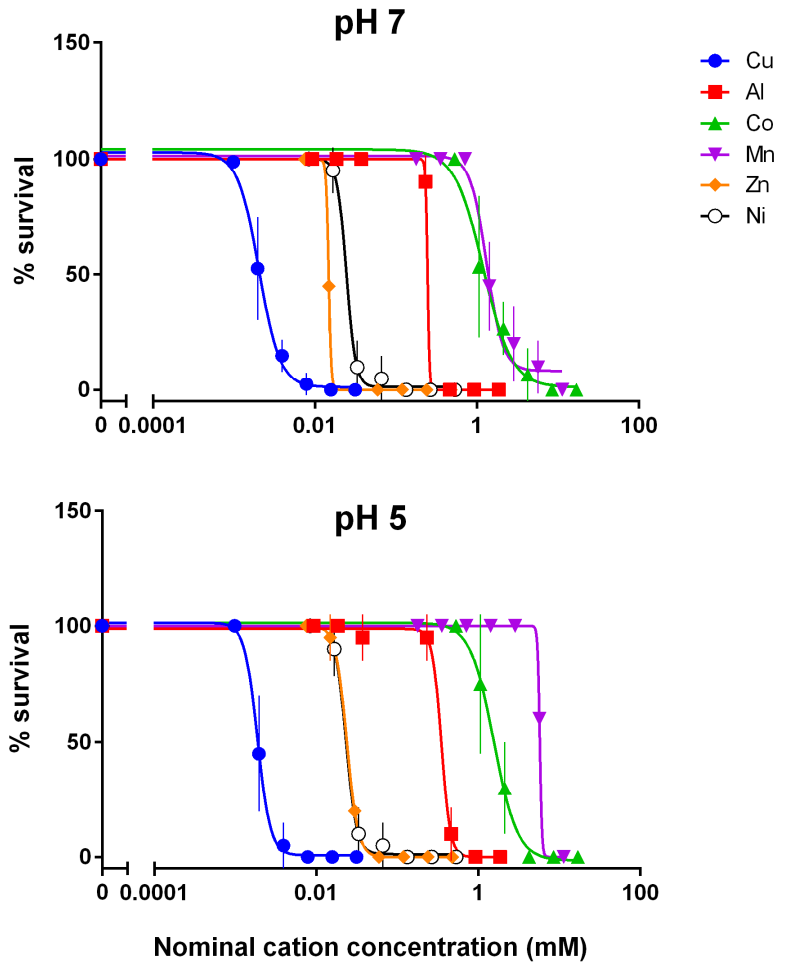


Figure 1. Concentration-response relationships for individual metals in 48 h snail acute toxicity tests conducted using freshwater snail, *Physella acuta*.

3.2 Mixture toxicity modelling and comparison with experimental data

For the present study, the aims were to investigate whether the toxicity of specific combinations of metals 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 whether lower pH could alter the toxicity of metal mixtures. To this end, we undertook a laboratory-based approach that utilised controlled water chemistry to determine the short-term acute toxicity of selected metals and their binary combinations. In this approach, which 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, and conclusions were made regarding whether the toxicity of the mixtures was synergistic (greater than expected from an additive model) or antagonistic (less than expected from an additive model).

Concentration-response curves for binary mixtures were predicted using CA and IA and determined experimentally. The ability of each model to accurately predict toxicity of a mixture at a given EC values was determined using 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 (Arora and Kumar, 2015; Belden et al., 2007; Phyu et al., 2011).

A comparison of CA and IA models with experimental data for all 15 binary combinations at pH 7 and pH 5 is shown in Figure 2. In general, the predicted toxicity of mixtures according to IA was less than that predicted by CA, and for the majority of mixtures, CA was more accurate than IA in predicting the toxicity of binary combinations in 48 h acute toxicity tests.

