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Toxicity of copper, lead, and zinc mixtures to *Ceriodaphnia dubia* and *Daphnia carinata*

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ABSTRACT

Acute and chronic bioassays were conducted to determine the effects of copper, lead, and zinc mixtures on *Ceriodaphnia dubia* and *Daphnia carinata*. Copper, lead, and zinc combined at up to 5.2, 4.5, and 51.8 μg/L, respectively, did not cause significant mortality during acute exposures, although mixtures of 10.6, 9, and 101.1 μg/L and higher resulted in 65–100% mortality. Binary combinations of Cu+Zn (1.3+13.0 μg/L) and Cu+Pb (1.3+1.1 μg/L) and ternary combinations of Cu+Pb+Zn (1.3+1.1+13.0 μg/L) had a significant effect on reproduction of *C. dubia*. Toxic units and associated confidence intervals were calculated to characterize the nature of metal interactions. In most cases, and based on confidence intervals encompassing a value of 1, most of the metal interactions would be classified as additive. However, a more than additive effect was indicated by the acute tests for both species exposed to Cu+Pb, for *D. carinata* exposed to Cu+Zn, and for *C. dubia* exposed to all three metals.

1. Introduction

Industrial and municipal wastewaters and urban stormwater commonly contain combinations of metals such as copper, lead, and zinc in addition to organic residues, all of which may be directly or indirectly released into aquatic receiving systems (Logan and Wilson, 1995; Woods et al., 2002; Otitoloju, 2003; Ross et al., 2003; Birch et al., 2004; Gobeil et al., 2005). As a result, contamination of aquatic ecosystems by mixtures of pollutants is of increasing concern worldwide (Shaw et al., 2006). Unfortunately, water quality guidelines/criteria that establish limits to chemical releases are usually derived from acute and/or chronic bioassays with individual contaminants and so may fail to predict interactions and associated effects of chemicals in mixture (Birge et al., 1992; Parrott and Sprague, 1993; Otitoloju, 2003; Shaw et al., 2006).

Several models have been developed to predict mixture toxicity for both organic and inorganic contaminants (Marking, 1977; Durkin, 1981; Konemann, 1981; Birge et al., 1992; Haas and Stirling, 1994; Logan and Wilson, 1995), and these have commonly indicated additive effects (mixture toxicity is equal to the toxicity that would be expected if the proportional, independent contributions of each toxicant were simply added; Mahar and Watzin, 2005). Recent studies however, have demonstrated that while concentration addition is common, synergistic (more than additive) effects are also often being reported (Franklin et al., 2002; Otitoloju, 2002; Woods et al., 2002). For example, Forget et al. (1999) determined that binary and ternary combinations of pesticides (carbofuran, dichlorvos, malathion) and metals (arsenic, cadmium, copper) exhibited synergistic lethal effects on the marine microcrustacean, *Tigriopus brevicornis*, and Woods et al. (2002) found that chlorpyrifos, profenofos, and endosulfan in binary and ternary combinations had synergistic effects on acute survival of *Ceriodaphnia dubia*. In addition, Kraak et al. (1994) assessed the impact of copper, cadmium, and zinc on the filtration rate of the zebra mussel, *Dreissena polymorpha*, and found synergistic effects for copper and cadmium in mixture. It is the enhanced/synergistic mixture effects that are of the greatest concern for exposed organisms, since these results indicate that toxicity guidelines for individual chemicals could underestimate the overall exposure effect, and therefore would not be protective.

The purpose of this study was to evaluate the acute and chronic single-chemical and mixture effects of three common trace metal contaminants—copper, lead, and zinc—on the cladocerans, *Daphnia carinata* and *C. dubia*. The metal concentrations used bracket the current water quality guidelines for Australia and New Zealand (ANZECC, 2000) and the United States (2002a), and are also similar to levels previously observed in urban stormwater samples collected from selected locations in Adelaide, South Australia (Bidwell and Kumar, unpublished data; Kumar et al., 2002). The objective was then to compare bioassay results with metal guidelines for each region to determine the degree of protection the guidelines would have afforded to the species.

