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NICKEL AND COPPER MIXTURE TOXICITY TO DAPHNIA IN SOFT WATER

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by

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Abstract:

Industrially important metals, such as Cu and Ni, sometimes are present at elevated concentrations in lakes, including those in the Sudbury, ON region. Although they are essential metals, their divalent-cation state (Cu^{2+} and Ni^{2+}) can be toxic at high concentrations in the water. The free-ion toxicity of each of these metals has been studied in isolation, but rarely as a mixture. The economic importance of Cu^{2+} and Ni^{2+} makes them essential to study in the context of mixture toxicity. The objectives were to: (1) determine Cu and Ni mixture toxicity to *Daphnia* through acute LC50 tests; (2) determine the appropriate model (concentration addition, independent action, or toxic units) to analyze mixture effects; (3) determine how the toxicity modifying factor, dissolved organic carbon (DOC), influences toxic responses. These metals are transported across the membrane through different mechanisms, therefore mixture effects were hypothesized to be additive and follow an independent action (IA) model. Results indicate that Ni-Cu mixtures can be additive, synergistic or antagonistic depending on the concentration of metals. Most combinations tested produced a less-than-additive effect according to the IA model. This finding was also supported by the toxic unit approach. Single-metal acute tests revealed that the 48h LC_{50} for Cu was 2.43 µg/L (95% CI 2.15-2.82 µg/L) while Ni LC_{50} was 995 µg/L (877- 1125 µg/L). DOC was protective against Cu only and Cu+Ni mixture exposures but not Ni alone. DOC protection for mixtures varied by source composition. Clearwater Lake DOC was the most protective, Daisy Lake was intermediate, and Luther Marsh was least protective against Ni-Cu mixtures.

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1. Introduction:

1.1 Copper and Nickel in the Environment

In natural environments, organisms are frequently exposed to mixtures of contaminants. Over 7000 lakes around Sudbury (Ontario, Canada) were contaminated by metal and acid emissions from long term mining and smelting activity (Keller et al., 2007). Consequently, many lakes in the region became unsuitable for aquatic life. For example, in 1974 the Cu and Ni concentrations in Hannah Lake, Sudbury, were over 1000 μg/L (Yan et al, 1996). Over the last three decades emission controls as well as whole lake and watershed treatment (e.g. liming) have improved water quality and much of the aquatic life, including zooplankton, has returned. However, metal contamination in this region is still ongoing and Cu and Ni concentrations remain elevated in some lakes (Keller et al, 2007). Cu and Ni have been identified as factors limiting growth and the recovery of zooplankton diversity to return to levels found in reference lakes (Yan et al., 1996, 2004).

It has been widely established that Cu and Ni are both essential micro-nutrients for the biological functioning and growth of organisms, particularly within enzymatic and metabolic reactions (Rainbow, 2002; Muyssen et al, 2004). Cu plays a functional role in the respiratory protein haemocyanin, and thus is required in metabolically available form (Rainbow, 2002), while Ni is an essential component of enzymes (e.g. urease) and aids in processes such as lipid metabolism (Anke et al, 1984; Phipps et al, 2002; Anke et al, 1995; Stokes, 1988).

Cu and Ni are naturally occurring elements that can be found in all environments and biota. It is well-known that Cu speciation affects bioavailability and toxicity in a variety of

aquatic organisms. The free ion ($Cu^{2+)}$ and $Cu(OH)_2$ are considered to be highly toxic forms, whereas other complexes and particulate bound Cu are significantly less toxic (Cuppet et al, 2006). In freshwater, naturally occurring Cu concentrations range between 0.2 µg/L to 30 µg/L (USEPA, 2012). The exposure concentration associated with 50% lethality (LC_{50}) ranges from 0.005 to 1 mg/L depending on the aquatic organism and its life stage (Hodson et al, 1979; USEPA, 2012). Similar to Cu, the divalent form of dissolved nickel (Ni^{2+}) is the most toxic form found in surface waters (ATSDR, 2011b). Naturally occurring concentrations of Ni in surface waters are between 0.5 and 10 µg/L (CCME, 1987; ATSDR, 2011b; Astrom and Bjorklund, 1996; Zwolsman and van Bokhoven, 2007). The Canadian Water Quality Guidelines (CWQG) for Ni and Cu vary according to water hardness. The CWQG is 25 µg/L and 2 µg/L for Ni and Cu respectively, when water hardness is not known (CCME, 1987a, b).

Both Cu and Ni are industrially important metals. They are released into water systems from industrial and agricultural wastes leading to elevated metal concentrations in the environment. There are many sources of anthropogenic Cu inputs to the environment. For example, elevated Cu comes from mining and smelting for the production of metals and alloys (ATSDR, 2011 a). Agricultural inputs of Cu include $Cu(SO_4)$, which is used in fungicides, algaecides, and nutritional supplements (ATSDR, 2011a). Ni is used in conjunction with Cu, zinc, chromium, and iron for the production of nearly 3000 alloys, which have over 250,000 applications including coins and jewelry (ATSDR, 2011b; CCME, 1999). Examples of other Ni uses are batteries, electroplating and ceramic colours (ATSDR, 2011a).

1.2 Mechanisms of toxicity

The mechanism of toxicity differs for both metals, and in general mechanisms of chronic toxicity are not well understood. The acute toxicity of Cu is associated with disruption of Na balance. Cu crosses apical membranes through the Na^+ channel, there is competition between Cu^{2+} and Na^+ ions and therefore increased Cu results in reduced Na uptake (Grosell and Wood, 2002; Leitao et al, 2013). While the uptake mechanisms for Cu are well studied in terms of toxicity, mode of action and bioavailability, these measures are not well understood for Ni (Keithly et al., 2004). Ni disrupts Mg^{2+} balance in *D. magna*, for example exposure to 694 µg/L Ni in moderately soft water (45 mg/L CaCO3) for 48 h resulted in significant reduction in whole-body Mg (Pane, 2003). Chronic Cu exposure can induce the generation of reactive oxygen species (ROS) that are responsible toxic responses (Pourahmad and O'Brien, 2000). Ni is also identified as an oxidative stress inducer which causes depletion of glutathione (Rodriguez et al., 1996). It causes gene expression changes in cell growth, differentiation and apoptosis in *Xenopus* oocytes as a result of changes to intracellular Ca^{2+} balance (Valko et al., 2005). In oocytes Ni is recognized as a Ca^{2+} channel blocker in (Zamponi et al., 1996; Lee et al., 1999). The influence of external factors on toxic responses will vary as a result of different mechanisms of uptake and toxicity.

1.3 Natural Organic Matter (NOM)

Metals associated with inorganic or organic ligands are less bioavailable due to complexation, and thus are less toxic. Natural organic matter (NOM) plays an important role in controlling metal speciation and the potential for effects (Luider et al, 2004). NOM can sequester metals and determine their fate and transport throughout the aquatic system (Steinberg et al,

2003; Winter et al., 2007). The sequestration of metals such as Cu and Ni makes them less available for uptake through cellular membranes (Mandal et al, 2002; Meyer et al, 1999).

The toxicity mitigating properties vary by NOM source because each ecosystem is unique, and the composition of NOM is linked to terrestrial characteristics as well as seasonal variation (Schwartz et al, 2004; Wood et al, 2012; Livingstone et al, 2013). Photochemical changes are also known to destroy NOM by reducing dissolved organic carbon (DOC) concentration and affecting NOM quality (Winter et al., 2007). In addition to mitigating metal toxicity, NOM can also affect light conditions in the water by absorbing ultraviolet (UV) and visible light (Jones and Arvola, 1984; Huovinen et al., 2000). Absorbing UV light can cause photodegradation, which alters the NOM composition (Steinberg et al, 2003; Winter et al., 2007; Reddy and De Laune, 2008).