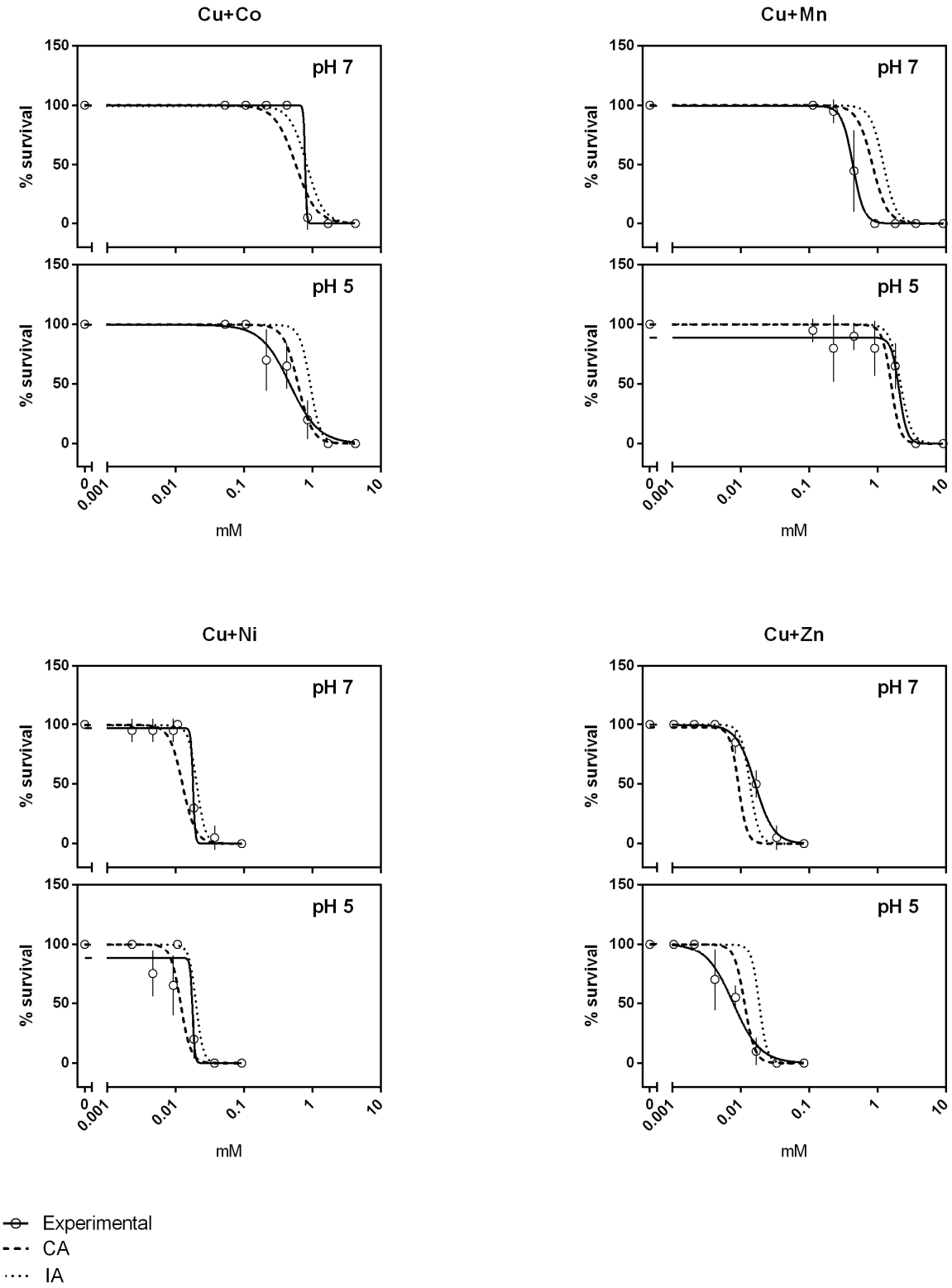
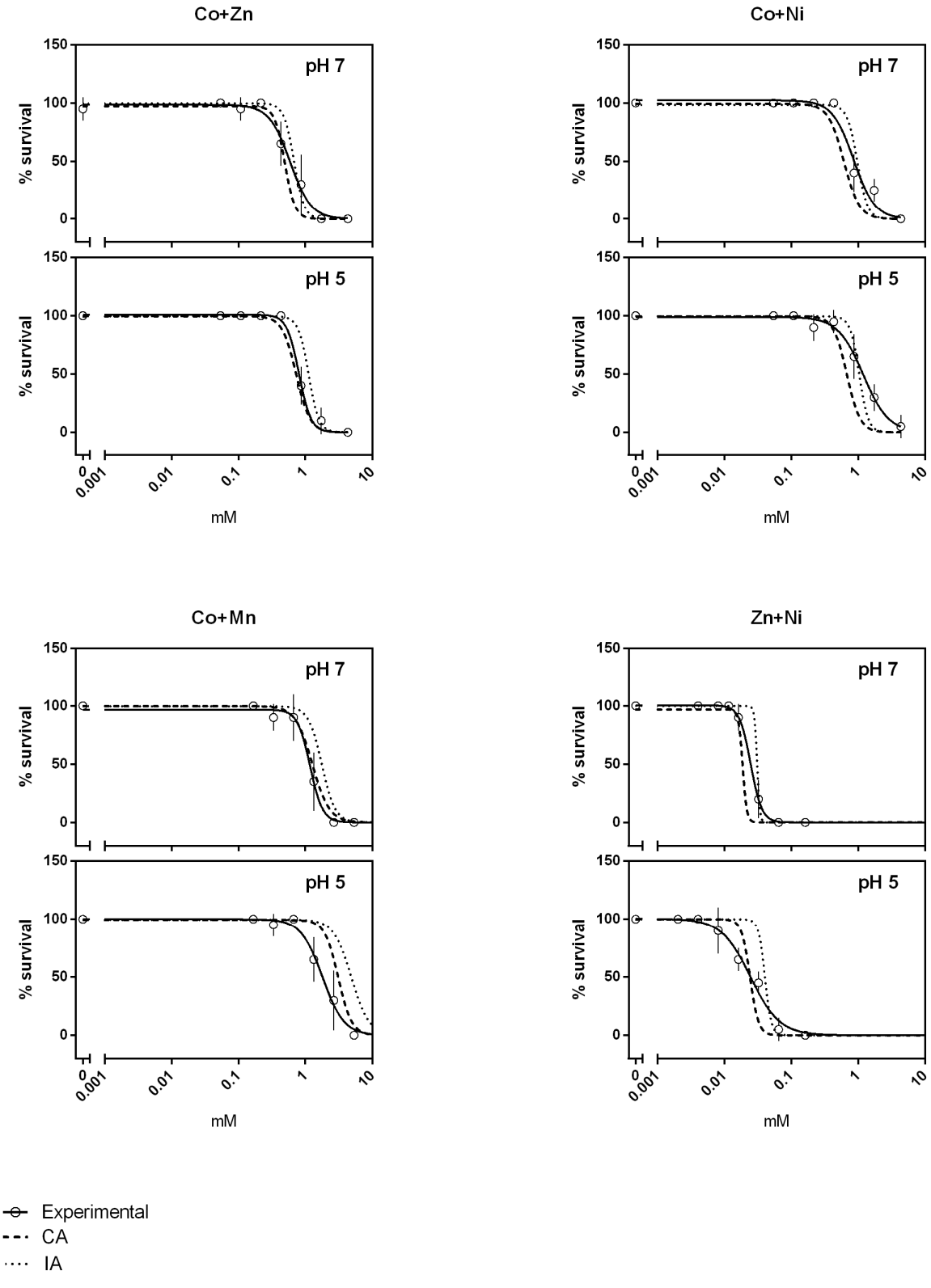


Figure 2 Comparison of concentration addition (CA) and independent action (IA) predictive models with experimental data for all 15 possible binary combinations of Al, Cu, Co, Mn, Ni, and Zn. (cont'd over next 4 pages)