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2. Materials and methods

2.1. Metal exposures

Acute (48-h) bioassays with copper, lead, and zinc alone and in binary and ternary mixtures were conducted with *C. dubia* and *D. carinata*. Chronic bioassays (7 d) using the metal combinations were also conducted with *C. dubia*. Bioavailable metal exposure concentrations ranged from 1.3 to 81 μg/L for Cu, 1.1 to 69 μg/L for Pb, and 13.0 to 821 μg/L for Zn, with moderately hard water as the diluent. At comparable water hardness (100 mg/L $CaCO_3$), the current water quality guideline level concentrations for these metals in Australia and New Zealand (trigger values) and the United States (Criterion Continuous Concentrations—CCCs) are 3.5 and 9 μg/L Cu, 13.6 and 2.5 μg/L Pb, and 20 and 120 μg/L Zn, respectively (ANZECC, 2000; USEPA, 2002a).

2.2. Test procedures

Mass cultures of *C. dubia* and *D. carinata* were maintained in 1 L beakers in a constant-temperature room (25 ± 1 °C) with a 16 h light: 8 h dark photoperiod using white fluorescent lamps. Cultures of *C.* cf. *dubia* were originally obtained from the Centre for Ecotoxicology, Environment Protection Authority, New South Wales, Australia, and cultures of *D. carinata* were collected from the Department of Environment and Conservation, New South Wales, Australia. The cladoceran species used in this study are native to Australia. Both species were maintained in formulated moderately hard water (80–100 mg/L as $CaCO_3$) prepared according to USEPA (2002b). The culture media for *C. dubia* was supplemented with 2 μg/L selenium (as Na_2SeO_4). Daphnids were fed 3 mL of tri-algal mixture of *Pseudokirchneriella subcapitata*, *Chlamydomonas* sp., and *Ankistrodesmus* sp., (10^6–10^7 cells/mL) in addition to 2 mL of a mixture of yeast, cereal leaves, and trout chow (YCT; USEPA, 2002b). Culture water was renewed three times weekly.

Toxicity tests were conducted under the same controlled environmental conditions as used for culturing. Water quality parameters (pH, temperature, dissolved oxygen, conductivity, and water hardness as total $CaCO_3$) were measured at the beginning and the end of acute tests and before and after each renewal during chronic tests. At least three replicates from each concentration and controls were selected randomly for these measurements.

Procedures for conducting acute lethal and chronic tests were based on those described by the USEPA (2002b, c). Static acute tests were initiated by randomly distributing five neonates in each of four replicate beakers per concentration. Chronic tests were initiated by placing one neonate in each of ten replicates per concentration. Survival of brood females and production of young were used as the response variables in the chronic tests.

Metal stock solutions for all tests were prepared by dissolving analytical grade copper sulfate, zinc sulfate, and lead nitrate (Fisher Scientific Inc, Pittsburg, PA) in distilled water. The same stock solutions were used throughout the tests. For mixture toxicity tests, stock solutions of each metal were mixed just prior to renewals to give desired concentrations. Water samples were collected on Day 1, 4, and 7 to determine total metal concentrations during various mixture chronic exposures. Water samples were analysed using a Perkin Elmer Analyst 700 atomic absorption spectrometer (Perkin Elmer Inc., Wellesley, MA). Calibration standards for each metal were made by serially diluting stock solutions with reagent grade water (Perkin Elmer Inc.) and check standards were run along with samples. Detection limits for lead, copper, and zinc were 0.5 μg/L. Bioavailable metal concentrations were calculated based on measured total metal concentrations using the methods outlined in Appendix A of the USEPA Water Quality Criteria (USEPA, 2002a).