Natural organic matter (NOM) is found in water systems and is formed by the decomposition of plant and animal materials (Steinberg et al, 2003). Aquatic dissolved organic matter (DOM) primarily contains fulvic and humic acids (50-90%, Thurman, 1985). DOM is quantified as dissolved organic carbon (DOC) and is a term used to describe dissolved compounds below 0.45 micrometers. DOC can be classified as allochthonous (terrigenous) or autochthonous. Allochthonous DOC is primarily composed of humic and fulvic acids (McKnight et al., 2001). Autochthonous DOC is derived from bacteria and algae in the water column. This type of DOC has a lower aromatic content and is made from aliphatic and nitrogenous groups (Wood et al., 2011; McKnight et al., 2001). The aromatic groups are associated with stronger binding of metals, hence allochthonous DOC is considered to be more protective against metal toxicity (Klink et al., 2005; Schwartz et al., 2004).

Although the mitigating effects of DOC are recognized, they are still poorly understood. Cu has stronger binding affinity for DOC (Schwartz et al, 2004; DeSchamphelere et al, 2002) than Ni (Kozlova et al, 2009; Deleebeeck et al, 2008). Numerous studies have shown that DOM source can cause up to a 4-fold difference in toxic effects of Cu (Al-Reasi et al., 2012; Richards et al., 2001; Schwartz et al., 2004; Gheorghiu et al., 2010). Conversely, Doig and Liber (2006) showed that acute toxicity of Ni to *H. azteca* was not significantly affected by DOC source or composition. Algae is also used in *in vitro* studies and acts as an organic ligand thus playing an important role in regulating metal toxicity by binding to metals and reducing their bioavailability (Komjarova and Blust, 2009). Therefore, increasing the amount of carbon in a system through algae or DOC can reduce metal toxicity to daphnids.

1.4 Mixture Toxicity

Although free ion concentrations of Ni^{2+} and Cu^{2+} have been studied, the toxic effects of those two metals as a mixture is not well understood. The toxic effects of metal mixtures can be additive, synergistic, or antagonistic (ECETOC, 2001). The term additive is defined as an effect in which the combination of two substances produce a total effect which the same as the sum of the individual effect (Meyer et al, 2014). A synergistic interaction occurs when the effect is greater than additive, whereas an antagonistic interaction means that it is less than additive (ECETOC, 2001). Mixtures make environmental hazard assessment difficult due to possible interactions that can occur between chemicals (Loureiro et al, 2010).

To understand mixture toxicity, there are six terms that are frequently used:

(1) **Interactive**: one or more chemicals influence the biological activity of the other substance in

the mixture. Responses can be synergistic or antagonistic (Meyer et al., 2014).

(2) **Non-interactive**: none of the chemicals in the mixture influence the biological activity of the other. Responses are additive and can follow a concentration addition or independent action model (Meyer et al., 2014).

(3) **Similar joint-action**: both metals in the mixture have a similar site of toxic action (Olmstead and LeBlanc, 2005; Jonker et al., 2005).

(4) **Dissimilar joint-action**: both metals in the mixture have different sites of toxic action (Olmstead and LeBlanc, 2005; Jonker et al., 2005).

(5) **Concentration addition**: Occurs if the two metals are interactive with similar joint-action (Barata et al., 2006; Ferreira et al., 2008; Loureiro et al., 2009; Olmstead and LeBlanc, 2005).

(6) **Independent action**: Also known as response addition, this occurs if the two metals are non-interactive with dissimilar joint-action (Barata et al., 2006; Ferreira et al., 2008; Loureiro et al., 2009; Olmstead and LeBlanc, 2005; Jonker et al., 2005).

As mentioned previously, there are few studies available on the effects of Cu and Ni mixtures. In a recent study with the amphipod *Gammarus pulex*, Charles et al. (2014) showed that mixtures under some exposure conditions Ni-Cu mixtures behaved synergistically. However, under low Ni exposure conditions, the response was antagonistic (Charles et al, 2014). Therefore, understanding mixture toxicity becomes difficult because of the different interactions that can take place between the two metals and their respective ligand sites, or amongst the metals themselves. Two modes of action are proposed in Fig 1.1 and 1.2. If Ni^{2+} and Cu^{2+} enter the cellular membrane through different transport sites (Fig 1.1), then their toxicity is thought to be additive and non-interactive between metals. In this case, an independent action model may be used. If they enter through the same site (Fig 1.2) then their toxicity is can be synergistic or

antagonistic with a competitive interaction. In this scenario a concentration addition model may be used.

1.5 . Test Organism

Metal toxicity has been well documented in *Daphnia* spp. and they are a useful ecological model organism for toxicology testing (Lampert, 2010). They are found in both lakes and ponds and considered keystone species in aquatic ecosystems. As *Daphnia* hybrids are common in nature (Hebert and Flinston, 1996) a *Daphnia pulex-pulicaria* hybrid native to McFarlane Lake in Sudbury was used in this study. This study is directed towards understanding the ongoing recovery of sensitive invertebrates in Sudbury lakes, and therefore this hybrid provides a relevant model for study. Sudbury lakes are soft-water lakes and none of the commercially available invertebrate organisms can tolerate the low levels of calcium associated with these lakes. It is standard knowledge that Ca and other hardness cations (e.g. Mg), can ameliorate toxic effects. Therefore conducting toxicity tests with low Ca levels allows a better representation of toxic effects in Sudbury and Canadian boreal lakes.

This hybrid satisfies the other requirements for a good model organism for toxicity testing; it has a high survival rate, high reproduction rate, and good brood size (Environment Canada, 1999). It becomes a mature adult around day 5-7 and has its first brood at day 10. Day 10 onwards, it reproduces every second day with a brood size of approximately 8-10 neonates when fed algae at 2 mg C/L daily. Therefore, it is a good test species for acute and chronic bioassays.

1.6 Purpose and Hypotheses

Sudbury has a history of mining and smelting activity which began before the turn of the 20th century and grew into one of the largest metal-producing complexes in the world (Keller et al, 2007) and lakes in and around Sudbury have been contaminated by Ni^{2+} and Cu^{2+} (Keller et al. 1999, 2007). The overarching goal of this study is to understand the effects of Ni and Cu mixtures in the context of these lakes. The objectives of this study are to:

(1) Determine Ni and Cu mixture toxicity to *Daphnia pulex-pulicaria* hybrids. Ni and Cu are transported across the membrane through different transport channels, therefore their effects as a mixture are hypothesized to be additive.

(2) Determine the type of mixture model (concentration addition or independent action). Cu uptake and toxicity will follow a Na^{+} channel pathway, whereas Ni toxicity will follow a Ca^{2+} pathway. If Ni^{2+} and Cu^{2+} enter the cellular membrane through different transport sites (Fig 1), then their toxicity is hypothesized to be additive and non-interactive between metals. Therefore, an independent action model should be used.

(3) Determine how the toxicity modifying factor, DOC, influences responses. Cu has a higher binding affinity to DOC than Ni, therefore, DOC should be more protective against Cu toxicity.