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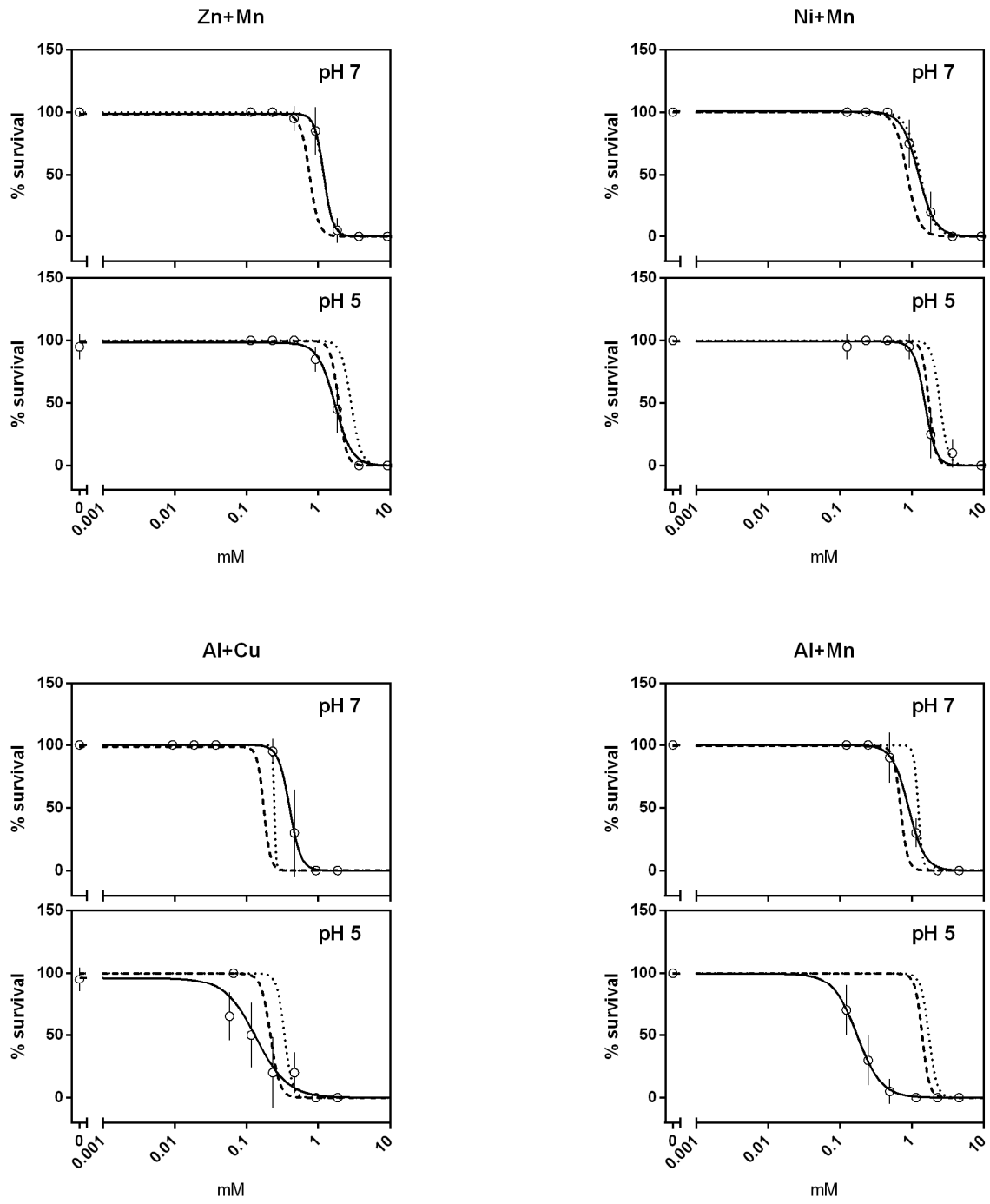

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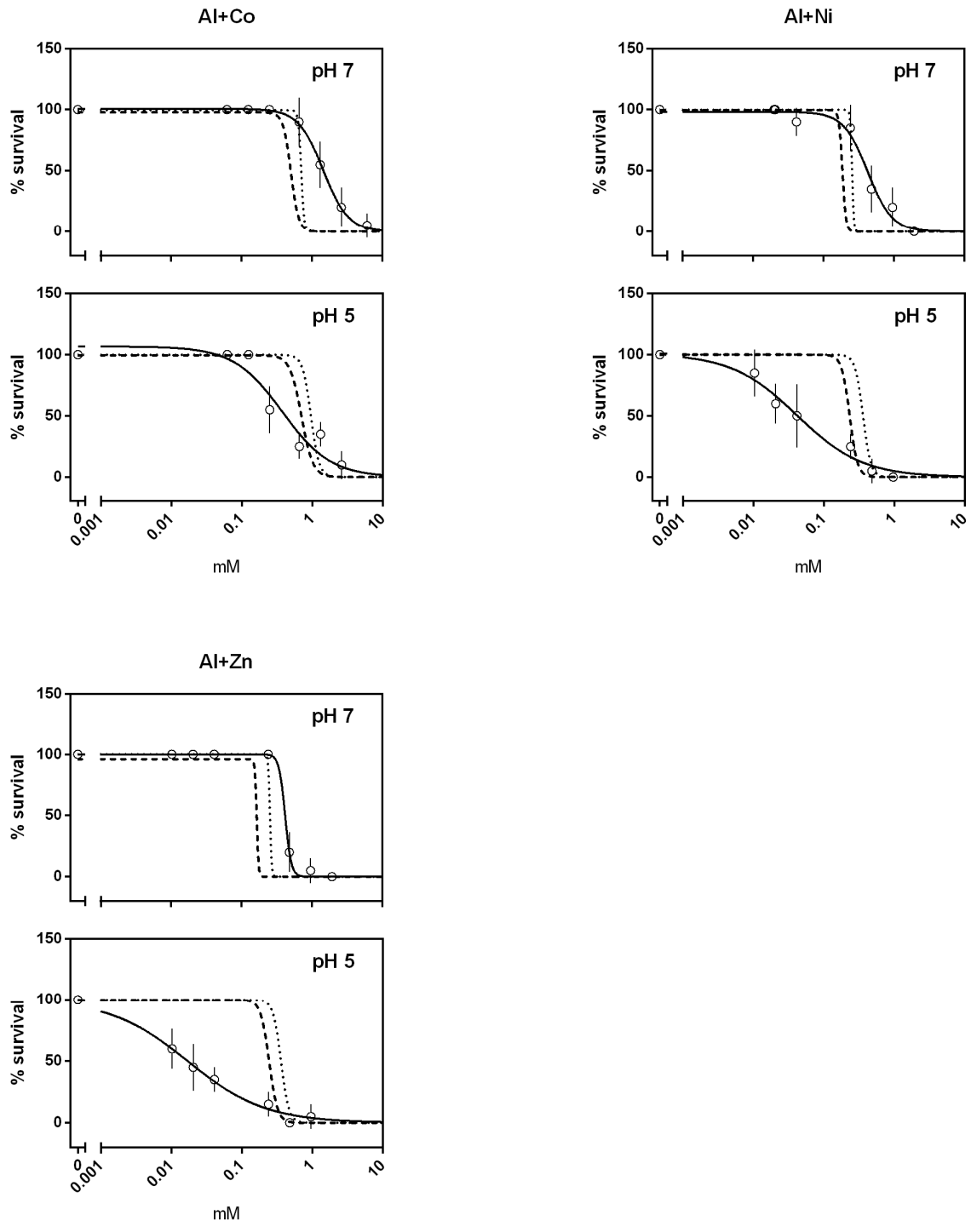