2.3. Statistical analyses

Median lethal concentrations (LC50 values) were calculated using the trimmed Spearman–Karber method (Hamilton et al., 1977). For chronic tests, the numbers of neonates from the first three broods were expressed per individual and these mean values analysed. Data were tested for normality and homogeneity of variance using Toxstat (1994). Statistical significance was determined at $\alpha = 0.05$. An analysis of variance (ANOVA) with Bonferroni (unequal replicates) or Dunnetts tests (equal replicates) was used to compare treatments and controls. An estimate of the value causing 50% reduction in the number of young produced per female (EC50) was also calculated (Toxstat, 1994).

Toxic interactions were characterized by calculating toxic units (and their corresponding confidence intervals) based on the LC50 or EC50 estimates from bioassays with mixtures and single metals (Spehar and Fiandt, 1986). Specifically, toxic units were derived by dividing the LC or EC50 estimate from the mixture by the corresponding estimate from the individual metal test. If fractions of the individual toxic effects in a mixture equal 1.0, a strictly additive action is indicated; if the total is less than 1.0, the action is more than additive and if it is greater than 1.0, the action is less than additive (Spehar and Fiandt, 1986). For example, the 48-h LC50 for *C. dubia* exposed to copper and zinc alone and in mixture was 18.0 and 208.8 and 6.11 and 72.4 μg/L, respectively. Toxic unit values for this mixture were calculated by dividing 6.11 by 18.0 for copper and 72.4 by 208.8 for lead resulting in values of 0.34 for copper and 0.35 for lead. When combined (0.34+0.35 = 0.69) the value is less than 1.0, indicating that the mixture is more than additive (synergistic). Categorization of the toxic interactions (antagonistic, additive, synergistic) in this study was determined based on whether the confidence intervals for toxic units overlapped 1.0. The LC50/EC50 values and toxic unit analyses were based on bioavailable metal concentrations.

3. Results

3.1. Water chemistry

Water quality parameters were consistent throughout the tests, ranging as follows: temperature—25.3 ± 0.5 °C; DO—88.3 ± 17.5% saturation; pH—7.5 ± 0.3; conductivity—350.2 ± 38.7 μS/cm; hardness—82.4 ± 6.1 mg/L as $CaCO_3$. For all bioassays, the measured total metal concentrations were within 10% of the nominal concentrations at all times. Bioavailable metal concentrations are used in all reported data that follow (Table 1).

3.2. Acute survival

Survival of *C. dubia* and *D. carinata* in acute exposures to metals mixed at different concentrations is presented in Table 2. No significant effects on survival of the daphnids were observed in exposures with all three metal concentrations at or below the current Australian and US water quality values. There was also no significant effect observed at the 5.2 μg/L Cu–4.5 μg/L Pb–51.8 μg/L Zn treatment even though the copper and zinc concentrations exceeded the Australian limits and the lead concentration exceeded the US limits. Survival was significantly reduced in all treatments containing 10.6 μg/L Cu–9.0 μg/L Pb–101.1 μg/L Zn and higher. In these mixtures, at least two of the metals, exceeded the Australian and US limits.

3.3. Chronic survival

As with the acute exposures, survival of *C. dubia* was not significantly affected by chronic exposure to any treatments (single metals or binary and ternary mixtures) in which the concentration of the constituents did not exceed either the Australian or the US water quality criteria (Table 3). In some treatments, there was also no effect on survival even though individual metal levels significantly exceeded one or both of the limits. For example, 90% of the test organisms survived the 7-d exposure to 9.0 μg/L Pb, which is 3.6 times higher than the US guideline for that metal. Survival in the 5.2 μg/L copper+51.8 μg/L zinc mixture was also 90% even though both of these levels exceed the respective Australian guideline values. In contrast, survival

Table 1
Average nominal, measured, and bioavailable concentrations (μg/L) of copper, lead, and zinc during various exposures.