2. Methods

2.1 Cultures

Daphnia pulex-pulicaria were obtained from existing cultures at The Field Laboratory for the Assessment of Multiple Ecological Stressors (FLAMES) lab, Dorset Environmental Science Center, Ontario Ministry of the Environment, Dorset, Ontario. These cultures were established from samples collected from McFarlane Lake in Sudbury, ON in 2006. The cultures were

renewed with new neonates (<24 hrs old) every 2 weeks so that only third brood neonates were used in studies. The cultures were kept at a constant temperature of 21°C with 16:8 hour light: dark photoperiod (TPCB-19, BioChambers Inc.,Winnipeg, Manitoba). The daphnids were cultured in FLAMES medium (Celis et al., 2008; Table 2.1). The pH of culture water ranged from 6.3 to 6.7. They were fed daily with 70:30 ratio of *Pseudokirchneriella subcapitata* to *Ankistrodesmus falcatus* algae. The algal food was prepared to contain 3.5 $x10^7$ cells/ml of *Pseudokirchneriella*, and 1.5$x10^7$ cells/ml of *Ankistrodesmus* and it was fed to achieve 1mg C/L on days 1 and 2, 1.5 mg C/L on days 3 to 7, and mg C/L after the first week. The relationship between cell density and absorbance at 660 nm was used to establish the equivalent carbon count in order to calculate the volume of algae to feed (Porter et al, 1982; Mitchell et al, 1992; Goulet et al, 2007).

The required optical density (OD) was calculated from the equation derived by monitoring the daily cell count for each algae species:

Selenastrum:	y = 0.0063 x	*Ankistrodesmus:*	y = 0.0092 x	**(1)**
	y = 0.0063*35		y = 0.0092*15	
	y = 0.22		y = 0.14	

Where 'x' is the required cell count (in 10^6 cells/ml) for each algal species based on the 70:30 ratio mentioned above and the constants (0.0063 and 0.0092) are slopes derived from the daily cell count monitoring.

The algae concentrate was resuspended into FLAMES culture medium when fed to cultures and into FLAMES test medium when fed to the chronic test subjects. The resuspension volume (RV) was calculated as:

$$RV = \frac{\text{Volume of algae from stock culture(ml)} * \text{Measured OD}}{\text{Required OD}} \quad \textbf{(2)}$$

2.2 Acute Tests

In order to determine the toxicity of these metals to *Daphnia*, a series of acute 48 h LC50 tests were done generally following standard methodologies. A matrix of concentrations was tested to determine 5 things: (1) acute toxicity of Ni and Cu individually where daphnia were exposed to each metal separately; (2) acute toxicity of Ni:Cu mixtures, where daphnia were exposed to a combination of the two metals; (3) acute toxicity of single metals with DOC (4) acute toxicity of mixtures with DOC.

The general procedure for acute tests was to expose 5 neonates (< 24 hr old) in drosophila culture vials (Fisher Scientific, Mississauga, ON) with 30 ml of test solution without food. The FLAMES medium was modified for test solutions by removing the EDTA (~ 1mg/L EDTA in culture media). Ni solutions were made with $NiCl_2$ $6H_2O$ salt, and Cu solutions were made with $CuSO_4$ $5H_2O$ salt (Sigma Aldrich, Oakville, ON). Test solutions were equilibrated for 24 hrs prior to the test start. Samples of 10 ml were filtered using a 0.45 µm filter (Acrodisc HT tuffryn membranes, Pall Corporation, Ann Arbor, MI) to measure the dissolved metal content at the beginning and end of tests. To measure the total metal concentrations at test initiation and

completion, 10 ml water samples were obtained and not filtered. After 48h of exposure, mortalities were counted and recorded.

There were 6 Cu (0.25, 0.5, 1, 3, 6, 12 µg/L Cu) and 7 Ni (0, 150, 250, 750, 500, 1000, 2000 µg/L) concentrations with 8 replicates per concentration. The concentrations used in the mixture test were derived based on the survival response from the single-metal acute LC50 tests. In order to distinguish the potential effects of mixtures, only concentrations that had resulted in less than 50% mortality in the single-metal tests were used for the mixture test.

Acute toxicity tests with the same Ni-Cu combinations were tested with a constant concentration of DOC at 4 mg/L. Three sources of DOC were used: Daisy Lake, Sudbury, Clearwater Lake, Sudbury, and Luther Marsh, Grand Valley (Table 2.2). All collections were done in October and November 2014. The NOM was collected using a reverse-osmosis unit with 400 Da molecular mass-cutoff membranes (FilmTec FT30, Minneapolis, MN). Collected surface water was reduced to concentrate. These concentrates were resinated using H^+ cation-exchange resin (USF C-211 H cation resin, U.S. Filter Corporation, Rockford IL) to remove all residual metals and cations from DOM binding sites. After resinating, the concentrate was reduced to pH 2 and stored in the refrigerator in polyethylene acid-washed containers (Schwartz et al., 2004).

DOC was characterized using absorbance at 340 nm (SAC_{340}) and fluorescence excitation-emission matrix spectroscopy (FEEM). SAC_{340} measures the aromatic content in the solution. The absorbance was measured using a SpectramaxPlus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA) and was converted to a specific absorbance coefficient (SAC) using the following formula from Curtis and Schindler (1997).

$$\mathbf{SAC_{340}} = \frac{(2.303 * \text{Absorbance at 340 nm})/\ \text{pathlength}}{\text{NOM}/\ 1000} \quad \textbf{(3)}$$

Where pathlength is in cm and NOM refers to the concentration of DOC in mg/L

FEEM contour plots show the presence of tyrosine, tryptophan, fulvic and humic acids depending on the intensity peaks. Since these contour plots could not be coupled with PARAFAC analysis, only the qualitative observations were recorded based on the appearance of peaks at the following locations on the excitation-emission diagrams (Gheorghiu et al, 2010):

Emission (nm)	Excitation (nm)	Interpretation
400 - 450	320 - 340 230	Fulvic peak
460 - 520	360 - 390 265	Humic peak
340 - 350	230 and 280	Tryptophan
300	230 and 280	Tyrosine

The DOC was also characterized using Fluorescence Indices (FI). FI can determine the original of the NOM, whether it is aquatic or terrestrially derived. FI is the ratio of fluorescence intensities at 370:450 and 370:500 (excitation: emission wavelengths in nm; McKnight et al., 2001):

$$\mathbf{FI} = \frac{I\ (370{:}450)}{I\ (370{:}500)} \quad \textbf{(4)}$$

2.3 Cu Ion Selective Electrode

Cu free ion (Cu^{2+}) measurements were conducted by using a Cu ion selective electrode (ISE; Orion Ionplus, Thermo Electron Corporation, Beverly, MA). A two-point calibration was conducted prior to the measurements. Two buffers were used for the calibration: glycine (0.001M) and ethylene diamene (0.001M) following the methods outline by Belli and Zirino (1993). The test solutions were passed through the electrode using a flow-through system. The millivolt (mV) response was documented when readings stabilized to ± 0.1 mV/min.

A total of 6 solutions were prepared and tested. The objective was to determine the interactions between the metals and DOC at the corresponding Ni and Cu LC50s. Each of the two DOC sources had a solution of DOC + Cu, DOC + Ni, and DOC + mixture. Solutions were prepared by spiking FLAMES medium with nominal concentrations of 3.7 µg/L Cu, 1000 µg/L Ni and 4 mg/L DOC from Luther Marsh and Clearwater Lake. Total Cu concentrations were then measured by GF-AAS. Test solutions were equilibrated for 24h prior to Cu^{2+} measurements.

2.4 Chronic Tests

In the chronic study, only 1 neonate was placed in 30 ml of solution per vessel, and each concentration had 10 replicates. The neonates were less than 24 hr old at the start of the 21-day test. Each animal was fed daily with 2 species of algae, *Ankistrodesmus and Selenastrum spp.* The amount of algae fed to the daphnia varied over time depending on its age: 1 mg C/L on Day 1-2, 1.5 mg C/L on Day 3-7, and 2 mg C/L on Day 8 onwards. Solutions were prepared 24 hr prior to start date and daphnids were placed in new solution every other day during the 21day testing period. There were 4 Cu (0.32, 1.0, 1.78, 3.18 µg/L) and 4 Ni concentrations (1.8, 5.6, 18,

56 µg/L) as well as control. The FLAMES medium was modified for test solutions by removing the EDTA (~ 1mg/L EDTA in culture media).