(Figure 2 cont'd)

This is perhaps most apparent from the mean MDR values determined for all binary combinations shown in Figure 3A (with the exception of mixtures containing Al, which exhibited markedly greater than expected toxicity at pH 5 and is therefore considered as a special case and omitted from the mean MDR calculated). Significant deviation of mean MDR values for from 1 indicates that a given model was not a good overall predictor of mixture toxicity for the mixtures studied. The mean MDR for CA (disregarding Al-containing mixtures) was not significantly different from 1 ($p = 0.69$), while the mean MDR for IA deviated significantly from 1 ($p < 0.001$). Since the mean MDR for the IA model was significantly greater than 1, it can be considered that the IA model generally underestimated the toxicity of the binary metal mixtures investigated in the present study, while the CA model was an adequate predictor of mixture toxicity. This is not unexpected, since metal ions in solution are generally 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), implying that CA is conceptually a more appropriate model for toxic metal ions than IA. More detailed evidence that CA was a more accurate predictive model than IA in the present study is shown in Figure 3B (CA model) and Figure 3C (IA model), which reveal that IA underestimated the toxicity of binary mixtures at both pH 5 and pH 7, resulting in MDRs greater than 1, while CA tended to slightly overestimate toxicity at pH 7 and slightly underestimate toxicity at pH 5.

A generalised enhancement of metal toxicity at pH 5 may be related to increased stress on the snails at the lower pH of 5, which is close to the pH range that the snails were adversely affected in the absence of metals (Figure 4), however because the individual toxicity of each metal was also conducted at pH 5 and the resulting concentration-response profiles used to predict toxicity at the same pH, the predictive models should in theory operate with similar efficiency at either pH. A more detailed representation of the MDR distribution is presented in Figure 5, which shows a plot of the MDRs for each mixture at EC10, EC25 and EC50 at pH 7 and pH 5. The complete lists of predicted and experimental values for EC10, EC25 and EC50 at pH 5 and pH 7 and MDRs are shown in Table 4 to Table 9. Disregarding the Al combinations, MDRs for the CA model at pH 5 were generally higher than 1 and there was a greater number of significant MDRs at pH 5 compared to pH 7. This indicates a general trend towards greater-than-additive toxicity of binary metal mixtures at pH 5.

3.3 Significant deviations from the concentration addition model

Due to some logistic curve fits for individual toxicity data returning ambiguous parameter values, a number of combinations did not pass significance testing; this was despite MDRs outside the accepted range for additivity (e.g. Cu+Zn EC50 at pH 7, MDR 0.56). Combinations that differed significantly from the CA model are summarised below:

- Cu + Mn, synergistic at pH 7 (EC50 MDR 1.93 and EC25 MDR 1.8)
- Ni + Mn, antagonistic at pH 7 (EC50 MDR 0.69)
- Cu + Zn, synergistic at pH 5 (EC50 MDR 1.41, EC25 MDR 1.99 and EC10 MDR 2.83)
- Co + Ni, antagonistic at pH 5 (EC50 MDR 0.58)

Of all the combinations tested, those containing aluminium exhibited the highest MDRs for both CA and IA. For Al-containing combinations, both models at pH 7 were conservative, i.e. the predictive models overestimated toxicity of binary mixtures containing Al. This may be due to changes in speciation or poor solubility at neutral pH. Conversely, at pH 5 the predictive models significantly underestimated the toxicity of Al-containing mixtures. The reason for the observed enhanced toxicity of Al at pH 5 may be due to a decrease in pH concomitant with increasing Al concentration, which occurred despite buffering with MES. Although this decrease in pH was also observed in the individual Al exposures at both pH 5 and pH 7, which suggests that mixture toxicity should be predictable based on single exposures despite changes in pH, for reasons that are currently not clear the MDR values for Al-containing combinations were high. Because *P. acuta* is highly sensitive to low pH at this developmental stage (**Error! Reference source not found.**), it is recommended that the results presented here for metal mixtures containing Al be taken with caution, and that an improved buffering system be incorporated in future experiments to prevent the drop in pH in solutions containing relatively high concentrations of Al. Because Al is an important stressor potentially generated by acid sulfate soils, low pH is not necessarily an undesirable condition for laboratory-based toxicity testing relevant to ASS-affected waters. However, the problem of separating the confounded effects of low pH and toxicity of Al ions is compelling and will require further study.

The present study focused on mixture toxicity under laboratory conditions at different pH. It should be noted that factors such as complexation with negatively charged species in natural waters will affect bioavailability of metal ions under typical environmental conditions. The toxicity of individual metals in under environmental conditions is commonly predicted using one of two approaches. The WHAM-F_{TOX} model (Stockdale et al., 2010) uses the Windermere Humic Aqueous Model (Tipping, 1994) combined with species-specific toxicity functions to predict the effects of metals to aquatic organisms. The biotic ligand model (BLM) (Di Toro et al., 2001; Paquin et al., 2002) takes into account complexation of metal ions with dissolved organic matter (DOM), competition for toxic metal ion biding sites on the susceptible target in the organism (i.e. the 'biotic ligand', e.g. the gills of fish) by non-toxic metal ions such as calcium, and changes in the speciation of toxic metal ions with changes in the physico-chemical parameters of water, e.g. pH and hardness. The accumulation of the toxic metal ion(s) on the biotic ligand of interest is deemed to be the key parameter in predicting lethality, and the median lethal accumulation is referred to as the LA50 value. Many BLM derivatives have recently been developed that extend the model to predict the toxicity of metal mixtures in natural waters (Farley and Meyer, 2015; Farley et al., 2015; Iwasaki and Brinkman, 2015; Iwasaki et al., 2015; Meyer et al., 2015a; Meyer et al.,

2015b; Naddy et al., 2015; Santore and Ryan, 2015; Tipping and Lofts, 2015; Van Genderen et al., 2015). These models should be considered when assessing risk of metal mixtures in real-world scenarios. However, 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 in the early development of metal mixture toxicity models suitable for freshwater snails and other invertebrates because this information is required for the development of class- or species-specific models.