Nominal concentration	Measured total concentration (bioavailable concentration)		
	Copper	Lead	Zinc[a]
Control	1.0 (0.8)	<0.1	2.0 (2.0)
1.25/12.5[a]	1.3 (1.3)	1.3 (1.1)	13.2 (13.0)
2.5/25[a]	2.7 (2.6)	2.5 (2.1)	25.5 (25.1)
5.0/50[a]	5.4 (5.2)	5.5 (4.5)	52.5 (51.8)
10.0/100[a]	11.0 (10.6)	11.0 (9.0)	102.5 (101.1)
20.0/200[a]	21.3 (20.5)	21.8 (17.9)	219.5 (216.4)
40.0/400[a]	40.7 (39.1)	42.6 (34.9)	410.7 (405.0)
80.0/800[a]	84.1 (80.7)	83.9 (68.7)	832.5 (820.9)

[a] Values followed by slash represent nominal concentrations for zinc. Values in parenthesis are the calculated bioavailable concentrations of each metal.

Table 5
48-h LC50 values (*C. dubia* and *D. carinata*) and 7-d EC$_{50}$ values (*C. dubia* reproduction) for copper, lead, and zinc individually and in binary and tertiary mixtures.

Metal combinations	*C. dubia*		*D. carinata*
	48-h LC50 (μg/L)	7-d EC50 (μg/L)	48-h LC50 (μg/L)
Copper	18.0 (14.7–21.8)	1.8 (1.6–2.1)	37.3 (29.1–47.5)
Lead	208.8 (160.1–272.2)	5.1 (3.5–7.5)	444.0 (330.2–597.1)
Zinc	173.5 (130.6–232.4)	21.8 (11.5–30.3)	339.8 (263.4–438.6)
Copper+lead	6.11 (4.9–7.6)	1.1 (0.8–1.6)	10.2 (7.9–13.2)
	72.4 (54.4–98.5)	0.9 (0.6–2.2)	114.4 (87.4–159.4)
Copper+zinc	6.72 (3.7–12.3)	1.1 (0.9–1.4)	16.7 (12.3–22.6)
	67.5 (37.0–123.3)	11.2 (5.4–16.9)	145.8 (107.9–197.3)
Lead+zinc	109.7 (83.4–147.8)	1.6 (1.0–2.5)	240.1 (174.8–326.5)
	93.0 (67.7–129.2)	20.0 (9.6–29.2)	182.0 (138.7–238.8)
Copper+lead+zinc	3.9 (3.1–5.0)	(0.9–1.3)	13.1 (9.5–18.0)
	32.6 (24.7–49.9)	0.9 (0.6–1.5)	148.2 (106.6–205.9)
	55.8 (39.7–71.2)	10.6 (5.1–15.2)	114.2 (83.1–157.0)

Numbers in parentheses are 95% confidence intervals.

Table 6
Toxic units derived from acute and chronic bioassays with *C. dubia* and *D. carinata* exposed to binary and tertiary combinations of metals.

Metal combinations	*C. dubia*		*D. carinata*
	Acute (μg/L)	Chronic (μg/L)	Acute (μg/L)
Copper+lead	0.34 (0.33–0.35)	0.58 (0.47–0.77)	0.27 (0.26–0.28)
	0.35 (0.34–0.36)	0.18 (0.16–0.29)	0.26 (0.26–0.27)
Sum	0.69 (0.67–0.71)	0.76 (0.63–1.06)	0.53 (0.52–0.55)
Copper+zinc	0.37 (0.25–0.56)	0.58 (0.53–0.68)	0.45 (0.42–0.48)
	0.39 (0.28–0.53)	0.52 (0.47–0.56)	0.43 (0.41–0.45)
Sum	0.76 (0.53–1.09)	1.10 (1.00–1.24)	0.88 (0.83–0.93)
Lead+zinc	0.53 (0.52–0.54)	0.32 (0.28–0.34)	0.54 (0.53–0.55)
	0.54 (0.52–0.56)	0.92 (0.83–0.96)	0.54 (0.53–0.55)
Sum	1.07 (1.04–1.10)	1.24 (1.11–1.30)	1.08 (1.06–1.10)
Copper+lead+zinc	0.22 (0.21–0.23)	0.58 (0.53–0.64)	0.35 (0.32–0.38)
	0.16 (0.15–0.18)	0.18 (0.16–0.20)	0.33 (0.32–0.34)
	0.32 (0.30–0.35)	0.48 (0.44–0.50)	0.34 (0.32–0.36)
Sum	0.70 (0.68–0.76)	1.24 (1.13–1.34)	1.02 (0.96–1.08)

Numbers in parentheses are 95% confidence intervals.

was suggested for reproduction of *C. dubia* exposed to Cu+Zn; however the confidence intervals for these interactions also overlapped with 1.0; so an additive effect would be assigned to this interaction as well.