To determine exposure concentrations for the chronic test, four factors were considered: CWQG, natural background range, Daisy Lake concentrations of Ni and Cu, and the results of the single-metal acute tests. CWQG for Cu is 2 µg/L and Ni is 25 µg/L. Natural background concentration for Cu ranges from 0.2-30 µg/L (USEPA, 2012) and for Ni it ranges from 0.5-50 µg/L (WHO, 2005; ATSDR, 2011b). The Ni and Cu concentrations from Daisy Lake in Sudbury were obtained from a lake survey (unpublished personal communication from Szkokan-Emilson, 2014). Cu concentration in Daisy Lake is 8.4 µg/L while Ni is 43.7 µg/L. The 48h LC50 as derived from the single-metal acute tests was 2.425 µg/L Cu (CI 2.145-2.823 µg/L) and 995 µg/L Ni (95% CI 877- 1125 µg/L). Hence for the chronic test, the four Cu concentrations that would encompass those four factors are: 10, 3.16, 1.0 and 0.32 µg/L; the Ni concentrations are: 56, 18, 5.6, and 1.8 µg/L.

2.5 Processing Samples

Cu samples were measured using graphite furnace atomic absorption spectroscopy (GF-AAS: PinAAcle 900T, Perkin Elmer, Waltham, MA). Samples for the AAS were acidified with 1% volume of 16N HNO_3 (Trace Metal Grade, Fisher Scientific, Nepean, ON). Ni along with the concentration of ions (Ca^{2+}, Na^{2+}, and Mg^{2+}) was measured via flame (AAS, PinAAcle 900T, Perkin Elmer, Waltham, MA). Certified multi-element standard reference solution (TM 23-4, Environment Canada, Burlington, ON)was tested in between samples to assure correct concentrations by the AAS. For DOC analysis, 30 ml water samples were collected and filtered with the 0.45 µm filter (same as above). These samples were stored in the fridge at 4 °C until

measurement with a TOC analyzer (TOC-$L_{CPH/CPN}$, Shimadzu, Kyoto, Japan).

2.6 Statistical Analysis

For the single-metal acute tests, a one-way ANOVA was conducted using IBM SPSS v. 22 to determine if Ni and Cu had a significant effect on Daphnia survival for the acute and chronic mixture experiments. This was followed by a Post-hoc Tukey-HSD test to differentiate between treatments when the ANOVA results displayed a significant effect. For acute and chronic mixture tests, a two-way ANOVA was conducted to determine whether there was a significant interaction between the two metals. This was also followed by a Post-hoc Tukey test to differentiate between treatments. SPSS was also used to derive the LC50 values with 95% confidence intervals through Probit analysis.

The degree of additivity was determined using three models: (1) Toxic units (TU), (2) Concentration Addition (CA) model, and (3) Independent Action (IA) model. As mentioned earlier, if Ni^{2+} and Cu^{2+} enter the cellular membrane through different transport sites, an IA model should be used. In addition to the IA and CA model, to understand whether the mixture effects are additive, the mixture LC_{50}'s from the matrix were compared to the single metal LC_{50} concentrations of Ni and Cu using the toxic unit approach (Khan et al., 2012).

In simple mixture tests, the TU sum is used as the expected response if additivity occurs and the actual mortality associated with the solutions is measured in an acute toxicity test to indicate whether the response is additive, synergistic or antagonistic (by comparing actual to calculated sum of TUs). For example, a sum of 1 TU means an expectation of 50% mortality if the response is additive, while if the actual test results for the mixture show less mortality occurs

then the response is antagonistic, and if greater than 50% mortality it indicates a synergistic response. If the mixture concentrations sum to 0.5 TUs then there is an expectation of 25% (i.e. 0.5 x 50%) mortality in the acute toxicity test with this solution if the response is additive and less or greater mortality if the response is antagonistic or synergistic (respectively). In this series of tests it was possible to calculate the LC50 for each of the 6 different mixture combinations and from that determine whether the sum of the toxic units is greater than, less than or equal to 1. The formula for toxic units is given below (Khan et al., 2012):

$$\mathbf{\Sigma TU} = \frac{Concentration\ of\ Cu}{LC50\ of\ Cu} + \frac{Concentration\ of\ Ni}{LC50\ of\ Ni} \qquad \mathbf{(5)}$$

The formula for the CA model is as follows (Hadrup et al., 2013):

$$X_{mix} = \mathbf{(P_{Cu} / X_{Cu}) + (P_{Ni} / X_{Ni})} \qquad \mathbf{(6)}$$

X_{mix} = concentration of the mixture LC50

P = Fractions of Ni or Cu in each mixture pair

X = Single-metal Ni or Cu LC50

The formula for the IA model is as follows (Hadrup et al., 2013):

$$\mathbf{Y = 100*(1-[1-R_{Ni}]*[1-R_{Cu}])} \qquad \mathbf{(7)}$$

Y = model mortality prediction (%)

R_{Ni} = proportion of Ni in LC50

R_{Cu} = proportion of Cu in LC50

The calculation of R_{Ni} was calculated as the Ni concentration, used in Cu-Ni mixture pair, divided by the single-metal Ni LC50. Same was repeated to calculated R_{Cu} with the corresponding Cu concentrations.

3. Results

3.1 Acute Toxicity of Single Metals

All acute tests reported here had less than 10% mortality of controls, therefore met the validity criteria (Environment Canada,1990). Measured total Ni concentrations were within 92 ± 1.1% SEM (n= 55) of nominal values while Cu was within 86 ± 7.5% (n = 44). Dissolved Cu was 100.6 ± 3.4% (n = 10) of total Cu, and Ni was 99.0 ± 1.4% (n = 10) of total measurements. Since total and dissolved were very similar, the total measured concentrations are reported.

In the tests with single metals, mortality increased as metal concentration increased (Fig 3.1 and 3.2). The 48h LC_{50} for Cu was 2.43 µg/L (95% CI 2.15-2.82 µg/L) while 48h Ni LC_{50} was 995 µg/L (877- 1125 µg/L; Fig 3.1 and 3.2 respectively). There was a significant effect of Cu on daphnid mortality ($p < 0.05$). There was also a significant effect of Ni on daphnid mortality (< 0.05). These tests were repeated 3 times, every 8-10 months, and the LC_{50} ranged from 2.43 - 2.65 µg/L (n= 3) for Cu , and 995-4680 µg/L Ni (n= 3, Table 3.1).

3.2 Acute Toxicity of Mixtures

The Ni and Cu mixture mortality response was compared to the single-metal mortality from acute 48h tests. The mortality within the mixture treatments was significantly higher than the mortality of single-metal treatments in some cases (Fig 3.3 B and C, and 3.4 B). Further exploration of additivity is made using the concentration addition and independent action models (Section 3.3). Cu has a marked effect on mixture mortality. As Cu concentration increases at fixed Ni concentrations, the mixture mortality increases (Fig 3.3). The effects of Ni on the mixture mortality are less pronounced at low Ni concentrations. However, there is an increase in toxicity with increasing Ni (Fig 3.3 and 3.4).