4 Conclusions

In this study, we investigated the toxicity of Al, Cu, Co, Mn, Ni and Zn and their binary combinations to the freshwater snail *Physa acuta* at pH 7 and pH 5. While Al toxicity was greater than expected at pH 5, solutions containing high concentrations of Al were found to exhibit low pH despite the presence of a buffer, and as such a confounding contribution of low pH to the observed toxicity of Al-containing mixtures cannot be ruled out.

While the CA model was generally a good predictor of mixture toxicity in the present study, two binary mixtures resulted in greater-than-additive toxicity and further study is recommended. These mixtures were copper plus manganese at pH 7 and copper plus zinc at pH 5.The synergistic toxic effects of copper and zinc at pH 5 to *P. acuta* may have implications for ASS-affected surface water and sediment pore water, since both metals have been shown to occur in ASS in the lower Murray region and can be rapidly mobilised under acidic conditions (Simpson et al., 2010).

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.

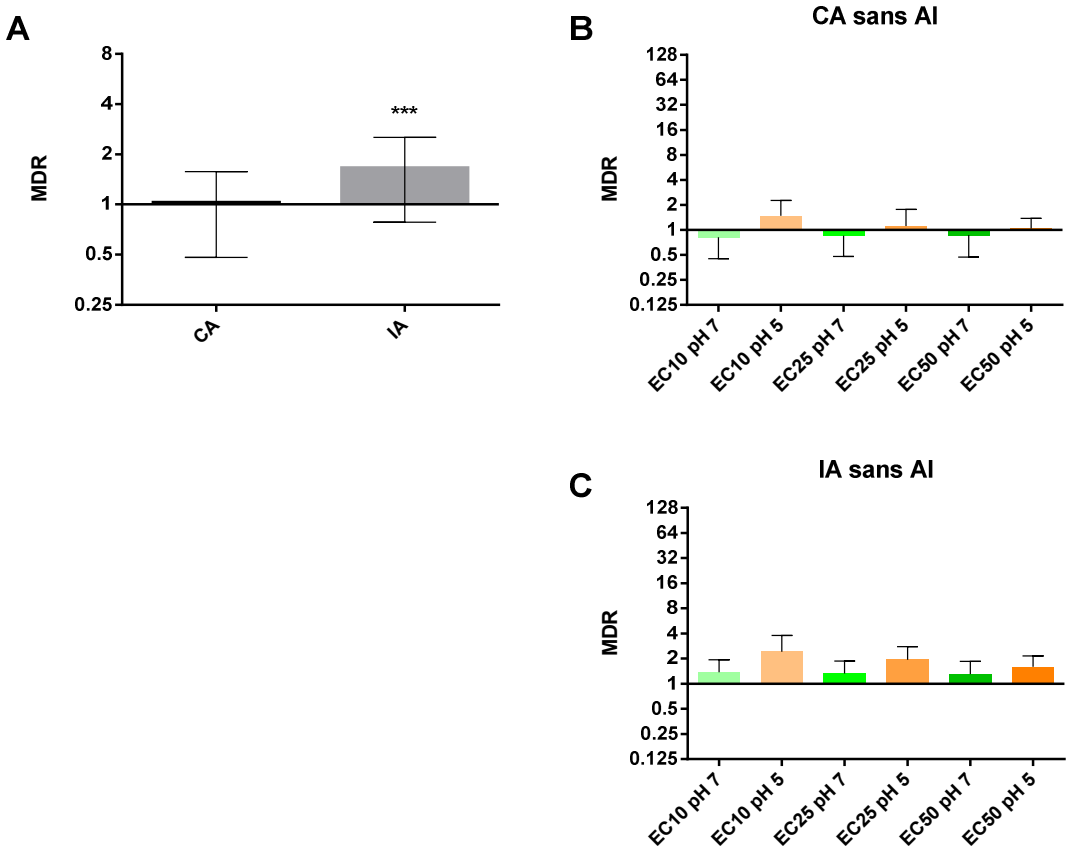

Figure 3 (A) Mean MDRs of all binary combinations except those containing Al. Error bars represent standard deviation; ***p < 0.001 according to a one-sample t-test comparing mean MDR values with a theoretical value of 1 representing no deviation from the model. (B) Mean MDRs for all binary combinations except aluminium-containing mixtures, using the concentration addition (CA) model at pH 5 and pH 7 for EC10, EC25 and EC50 values. (C) Mean MDRs for all binary combinations except aluminium-containing mixtures, for the independent action (IA) model at pH 5 and pH 7 for EC10, EC25 and EC50 values.

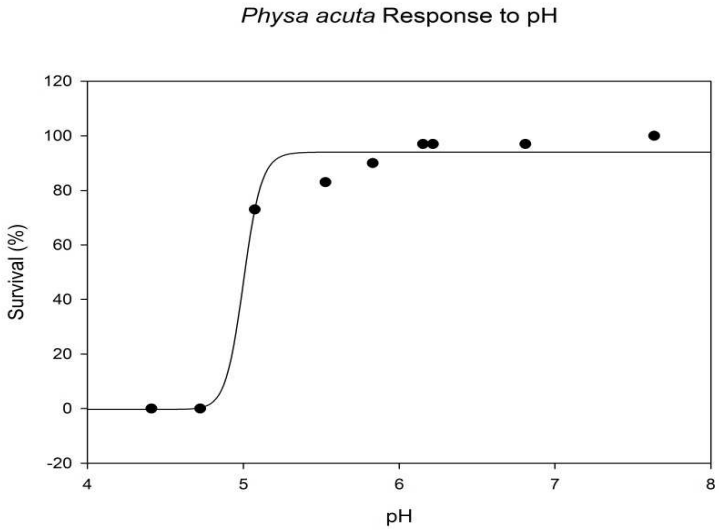

Figure 4. Mean percentage of juvenile *P. acuta* surviving 48 h in solutions of pH 4.4, 4.7, 5.1, 5.5, 5.8, 6.2, 6.2, 6.8 and control 7.6.