4. Discussion

In the present study, no significant effects on acute survival occurred for either *C. dubia* or *D. carinata* when exposed to single metals at or below the Australian trigger or US water quality criteria values (CCC), indicating protection of these organisms under single exposure conditions for short durations (48 h). As with the acute studies, no effects on *C. dubia* 7-d (chronic) survival occurred below the ANZECC trigger or USEPA CCC values. The effect concentrations determined in this study are within ranges found in previous studies assessing the impact of metals on cladocerans; 7–78 μg/L Cu (Naddy et al., 2002; Banks et al., 2003; Mahar and Watzin, 2005; Boeckman and Bidwell, 2006), 19–>2700 μg/L Pb (Spehar and Fiandt, 1986; Schubauer-Benigan et al., 1993; Jak et al., 1996), 1.2–416 μg/L Zn (Schubauer-Benigan et al., 1993; Gillespie et al., 1999; Hyne et al., 2005; Mahar and Watzin, 2005; Shaw et al., 2006).

Of interest to note is the wide range of toxicity values determined in this and prior studies assessing the impacts of metals on cladoceran species. Shaw et al. (2006) attributed some of this inter-laboratory variability to different source populations and differences in culture and test design/techniques. Another potential cause, water hardness (which was not standardized in the results presented from previous research), has also been observed in previous studies to contribute to large variations in toxicity data, with sensitivity to organisms increasing as hardness decreased (Naddy et al., 2003; Sciera et al., 2004). Other water chemistry factors, including pH, alkalinity, and the presence of dissolved organic carbon have also been determined to have a significant effect on the toxicity of metals to cladocerans (De Schamphelaere and Janssen, 2002, 2004; Paquin et al., 2002; Santore et al., 2001; Hyne et al., 2005), with these effects differing significantly between metals and water chemistry parameters (Hyne et al., 2005) Additionally, species sensitivity could be a factor, as previous studies comparing toxicant sensitivity between cladocerans have determined that *Daphnia* sp. are generally more tolerant to pollutants than *Ceriodaphnia* sp. This increase of tolerance has been attributed to *Daphnia* possibly being adapted to withstand more severe fluctuations in environmental conditions that may provide a greater ability to tolerate toxicant stress (Koivisto, 1995; Shaw et al., 2006).

In the present study, 48-h acute survival of *C. dubia* and *D. carinata* exposed to mixtures of lead, zinc, and copper was not significantly reduced when levels of all metals fell below either the US or Australian regulatory limits. Survival was also not affected in some exposures that had a single metal concentration exceeding either the Australian or US regulatory values. A similar result was observed for the *C. dubia* 7-d survival data, with no effects on survival occurring when all metal exposure concentrations fell below the CCC and trigger values.

Of greater importance are those cases in which the concentration of a mixture constituent was below its regulatory value but the mixture had an effect. For example, the treatment at which acute survival effects were first observed, 11 μg/L Cu+11 μg/L Pb+102.5 μg/L Zn, included at least one metal that fell below a trigger value or a CCC (copper and zinc for the Australian trigger values and copper and lead for the US CCC). Survival was also impaired in the 5.5 μg/L Cu+5.4 μg/L Pb+52.5 μg/L treatment from the 7-d *C. dubia* chronic test, even though the levels of both copper and zinc in this mixture were below their respective trigger values and lead was below the CCC value.