Overall, 7 Ni treatments were tested with 5 Cu concentrations (Fig 3.5). As expected, mixture mortality increases as metal concentrations increased. With the addition of more Ni, the toxicity curve is shifted further to the left when compared to the Cu-only mortality, thus indicating that the mixtures are more toxic in the Ni-Cu combinations tested (Fig 3.5). There are some portions of the curve which dip below the Cu-only toxicity curve, indicating an anomaly likely caused by inherent variability. The grey box indicates the LC50 range for Cu without added DOC. Within this range, the 56 µg/L Ni curve spikes up to 100% mortality. This is likely due to a Cu effect since 6 and 12 µg/L Cu is nearly 3-6x higher than the Cu LC50.

A two-way ANOVA was used to assess the effect of Cu (5 concentrations) and Ni (7 concentrations) on Daphnia mortality (Table 5.1 Appendix). There is evidence of a significant interaction between Cu and Ni ($F(24, 275) = 15.82$, $p < 0.05$). Follow-up analyses using simple effects were conducted to understand the nature of the interaction (Table 5.1 Appendix). Differences in mortality among different Cu concentrations within each Ni treatment were

considered. Statistically significant differences across the Cu conditions were observed for all conditions of Ni (Table 5.1 Appendix). Pair wise comparisons among the cell means using a Bonferroni adjustment for multiple comparisons revealed that in general, low Cu concentrations were significantly different to high Cu, but not to each other (Table 5.1 b Appendix). Further details on significant differences between Cu concentrations at each Ni treatment are indicated by the letters (Table 5.1 b Appendix).

Another two-way ANOVA was conducted to test the effect of Ni at each Cu concentration (Table 5.1 c Appendix). There is evidence of a significant interaction between Cu and Ni ($F(4,725) = 15.82$, $p<0.05$). Analyses using simple effects were conducted to further understand this interaction. Statistical significant differences across the Ni conditions were observed for all concentrations of Cu (0, 1, 2, and 6 µg/L) except for 12 µg/L (Table 5.1 c Appendix). Pair wise comparisons among the cell means using a Bonferroni adjustment for multiple comparisons are reported in (Table 5.1 c Appendix). Significant differences between Ni treatments at each Cu concentration are indicated by the letters.

3.3 Modeling Approaches:

Three modeling approaches were explored to determine whether the mixtures were additive, or greater or less than additive: Toxic Units (TU), Concentration Addition (CA) model, and Independent Action (IA) model. The mixture combinations were additive, more than additive or less than additive depending on the individual metal concentration combinations. According to the toxic unit approach (Fig 3.6, Table 3.2), only one pair was greater than additive: 75 µg/L Ni at 1.392 µg/L Cu. The IA model predicted the mortality based on the fraction of each metal in the mixture. The predicted mortality was compared to the actual mortality observed in 48h acute

toxicity tests (Fig 3.7, Table 5.2 Appendix). Approximately 20% of pairs were more than additive. Their combinations are as follows: 500 µg/L Ni at 1 µg/L Cu, 1000 µg/L at 1, 3, and 12 µg/L Cu, and 2000 µg/L Ni at 3 µg/L Cu. Approximately 30% of pairs, their combinations listed: 75 µg/L at 1 and 3 µg/L Cu, 150 µg/L Ni at 1 and 3 µg/L Cu, and 250, 1000, and 2000 µg/L at 1 µg/L Cu. 50% of the pairs fall on the line of strictly additive. These are the pairs which had both predicted and observed mortality of 100%. The CA model predicts the concentration of the mixture at which 50% mortality will occur. This was compared to the calculated Probit LC50 of the mixture based on observed mortality from toxicity tests (Table 3.2). Based on this approach, only one pair was more than additive, 2000 µg/L Ni (Fig 3.8, Table 5.3 Appendix). All other combinations were less than additive: 75, 150, 250, 500, and 1000 µg/L Ni.

3.4 Acute Toxicity of Single-Metals with Added DOC

The *Daphnia* survived well in positive controls for tests containing a nominal concentration of 4 mg/L added DOC. There was a slight decrease in mortality compared to no added DOC when LM DOC was added to Ni solutions (Fig 3.10A). The 2 way ANOVA (Ni and DOC source) showed a significant interaction between Ni and DOC ($F(3, 56) = 446.43$, $p < 0.05$). As CWL DOC was added, there was a slight decrease in mortality caused by Ni at certain concentrations. When comparing the effect of DOC at different Ni concentrations, we see that there is a significant protection of CWL DOC (compared to no added DOC) at 1000 ug Ni/L. DOC did not have a significant effect on mortality at any other Ni treatments.

When comparing Ni treatments at different DOC concentrations (0 mg/L and 4 mg/L DOC; Fig 3.10), 2000 µg/L Ni treatment was significantly different to 250, 500 and 1000 µg/L

but those three concentrations were not significantly different from each other when there was added DOC. When there was no DOC added, 250 µg/L Ni was significantly different to 1000 and 2000 µg/L Ni, and 1000 and 2000 µg/L Ni were significantly different to each other. There was a significant effect of Ni on daphnid mortality with and without the presence of DOC ($F(3, 28) = 18.37$, $p < 0.05$, and $F(3,28) = 48.92$, $p < 0.05$ respectively; Fig 3.10 a).

There was a significant effect of Cu on daphnid mortality $F(3, 20) = 118.33$, $p < 0.05$ only when there was no DOC present (Fig 3.10 b). Cu did not have a significant effect on mortality in the presence of DOC, $F(3, 20) = 1.00$, $p > 0.05$. There was also a significant interaction between Cu and DOC ($F(3, 48) = 137.5$, $p < 0.05$). Cu treatments were compared to each other at different DOC concentrations. When there was no DOC added, Cu treatment of 1 µg/L was significantly different than 6 and 12 µg/L. 1 and 3 µg/L were not significantly different to each other and 6 and 12 were not significantly different to each other either, when there was no DOC.

When comparing the effect of DOC at different Cu treatments, there was a significant decrease in mortality when DOC was added to Cu solutions (Fig 3.10 b). The treatment of 1 µg/L Cu was did not have a significant effect on mortality with and without the presence of DOC, according to the Post-Hoc Tukey test following a 2-way ANOVA. However, the Tukey test indicates that all other concentrations of Cu had a significant effect on mortality at the other three Cu concentrations (3, 6 and 12 µg/L Cu).

3.5 Acute Toxicity of Mixtures with added DOC

The *Daphnia* survived well in positive controls for tests containing added DOC. Three sources of DOC were compared to each other to observe source differences in protection: Luther Marsh (LM), Clearwater Lake (CL), and Daisy Lake (DL; Fig 3.9). For tests done at 1 mg Ni/L the CWL source was the most protective and LM is the least with DL being intermediate. It is clear that the solutions with 2000 µg/L Ni resulted in very high mortalities at all Cu concentrations as well as Ni only and therefore meaningful comparisons of the relative protection of different sources was not possible (Fig 3.9). LC50 values were also calculated for each mixture toxicity curve (Table 3.3). The LC50 value for CWL + 2 mg Ni and DL + 2 mg Ni could not be calculated since the mortality was greater than 50% in all treatments. As indicated by a decrease in LC50, it can be inferred that the toxicity increases with increased Ni for LM.

3.6 Cu Free Ion Measurements

The addition of Ni to solutions containing DOC and Cu resulted in an increased of Cu free ions (Table 3.4). Of the two DOC sources tested (CWL and LM), LM had approximately 10x more Cu free ions when the same concentration of Ni was added to the solution containing DOC + Cu (Table 3.4). This increase in Cu free ions also corresponded to an increased mortality observed in the acute mixture tests (Fig 3.9).