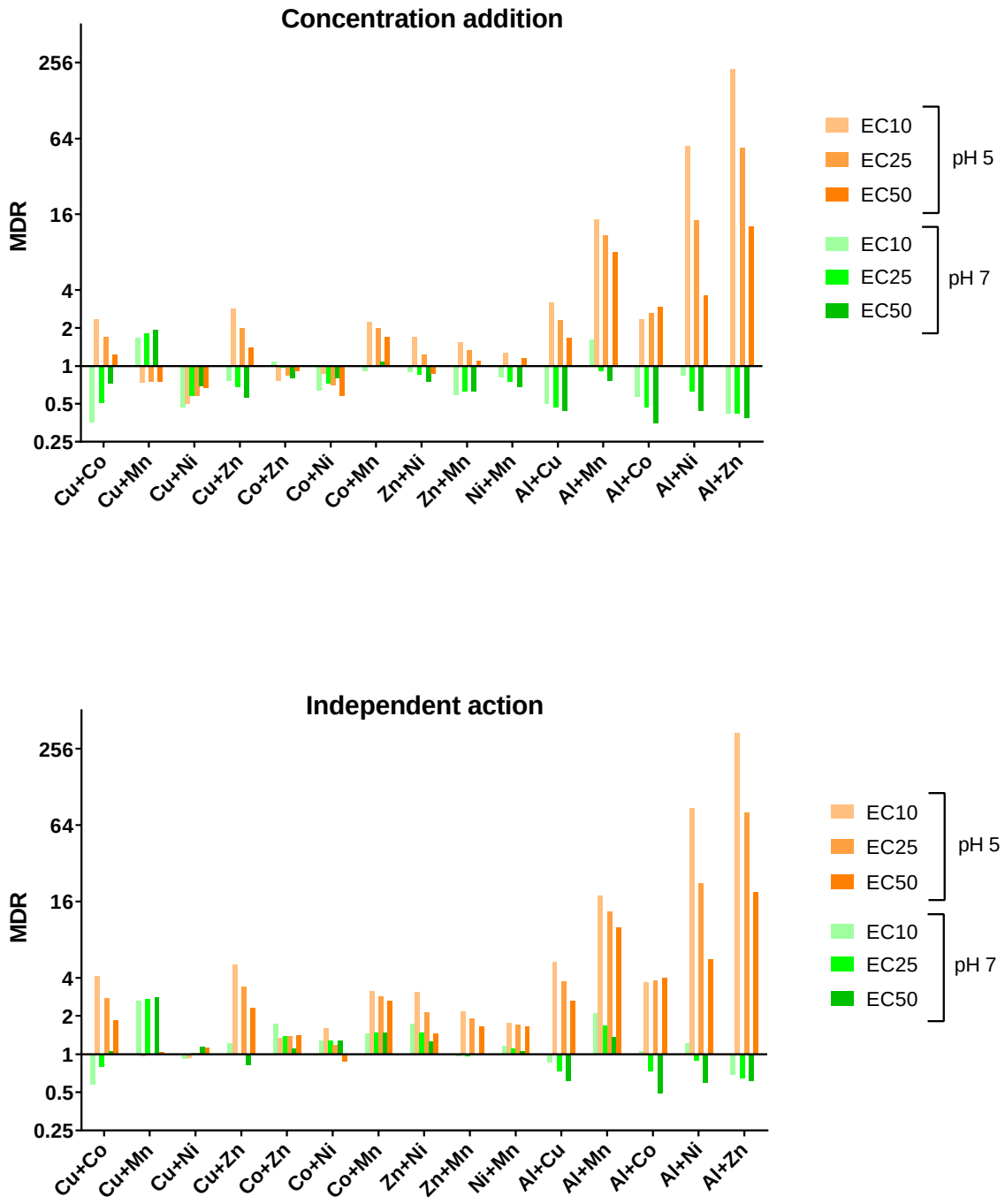

Figure 5. Model deviation ratios (MDR) for concentration addition (CA) or independent action (IA) models. MDRs for each binary combination are shown for 10%, 25% and 50% effect levels at pH 7 and pH 5 for CA (top) and IA (bottom) models.

Table 4 Determination of model deviation ratios (MDR) at 50% effective concentration (EC50) for *Physa acuta* 48 h acute toxicity experiments at pH 7. Blue shading indicates significant deviation of experimentally-derived EC50 from the predictive model (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	0.785	NA	NA	0.574	0.469	0.699	0.73	N	0.832	0.677	0.996	1.06	N
Cu+Mn	0.439	0.393	0.490	0.846	0.747	0.959	1.93	Y	1.23	1.09	1.39	2.80	Y
Cu+Ni	0.0180	NA	NA	0.0127	0.0112	0.0143	0.70	N	0.0204	0.0189	0.0225	1.14	N
Cu+Zn	0.0167	0.0151	0.0184	0.00934	NA	NA	0.56	N	0.0137	NA	NA	0.82	N
Co+Zn	0.609	0.492	0.753	0.496	NA	NA	0.81	N	0.679	NA	NA	1.11	N
Co+Ni	0.761	0.674	0.860	0.616	0.504	0.748	0.81	N	0.969	0.816	1.11	1.27	N
Co+Mn	1.18	1.03	1.36	1.28	1.07	1.5	1.08	N	1.76	1.52	2.05	1.49	Y
Zn+Ni	0.0248	0.0227	0.0271	0.0187	NA	NA	0.75	N	0.0310	NA	NA	1.25	N
Zn+Mn	1.20	1.05	1.37	0.76	NA	NA	0.63	N	1.18	NA	NA	0.99	N
Ni+Mn	1.25	1.12	1.41	0.865	0.766	0.976	0.69	Y	1.31	1.15	1.49	1.05	N
Al+Cu	0.399	0.345	0.460	0.175	NA	NA	0.44	N	0.245	NA	NA	0.61	N
Al+Mn	0.904	0.808	1.01	0.700	NA	NA	0.77	N	1.23	NA	NA	1.36	N
Al+Co	1.45	1.24	1.71	0.507	NA	NA	0.35	N	0.705	NA	NA	0.49	N
Al+Ni	0.427	0.357	0.510	0.186	NA	NA	0.44	N	0.255	NA	NA	0.60	N
Al+Zn	0.417	0.285	0.609	0.164	NA	NA	0.39	N	0.254	NA	NA	0.61	N