This issue became even more pronounced for the *C. dubia* reproduction data, where effects were observed in metal combinations with all constituent levels below their guideline values. Effects of some individual metals were also observed even when their levels were below the US or Australian limits. For example, the metal concentrations in the binary mixture of copper and zinc were 7 × and nearly 10 × less than the hardness-adjusted CCC for these metals. The implication here is that regulatory limits for individual metals may not be sufficiently protective, particularly when the element is occurring in a mixture. It must be noted however, that in recent studies water hardness has been found to have negligible effects on copper toxicity to cladocerans (De Schamphelaere and Janssen, 2002; Hyne et al., 2005; Markich et al., 2005). As a result Markich et al. (2005) suggest that the hardness-corrected algorithm developed as part of the ANZECC guidelines (ANZECC, 2000) is not recommended for assessing copper toxicity to *Ceriodaphnia* or other sensitive freshwater species, including other cladocerans. If this algorithm is not used then the guideline value for copper in freshwater systems is 1.4 μg/L rather than the 3.5 μg/L used in this study. In terms of this was impaired in the 4.5 μg/L lead+51.8 μg/L zinc treatment, with the lead concentration below the respective Australian levels. Survival was also impaired in all ternary treatments that had a metal level exceeding the Australian or US regulatory limits.

3.4. *Chronic reproduction*

There was no significant difference in the number of neonates produced in the three broods by the *C. dubia* control groups from any of the bioassays conducted, with average production ranging from 18.6 and 20.5 neonates per daphnid (Table 4). Reproduction of *C. dubia* was significantly impaired in single metal exposures for both copper and zinc at concentrations below both the Australian and US guideline levels for these metals. Similarly, a reproductive effect from exposure to lead alone was observed at a concentration below the Australian value for this metal. Impaired reproduction was also observed in all binary mixtures of copper and lead ($\geqslant$1.3 μg/L for Cu and $\geqslant$1.1 μg/L for Pb) and copper and zinc ($\geqslant$1.3 μg/L for Cu and $\geqslant$13.0 μg/L for Zn) and in all ternary combinations of the metals. These effects were observed at concentrations well below the regulatory limits for both countries.

Table 2
Survival of *C. dubia* and *D. carinata* in 48-h acute exposures to copper, lead, and zinc.

Bioavailable metal concentration (μg/L)			48-h Survival (%)	
Cu	Pb	Zn	*C. dubia*	*D. carinata*
1.0	<0.1	2	100	100
1.3	1.1	13.0	90	90
2.6	2.1	25.1^{a}	100	80
5.2^{a}	4.5^{b}	51.8^{a}	90	85
10.6^{a,b}	9.0^{b}	101.1^{a}	35^{c}	45^{c}
20.5^{a,b}	17.9^{a,b}	216.4^{a,b}	5^{c}	30^{c}
39.1^{a,b}	34.9^{a,b}	405.0^{a,b}	0^{c}	0^{c}
80.7^{a,b}	68.7^{a,b}	820.9^{a,b}	0^{c}	0^{c}

[a] Concentration exceeds Australian Water Quality Guideline trigger value (95% level of protection, hardness of 100 mg/L $CaCO_3$).

[b] Concentration exceeds US Criterion Continuous Concentration.

[c] Survival significantly different from control at $\alpha = 0.05$.

3.5. *Metal interactions*

In order to evaluate the joint action of the metals, an estimate of the median lethal effect concentration (LC50 and EC50) was calculated for metals as single entities and in mixture (Table 5). For the acute exposures, copper was the most toxic to both species followed by zinc and then lead, with *C. dubia* being more sensitive than *D. carinata*. For chronic effects on *C. dubia* reproduction, copper was again the most toxic, followed by lead, then zinc.

The median effect concentrations were also used to calculate toxic units as a way to characterize metal interactions in the mixtures (Table 6). Using this approach, a more than additive effect (synergistic) effect (ΣTU for mixture constituents <1) was indicated by the acute tests for both species exposed to Cu+Pb and for *D. carinata* exposed to Cu+Zn, while a less than additive interaction was indicated for both organisms in acute exposure to Pb+Zn. Acute exposure to the mixture of all three metals leads to a more than additive effect on *C. dubia*. For reproduction of *C. dubia*, the sum of the toxic units indicated a less than additive effect for Pb+Zn, Cu+Zn, and Cu+Pb+Zn combinations during the chronic exposure. For acute survival of *C. dubia* exposed to Cu+Zn, the sum of toxic units was less than 1, but the confidence intervals for these interactions overlapped 1.0, so an additive effect would be assigned to the interaction. Similarly, a more than additive effect

Table 3
Survival of *C. dubia* in a 7-d chronic bioassay of copper, lead, and zinc alone and in combinations.