3.7 DOC Characterization

3.7.1 Optical Characterization Plots

Only a qualitative observation can be made using the FEEM optical characterization plots, since parallel factor analysis (PARAFAC) could not be done to confirm the identification of flourescent components and quantify their abundance. LM and CWL have fulvic substances since it peaks between 450-500 nm (Fig 3.11). DL has some humic compounds since it peaks around 360-390 nm, and a presence of tyrosine and tryptophan are also indicated by peaks at 300 and 350 nm respectively (Fig 3.11). CWL has fulvic, humic, tryptophan and tyrosine-like fluorphores (Fig 3.11). Tryptophan-like and tyrosine-like fluorophores are labeled as proteinaceous compounds. Due to the presence of these peaks, DL and CWL likely contain protein compounds (Fig 3.11).

3.7.2 Absorbance at 340 nm

The measured absorbance readings at 340 nm were converted to a specific absorbance coefficient (SAC) using equation 3. Luther Marsh has darker coloured DOC than Clearwater Lake. This can be confirmed from the SAC_{340} absorbance coefficients in Table 3.5. Higher absorbance reading corresponds to a darker coloured DOC.

3.7.3 Fluorescence Indices

Fluorescence indices were calculated to determine the origin of the DOC from the three sources, CWL, LM and DL. The FI and excitation-intensities are reported in Table 3.6. At an excitation of 370 nm, the maximal emission intensity ranged from 15.08 to 23.79. The FI for LM was 1.03,

CWL was 1.22, and DL was 1.41.

3.8 Chronic toxicity

For 21-day chronic Ni and Cu exposures, overall mixture mortality increased as Cu concentration increased. A similar trend is evident for Ni that shows increased mortality with increased Ni concentrations, although some anomalies exist (Fig 3.12). All Cu treatments were significantly different from controls ($p < 0.05$) for Ni concentrations of 1.8 ($F(4, 33) = 5.51$), 5.6 ($F(4, 39) = 2.79$) and 56 µg/L ($F(4, 41) = 77.99$).

No clear effects on reproduction were observed. The graph shows the average number of neonates produced by daphnids that survived after 21 days (Fig 3.13). The daphnids that survived produced similar number of neonates regardless of the metal concentration. The number of neonates produced in each Ni treatment were not significantly different from the control: $F(4, 19)= 1.69$ for 1.8 µg/L Ni, $F(4, 30)= 0.61$ for 5.6 µg/L Ni, and $F(4, 30) = 1.17$ for 18 µg/L Ni.

4.0 Discussion:

4.1 Ni-Cu interactions

Ni was less toxic than Cu, and this finding is consistent with the literature (Table 4.1, Fig 3.1 and 3.2). The *D. pulex-pulicaria* clone is a good organism to use in toxicity studies since it is very sensitive and survives in low Ca concentrations, which would provide a fairly conservative LC50.). Given the unique nature of the *Daphnia pulex-pulicaria* clone used in these studies, it was difficult to find comparable Cu and Ni LC50s conducted in similar water chemistry

conditions. Published Cu LC50s range from 2-249 µg/L, with Ca concentrations ranging from 2.5-80 mg/L. Published Ni LC50s range from 510- 466,000 µg/L with Ca concentrations between 2.5-421 mg/L. (Table 4.1). There was only one other study that which reported similar Cu LC50s as this study. Long et al., (2004) conducted her test in similar Ca concentration (2.8 mg/L Ca) at a fairly low pH (5.6) and reported a Cu LC50 of 2 ± 1.5 µg/L, which is consistent with the LC50, 2.43 µg/L, derived in the current study. There were only 2 studies that reported Ni LC50s lower than the present study: 510 µg/L (Biesinger and Christensen, 1972) and 750 µg/L (Leonard and Wood, 2013). Both of these studies were conducted in Ca and DOC concentrations greater than FLAMES media so it is surprising that the reported LC50s are lower. It is likely that the *D. magna* and *D. pulex* clones used were highly sensitive organisms, or that the higher pH of 7.3-8 affected the bioavailability of Cu and Ni. It is also possible that a daphnid from a Sudbury lake developed toxic resistance due to water contamination over a long period.

As expected, the Ni-Cu mixtures were more toxic than single-metal responses (Fig 3.3, 3.4, 3.5), with the exception of some anomalies. The addition of Ni produces left-shifted mortality curves, indicating a more toxic response which could be additive. This degree of additivity was evaluated by the three modelling approaches: CA, IA and TU models. Published studies have reported that Ni-Cu mixtures are considered additive at specific combinations in the water and a blanket statement cannot be applied to explain their interaction (Meyer et al, 2015; Charles et al., 2013). Conclusions about the additive toxic effects are dependent on the concentration of tested combinations and the form of the metal (e.g. dissolved, free ions, biotic ligand-bound; Meyer et al., 2015; Santore et al., 2015; Nys et al., 2015).

The three modelling approaches (CA, IA and TU models) were used to evaluate the Ni-Cu interaction, and each one gave slightly different answers as to which mixture combinations

were additive, more than additive or less than additive. In theory, it would be ideal to have a single model that is used universally and can be applied to all situations, especially since it is not possible to test all mixture combinations with every chemical in existence. This type of model would also be desirable in situations where the mechanism of action is unknown. Several reviews have been conducted to determine which of these approaches should be applied for risk assessment and predicting the outcomes of contaminants in the environment. The general consensus is that CA and IA modelling approaches give very similar outcomes (Hadrup et al, 2013; Backhaus et al, 2004; Faust et al, 2003; Cremazy et al, 2015; Cedergreen et al., 2008). Cedergreen et al., 2008, found that 20% of the mixtures adequately predicted by IA and 10% by CA, but half of their experiments could not be correctly predicted by either model. Cedergreen et al., 2008, also suggest that IA is not considerably better than CA model predictions. According to the CA model in this study, 1 out 6 pairs show more than additive toxicity, and for the IA model there are 3 out of 9 combinations that show this (Fig 3.7 and 3.9). The presence of Ni increased the overall mortality in all mixture treatments. This was expected since the two metals are known to have two different mechanisms of toxic action. Ni disrupts Mg balance, while Cu disrupts Na balance. Therefore, both metals are likely entering the body and disturbing the required ion balance.

4.2 Ni-Cu Interactions with DOC

The protective effect of DOC on single-metals and mixtures was explored. Both CWL and LM were significantly protective to Cu but not Ni (Fig 3.10). This is consistent with literature that states Cu binds strongly to DOC (Wood et al., 2011). All DOC sources were protective against Ni-Cu mixtures (Fig 3.9). As expected, there was variation of protection by source composition.

CWL is more protective than LM to the Cu-Ni metal mixture (Fig 3.9). A change in 1 mg of Ni made a significant difference in the mortality response in the presence of both DOC sources (Fig 3.9). This indicates that the concentration of Ni likely exceeded the binding capacity of DOC and/or bound weakly, thus leading to increased mortality.

The DOC composition was determined to better understand the protective effects with these metals. The optical characterization of DOC was done through SAC340, fluorescence indices (FI) and fluorescence excitation-emission matrices (FEEM). The amount of Cu free ions in solution was also used for comparing the binding interactions between the metals and DOC sources.

The fluorescence index is used to determine the origin of the DOC, either allochthonous (terrestrial) or autochthonous (aquatic). Typical freshwater FI values range from 1.3-1.8 (McKnight et al., 2001). High FI values indicate an autochthonous origin (McKnight et al., 2001). Daisy lake (DL) had the highest FI value at 1.41 and LM had the lowest, 1.03 (Table 3.4). From FI analysis, it can be inferred that DL DOC is of autochthonous origin while LM is of allochthonous origin since it had a low FI value of 1.03. Since CWL has an intermediate FI (Table 3.4), it can be inferred that CWL can have autochthonous and/or allochthonous inputs. The FI value for CWL is 1.22, which is on the lower side of the typical freshwater range of 1.3-1.8 (McKnight et al, 2001), thus meaning that it could be of allochthonous origin. The SAC340 value is also lower than that of the terrestrially derived LM DOC, at 15.99. This could mean that it has more fulvic content and is tyrosine rich if it is allochthonous DOC (Wood et al, 2011).