Table 5 Determination of model deviation ratios (MDR) at 50% effective concentration (EC50) for *Physa acuta* 48 h acute toxicity experiments at pH 5. Blue shading indicates significant deviation of experimentally-derived EC50 from the predictive model (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	0.501	0.367	0.684	0.617	0.536	0.709	1.23	N	0.930	0.828	1.036	1.86	Y
Cu+Mn	2.12	1.50	3.00	1.58	NA	NA	0.75	N	2.18	NA	NA	1.03	N
Cu+Ni	0.0177	NA	NA	0.0119	0.0108	0.0131	0.67	N	0.0200	0.0185	0.0216	1.13	N
Cu+Zn	0.00814	0.00646	0.0103	0.0114	0.0106	0.0124	1.41	Y	0.0187	0.0175	0.0201	2.30	Y
Co+Zn	0.795	0.728	0.867	0.730	0.644	0.822	0.92	N	1.12	1.01	1.24	1.41	Y
Co+Ni	1.18	0.93	1.50	0.679	0.587	0.783	0.58	Y	1.04	0.933	1.17	0.88	N
Co+Mn	1.85	1.5	2.27	3.11	NA	NA	1.68	N	4.86	NA	NA	2.63	N
Zn+Ni	0.0277	0.0220	0.0349	0.0242	0.0222	0.0263	0.87	N	0.0404	0.0372	0.0440	1.46	Y
Zn+Mn	1.74	1.55	1.95	1.92	NA	NA	1.10	N	2.86	NA	NA	1.65	N
Ni+Mn	1.50	1.29	1.73	1.73	NA	NA	1.16	N	2.47	NA	NA	1.65	N
Al+Cu	0.129	0.0853	0.196	0.216	0.196	0.251	1.67	Y	0.339	0.303	0.389	2.63	Y
Al+Mn	0.174	0.147	0.207	1.39	NA	NA	8.00	N	1.73	NA	NA	9.91	N
Al+Co	0.240	0.121	0.476	0.703	0.608	0.813	2.93	Y	0.957	0.845	1.088	3.98	Y
Al+Ni	0.0641	0.0131	0.313	0.233	0.207	0.262	3.64	N	0.361	0.318	0.409	5.63	Y
Al+Zn	0.0191	0.00817	0.0447	0.244	0.221	0.269	12.8	Y	0.360	0.319	0.406	18.8	Y

Table 6 Determination of model deviation ratios (MDR) at 25% effective concentration (EC25) for *Physa acuta* 48 h acute toxicity experiments at pH 7. Blue shading indicates significant deviation of experimentally-derived EC25 from the predictive model (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	0.762	NA	NA	0.389	0.290	0.518	0.51	N	0.599	0.480	0.729	0.79	N
Cu+Mn	0.355	0.267	0.470	0.637	0.514	0.789	1.80	Y	0.965	0.860	1.09	2.72	Y
Cu+Ni	0.0171	NA	NA	0.00990	0.00834	0.0117	0.58	N	0.0175	0.0163	0.0188	1.02	N
Cu+Zn	0.0116	0.0102	0.0131	0.00787	NA	NA	0.68	N	0.0116	NA	NA	1.00	N
Co+Zn	0.403	0.310	0.523	0.396	NA	NA	0.98	N	0.560	NA	NA	1.39	N
Co+Ni	0.621	0.489	0.789	0.452	0.337	0.597	0.73	N	0.795	0.603	0.911	1.28	N
Co+Mn	0.925	0.720	1.19	0.922	0.687	1.23	1.00	N	1.37	1.18	1.58	1.48	N
Zn+Ni	0.0200	0.0178	0.0226	0.0173	NA	NA	0.86	N	0.030	NA	NA	1.48	N
Zn+Mn	1.02	0.917	1.13	0.643	NA	NA	0.63	N	0.992	NA	NA	0.97	N
Ni+Mn	0.925	0.807	1.06	0.695	0.567	0.850	0.75	N	1.025	0.909	1.17	1.11	N
Al+Cu	0.327	0.250	0.428	0.154	NA	NA	0.47	N	0.237	NA	NA	0.73	N
Al+Mn	0.677	0.564	0.813	0.613	NA	NA	0.91	N	1.14	NA	NA	1.68	N
Al+Co	0.923	0.726	1.17	0.430	NA	NA	0.47	N	0.671	NA	NA	0.73	N
Al+Ni	0.279	0.216	0.362	0.175	NA	NA	0.63	N	0.248	NA	NA	0.89	N
Al+Zn	0.381	0.0947	1.54	0.159	NA	NA	0.42	N	0.246	NA	NA	0.65	N