Bioavailable metal concentration (μg/L)			7-d Survival (%)						
Cu	Pb	Zn	Cu	Pb	Zn	Cu+Pb	Cu+Zn	Pb+Zn	Cu+Pb+Zn
1.0	<0.1	2	100	100	90	100	90	100	100
1.3	1.1	13.0	100	100	100	80	90	100	100
2.6	2.1	25.1^{a}	80	100	100	90	90	90	90
5.2^{a}	4.5^{b}	51.8^{a}	80	100	80	80	90	60^{c}	60^{c}
10.6^{a,b}	9.0^{b}	101.1^{a}	60^{c}	90	70	50^{c}	70	50^{c}	30^{c}
20.5^{a,b}	17.9^{a,b}	216.4^{a,b}	40^{c}	30^{c}	10^{c}	50^{c}	70	40^{c}	0^{c}

[a] Concentration exceeds Australian Water Quality Guideline trigger value (95% level of protection, hardness of 100 mg/L $CaCO_3$).

[b] Concentration exceeds US Criterion Continuous Concentration.

[c] Survival significantly different from control at $\alpha = 0.05$.

Table 4
Average ($\pm$SD, $n = 10$) number of neonates produced per brood female *C. dubia* in a 7-d chronic bioassay of copper, lead, and zinc alone and in combination.

Bioavailable metal concentration (μg/L)			7-d Reproduction						
Cu	Pb	Zn	Cu	Pb	Zn	Cu+Pb	Cu+Zn	Pb+Zn	Cu+Pb+Zn
1.0	<0.1	2	19.3$\pm$2.6	20.5$\pm$3.4	19.9$\pm$2.2	19.8$\pm$2.3	18.9$\pm$2.6	18.6$\pm$2.7	19.2$\pm$2.6
1.3	1.1	13.0	13.1$\pm$3.1	17.4$\pm$3.5	12.1$\pm$5.1	8.3$\pm$5.6^{c}	8.3$\pm$3.9^{c}	13.4$\pm$4.5	8.0$\pm$3.7^{c}
2.6	2.1	25.1^{a}	5.4$\pm$4.1^{c}	16.6$\pm$6.9	9.3$\pm$5.9^{c}	8.4$\pm$4.1^{c}	5.3$\pm$3.2^{c}	6.8$\pm$5.4^{c}	5.8$\pm$4.1^{c}
5.2^{a}	4.5^{b}	51.8^{a}	8.0$\pm$5.0^{c}	11.6$\pm$5.6^{c}	8.5$\pm$5.8^{c}	4.2$\pm$3.9^{c}	4.7$\pm$1.9^{c}	5.6$\pm$6.4^{c}	1.7$\pm$3.2^{c}
10.6^{a,b}	9.0^{b}	101.1^{a}	3.4$\pm$3.4^{c}	6.2$\pm$7.3^{c}	8.1$\pm$0.7^{c}	2.3$\pm$2.8^{c}	2.1$\pm$1.9^{c}	2.1$\pm$3.2^{c}	0.9$\pm$1.5^{c}
20.5^{a,b}	17.9^{a,b}	216.4^{a,b}	2.7$\pm$4.5^{c}	1.7$\pm$3.4^{c}	1.8$\pm$5.3^{c}	2.0$\pm$2.5^{c}	1.3$\pm$1.3^{c}	4.3$\pm$5.7^{c}	0.0$\pm$0.0^{c}

[a] Concentration exceeds Australian Water Quality Guideline trigger value (95% level of protection, hardness of 100 mg/L $CaCO_3$).