SAC340 is an indicator of the aromaticity of the DOC sample and is also used to determine the sample origin. LM has a high SAC340 value, 38.86 (Table 3.3), while CWL is less

than LM at 15.99. A high SAC340 value indicates a higher humic content (Wood et al, 2011). This also corresponds to having darker coloured DOC, increased aromatic rings and phenolic groups as well as larger molecules (Wood et al, 2011). LM had a darker colour than CWL at the same DOC concentration, so a higher SAC340 was expected.

Usually, a darker DOC colour corresponds to an increased amount of humic fractions in the sample (Wood et al., 2011). LM has darker coloured DOC than CWL or DL. The composition of the DOC was determined through the FEEM analysis. The contour plots (Fig 3.11) of the two lakes indicates that CWL is very similar to but slightly more proteinaceous than LM, due to the presence of tyrosine and tryptophan amino acid peaks in CWL which are absent in the LM plot. CWL has fulvic-like compounds, indicated by the peaks at 400-450 nm emission, with a small peak at 300 nm emission indicating the presence of tyrosine. Tyrosine rich sources are more protective towards Ni toxicity (Cooper et al, unpublished, 2014; McKnight et al, 2001). Since CWL contains some protein compounds, in theory it should be more protective towards Ni toxicity than LM. Having an increased amount of humic fluorophores correlates to greater protection against Cu toxicity (Wood et al, 2011).

There were some inconsistencies with the optical characterization, Cu free ion measurements and toxicity tests. The single-metal toxicity tests agree with SAC340, FI, and FEEM because LM DOC was more protective against Cu than Ni toxicity. SAC340 and FI indicates that LM comes from allochthonous origin, meaning it contains humic content. Humic fluorophores were observed in LM DOC through the FEEM analysis so this agrees with the mortality results from single-metal toxicity tests. There was zero mortality when only Cu was added to LM DOC. When only Ni was added to LM DOC, the mortality was at 90% (Fig 3.9

and Table 3.2). Therefore, singe-metal toxicity tests are consistent with the optical characterization and free-ion measurements.

A conflicting picture emerges in the mixture toxicity tests with DOC. SAC340 and FI indicated that CWL comes from autochthonous and LM comes from allochthonous origin. The FEEM analysis indicated that LM had more humic content than CWL, therefore Cu was expected to bind more strongly to CWL than LM. The Cu free ion measurements indicated an increase in Cu^{2+} when Ni was added, with LM having more Cu^{2+} than CWL. Cu^{2+} ions in the 'mixture + LM DOC' solution are approximately 10x greater than the free ions in 'mixture + CWL DOC' solution. This is not typical since humic-rich sources, such as LM DOC, should bind more strongly to Cu than Ni. In the toxicity tests with LM DOC, the concentration of Cu^{2+} was 34x greater in the solution after Ni was added (Table 3.4). This could mean that the Cu was displaced by the addition of Ni, and the Ni was binding to the LM DOC. Two reasons can explain this: (1) The concentration of Ni was 360x higher than Cu (1800 ug/L Ni and 5 ug/L Cu; Table 3.4), therefore the competition favoured the binding of Ni to LM DOC; (2) Ni was binding more strongly than Cu to LM DOC, which would disagree with the literature that indicates Cu binds strongly to humic-rich DOC (Wood et al., 2011). An increase of Cu^{2+} in the LM solution was not expected. Therefore, the SAC340, FI, and FEEM results do not agree with toxicity tests. However, the Cu^{2+} measurements agree with the mixture toxicity tests. Increased Cu^{2+} in the 'LM DOC + mixture' solution correlates to an increased mortality as well since there was more Cu available to cause toxicity (Table 3.4).

The Cu free ions were also measured in the CWL DOC solutions (Table 3.4). It can be inferred that Cu binds strongly to CWL DOC as well since there was zero mortality in the acute tests with Cu-only + CWL DOC (Fig 3.9, Table 3.4). Similar to the LM solutions, the 'Ni + CWL

DOC' solution produced a similar mortality to the 'mixture + CWL DOC' solution (80% and 100% mortality respectively), at the Cu LC50 (Fig 3.10), which could mean that the Ni is causing the toxicity. The CWL DOC had a slightly lighter colour than the LM DOC at ~ 4 mg/L DOC. Lighter coloured DOC is characteristic of lower SAC340 values, is microbial-derived (autochthonous), has smaller molecules, and lower aromatic content (Wood et al, 2011). It is recognized that metals bind strongly to the phenolic (aromatic) groups found in darker DOC (Luider et al., 2004; Schwartz et al., 2004; Winch et al., 2002). It is also presumed that autochthonous DOC provides more protection against Ni toxicity in marine samples (Cooper et al., unpublished document, 2014). In two studies (Cooper et al, 2015 unpublished; McKnight et al 2001) it was reported that tyrosine rich DOC sources are characteristic of autochthonous origin, created by biological activity within the water. In contrast, humic acid rich DOC was allochthonous and created by the decomposition of plant material. Allochthonous DOC provides the least protection against Ni toxicity (Cooper et al, unpublished, 2014). Since CWL has fulvic and tyrosine content, it can be inferred that it is of autochthonous origin. This also matches what is found in the SAC340 and FI analysis. Being autochthonous, it should be the most protective towards Ni toxicity. However, the Cu free ion measurements indicate that there was more free Cu in the LM samples than CWL, indicating that Ni is likely binding more strongly to LM (Table 3.2). The presence of tyrosine indicates that the origin of DOC could be from sewage inputs (Baker et al., 2001; Her et al., 2003) or bacterial origin (Determann et al., 1998; Cammack et al., 2004).

Daisy lake (DL) also contained protein compounds (Fig 3.11). DL contains fulvic compounds as well as tryptophan and tyrosine. The presence of tryptophan indicates that the DOC could have come from algae (Determann et al., 1998). This matches the interpretation of

the FI value (1.41, Table 3.4) indicating an autochthonous origin. DL had the lightest coloured DOC yet it provided intermediate protection to the metal mixture as observed by the mortalities from acute toxicity tests (Fig 3.10). This matches the findings of Schwartz et al, (2004) who claims that colour does not always track with metal binding and this approach will not always work.

In conclusion, LM is of allochthonous origin while CWL and DL are autochthonous. The toxicity tests with mixtures and DOC (Fig 3.9) indicate that CWL was more protective to the metal mixtures than LM. However, this is inconsistent with the SAC340 and FI analyses. Both solutions of LM and CWL contained the same concentration of Ni (1800 µg/L). The presence of Ni displaced more Cu free ions in LM solution than CWL, meaning that it likely bound more strongly to LM. This is not typical since Ni is presumed to bind more strongly to autochthonous DOC. Therefore more Cu is bound to CWL DOC and perhaps more Ni is suspended in solution. This contradicts the findings of Cooper et al. (unpublished, 2014) and Wood et al, (2011). However, Schwartz et al., (2004) noted that colour does not always correlate with metal binding and this assumption does not always work. The binding of CWL DOC to Cu has also been noted by Taylor et al., (2016), who used the same *D. pulex-pulicaria* clone from this study. Further analysis should be done to quantify the amount of fulvic, humic and protein content in the DOC. Additional tests to measure the amount of total nitrogen should be conducted. Since proteinaceous sources bind strongly to Ni, this could explain the interactions of Ni to LM DOC.