Table 7 Determination of model deviation ratios (MDR) at 25% effective concentration (EC25) for *Physa acuta* 48 h acute toxicity experiments at pH 5. Blue shading indicates significant deviation of experimentally-derived EC25 from the predictive model (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	0.276	0.188	0.404	0.471	0.356	0.62	1.71	N	0.766	0.663	0.852	2.78	Y
Cu+Mn	1.79	1.54	2.09	1.35	NA	NA	0.75	N	1.78	NA	NA	0.99	N
Cu+Ni	0.0171	NA	NA	0.00985	0.00805	0.0120	0.58	N	0.0175	0.0162	0.0188	1.02	N
Cu+Zn	0.00473	0.00350	0.00640	0.00944	0.00776	0.0114	1.99	Y	0.0162	0.0150	0.0173	3.41	Y
Co+Zn	0.666	0.531	0.835	0.558	0.462	0.67	0.84	N	0.920	0.832	1.01	1.38	N
Co+Ni	0.737	0.581	0.935	0.524	0.431	0.632	0.71	N	0.873	0.780	0.973	1.18	N
Co+Mn	1.21	0.939	1.55	2.40	NA	NA	1.99	N	3.47	NA	NA	2.88	N
Zn+Ni	0.017	0.0130	0.0220	0.0206	0.0184	0.0231	1.22	N	0.0358	0.0333	0.0387	2.12	Y
Zn+Mn	1.26	1.08	1.48	1.68	NA	NA	1.33	N	2.40	NA	NA	1.90	N
Ni+Mn	1.26	1.00	1.59		NA	NA	0.00	N	2.14	NA	NA	1.70	N
Al+Cu	0.0791	0.0467	0.134	0.183	0.151	0.212	2.31	Y	0.296	0.265	0.338	3.74	Y
Al+Mn	0.113	0.0880	0.145	1.23	NA	NA	10.9	N	1.50	NA	NA	13.30	N
Al+Co	0.215	0.0135	3.44	0.569	0.464	0.694	2.64	N	0.826	0.732	0.934	3.84	N
Al+Ni	0.0142	0.00546	0.0369	0.202	0.173	0.236	14.2	Y	0.314	0.278	0.355	22.15	Y
Al+Zn	0.00390	0.00146	0.0104	0.209	0.183	0.239	53.7	Y	0.313	0.278	0.353	80.21	Y

Table 8 Determination of model deviation ratios (MDR) at 10% effective concentration (EC10) for *Physa acuta* 48 h acute toxicity experiments at pH 7. Blue shading indicates significant deviation of experimentally-derived EC10 from the predictive model (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	0.739	NA	NA	0.263	0.164	0.413	0.36	N	0.428	0.338	0.530	0.58	N
Cu+Mn	0.287	0.175	0.470	0.479	0.337	0.680	1.67	N	0.758	0.680	0.850	2.64	Y
Cu+Ni	0.0163	NA	NA	0.00766	0.00591	0.00991	0.47	N	0.0149	0.0141	0.0157	0.92	N
Cu+Zn	0.00803	0.00654	0.00985	0.00608	NA	NA	0.76	N	0.00979	NA	NA	1.22	N
Co+Zn	0.266	0.177	0.401	0.287	NA	NA	1.08	N	0.460	NA	NA	1.73	N
Co+Ni	0.506	0.347	0.738	0.324	0.202	0.501	0.64	N	0.642	0.435	0.741	1.27	N
Co+Mn	0.726	0.487	1.08	0.660	0.408	1.06	0.91	N	1.056	0.916	1.21	1.45	N
Zn+Ni	0.0162	0.0137	0.0191	0.0147	NA	NA	0.90	N	0.0282	NA	NA	1.74	N
Zn+Mn	0.867	0.769	0.978	0.512	NA	NA	0.59	N	0.831	NA	NA	0.96	N
Ni+Mn	0.683	0.558	0.835	0.557	0.402	0.765	0.82	N	0.801	0.715	0.908	1.17	N
Al+Cu	0.268	0.177	0.404	0.133	NA	NA	0.50	N	0.230	NA	NA	0.86	N
Al+Mn	0.504	0.383	0.662	0.530	NA	NA	1.62	N	1.06	NA	NA	2.10	N
Al+Co	0.611	0.411	0.906	0.351	NA	NA	0.57	N	0.638	NA	NA	1.05	N
Al+Ni	0.196	0.130	0.294	0.164	NA	NA	0.84	N	0.240	NA	NA	1.23	N
Al+Zn	0.347	0.0480	2.50	0.146	NA	NA	0.42	N	0.239	NA	NA	0.69	N

Table 9 Determination of model deviation ratios (MDR) at 10% effective concentration (EC10) for *Physa acuta* 48 h acute toxicity experiments at pH 5. Blue shading indicates significant deviation of experimentally-derived EC10 from the predictive model (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	0.152	0.082	0.281	0.356	0.226	0.560	2.35	N	0.629	0.530	0.699	4.14	Y
Cu+Mn	1.52	1.02	2.25	1.12	NA	NA	0.74	N	1.45	NA	NA	0.96	N
Cu+Ni	0.0163	NA	NA	0.00816	0.00582	0.0111	0.50	N	0.0153	0.0141	0.0164	0.94	N
Cu+Zn	0.0028	0.00170	0.00446	0.00779	0.00555	0.0106	2.83	Y	0.0139	0.0128	0.0149	5.06	Y
Co+Zn	0.558	0.382	0.813	0.422	0.313	0.557	0.76	N	0.754	0.681	0.831	1.35	N
Co+Ni	0.460	0.313	0.678	0.400	0.297	0.529	0.87	N	0.732	0.648	0.811	1.59	N
Co+Mn	0.788	0.531	1.17	1.76	NA	NA	2.24	N	2.47	NA	NA	3.13	N
Zn+Ni	0.0103	0.00684	0.0156	0.0176	0.0151	0.0204	1.70	N	0.0318	0.0297	0.0340	3.08	Y
Zn+Mn	0.919	0.706	1.20	1.42	NA	NA	1.55	N	2.02	NA	NA	2.19	N
Ni+Mn	1.06	0.756	1.47	1.34	NA	NA	1.27	N	1.85	NA	NA	1.75	N
Al+Cu	0.0484	0.0219	0.107	0.155	0.111	0.184	3.19	Y	0.258	0.232	0.293	5.32	Y
Al+Mn	0.0730	0.0499	0.107	1.071	NA	NA	14.7	N	1.30	NA	NA	17.8	N
Al+Co	0.192	0.00149	24.9	0.454	0.337	0.605	2.36	N	0.712	0.634	0.802	3.70	N
Al+Ni	0.00314	0.00073	0.0136	0.175	0.144	0.213	55.8	Y	0.274	0.243	0.309	87.1	Y
Al+Zn	0.000796	9.76E-05	0.00649	0.180	0.151	0.214	226	Y	0.272	0.242	0.307	342	Y

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