[b] Concentration exceeds US Criterion Continuous Concentration.

[c] Reproduction significantly different from control at $\alpha = 0.05$.

study this would result in no significant effects on *C. dubia* reproduction at copper (single metal exposure) concentrations below the Australian guideline levels. In the mixtures assessed in this study however, even at this lower guideline concentration of 1.4, there is still a significant impact on reproduction for all metal combinations. As one of the aims of this paper was to compare the current guidelines for each region as is, we decided to present our results with the hardness corrections in place.

In the present study, LC50 and EC50 data were used to determine the interactions of metals on the basis of reproduction for *C. dubia*. Using this approach, a more than additive effect was indicated by the acute tests for both species exposed to Cu+Pb, for *D. carinata* exposed to Cu+Zn, and for *C. dubia* exposed to all three metals in mixture. For *C. dubia* reproduction an additive effect was observed for Cu+Pb, while interactions tended toward less than additive for Cu+Zn, Pb+Zn, and Cu+Pb+Zn.

Our findings are similar to those determined in other studies on mixture effects in cladocerans. Mahar and Watzin (2005) assessed the impacts of mixtures of copper, zinc, and diazinon on *C. dubia* survival and reproduction, and determined that for the binary mixture of copper and zinc there was less than additive effects on survival, but for reproduction a more than additive effect was observed. Spehar and Fiandt (1986) assessed the impact of the maximum acceptable toxicant concentrations (MATCs, based on the 1986 EPA Water Quality Criteria; USEPA, 1986) of a mixture of six metals (arsenic, cadmium, chromium, copper, mercury, and lead) on *C. dubia* acute survival and reproduction. The authors found nearly strictly additive effects for the metal mixture on acute survival and reproduction at the MATC concentrations. However, adverse effects on daphnid reproduction were observed at one third the MATC concentrations when in a mixture, indicating that single-chemical water quality criteria were not sufficient to protect some species when other toxicants are present. Shaw et al. (2006) compared toxicity of four daphnid species (*C. dubia, D. magna, D. ambigua*, and *D. pulex*) to mixtures of zinc and cadmium. Differences in response were observed when zinc was held at the LC15 value and combined with cadmium at the LC50 and LC85 values. In general the responses indicated less than additive effects under these conditions with the exception of *D. magna*, which had greater than additive effects at the LC15 value for each metal and additive effects for all other combinations.

Metal interactions may be influenced by the species being tested, the combination of metals, or water quality (European Inland Fisheries Advisory Commission, 1980; Kraak et al., 1994; Preston et al., 2000; Hagopian-Schlekat et al., 2001; Otitoloju, 2002). Spehar and Fiandt (1986) indicated that the same combination of metals (arsenic, cadmium, chromium, copper, mercury, and lead) showed different interactive effects depending on both the species exposed and the endpoint tested. These authors found that acute exposure of fathead minnows (*Pimephales promelas*) to metal mixtures resulted in more than additive effects whereas chronic exposures were less than additive. However, when daphnids were used as the test organism the effects were almost strictly additive and nearly strictly additive for acute and chronic exposures, respectively (Spehar and Fiandt, 1986).

5. Conclusion

The development of water quality guidelines/criteria is a continuous process that needs to be revised periodically as new data become available. In the present study, adverse effects were observed in mixtures of metals at water quality guideline levels, which indicate the importance of considering chemical interactions in water quality management. The effects of mixtures at sublethal levels are of particular importance since chronic exposures may be allowed to persist continuously in some natural environments and these may affect some aquatic biota.

The data generated in this study reiterate the results of previous studies that have reported effects of metal mixtures on aquatic species even when individual metal concentrations are at maximum acceptable concentrations or are at no-observable effect concentrations (Spehar and Fiandt, 1986; USEPA, 2003). In addition, these results reinforce statements made by previous authors that longer duration exposures and toxicity tests examining mixture effects need to be considered when determining guideline levels for contaminants (Spehar and Fiandt, 1986; Biesinger et al., 1986).

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