4.3 Summary:

- Ni was toxic at higher concentrations than Cu, and this is consistent with the peer reviewed literature. As expected, the mixtures were more toxic than single-metal responses, with the exception of some anomalies.
- The three modelling approaches (CA, IA, and TU model) gave slightly different answers as to which mixture combinations were additive, more than additive or less than additive. This is consistent with other literature that report conclusions about the additive toxic effects are dependent on the concentration of tested combinations and the form of the metal (e.g. dissolved, free ions, BL-bound; Meyer et al., 2015; Santore et al., 2015; Cedergreen et al., 2008; Nys et al., 2015)
- DL had the lightest coloured DOC while LM had the darkest for the same concentration of 4 mg/L DOC. LM is of allochthonous origin, CWL can have allochthonous and autochthonous inputs, and DL is autochthonous. The presence of Ni displaced more Cu free ions in LM solution than CWL, meaning that it likely bound more strongly to LM. This is not typical since Ni is presumed to bind more strongly to autochthonous DOC.
- There were no clear trends seen regarding the effects on reproduction in chronic tests. Out of the daphnids that survived after 21 days, all were producing roughly the same amount of neonates regardless of metal treatment. The quality of those neonates could be different in each treatment. This hypothesis can be tested in future studies.

4.4 Significance:

The integrative aspect of this research was achieved through addressing the objectives by using a variety of biological and chemical tools. For example, acute toxicity tests with live animals was compared to the Cu free ion measurements obtained from chemical analyses. Chemical analyses were also used for characterizing the DOC and deriving the biological origins of the different sources. Being an ecotoxicology project, nearly all aspects of this work integrated biology with chemistry.

Furthermore, metal contamination was studied at the organism level but connections to the ecosystem level are made by applying this research in the understanding of ecosystem recovery processes in the Sudbury region. This research was part of a 5 year TALER (Terrestrial-Aquatic Linkages for Ecosystem Recovery) project. Understanding the connections between DOC and metal mixtures can be useful in advising industries and policy-makers regarding innovative remediation strategies to overcome the ecological stresses from metal contamination. The current study highlights an important area of research that needs to be further understood since metals in the environment are present as mixtures rather than in isolation. It was discovered through this work that, at certain concentrations, the toxicity of Ni and Cu can be greater when combined, in comparison to their individual metal toxicity. Metal mixtures may be integrated into modelling tools, such as the Biotic Ligand Model (BLM), used in environmental policy-making. By understanding toxicity of metal mixtures, it is likely that future harmful effects on aquatic ecosystems can be diminished.

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6. Figures

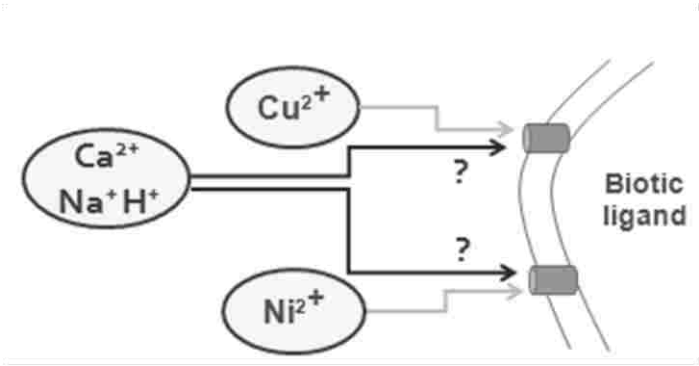


Figure 1.1: Proposed mode of action for nickel and copper cations entering the cellular membrane at the respiratory interface. In this diagram, nickel and copper enter at different ion transport sites. Question marks represent possible unknown sites of competition with other cations passing through the same channel.

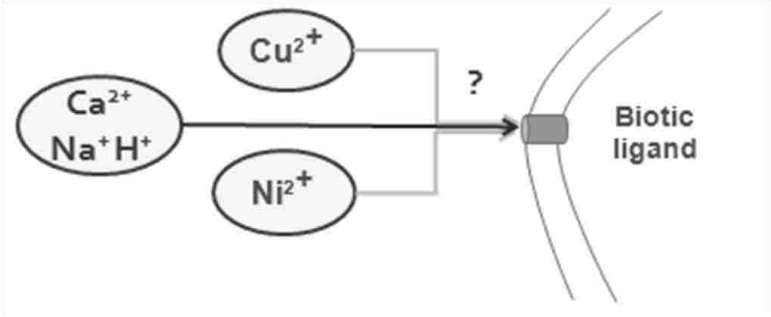


Figure 1.2: Alternative mode of action for nickel and copper cations entering the cellular membrane at the respiratory interface. In this diagram, nickel and copper are thought to enter through the same ion channel. Question marks represent possible unknown sites of competition with other cations passing through the same channel.

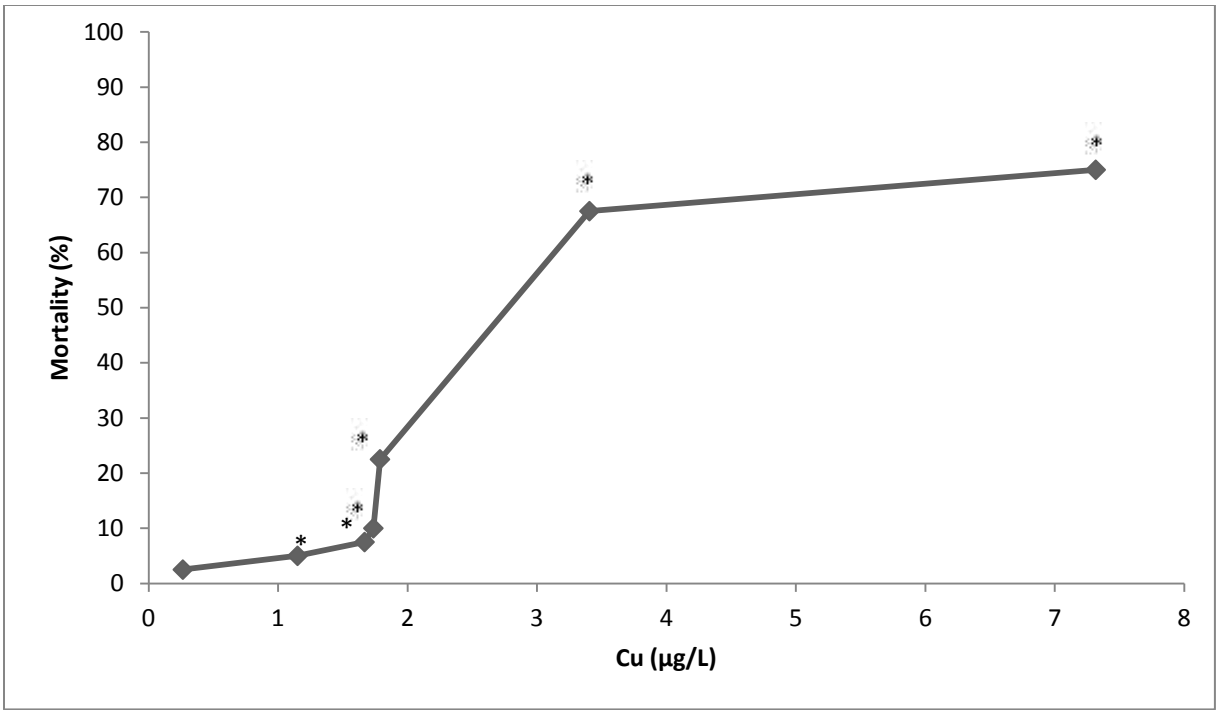


Figure 3.1: Mortality rate as a result of Cu exposure over 48h to *D. pulex-pulicaria*. Probit analysis yields a LC50 is 2.43 (95% CI 2.12-2.82) µg/L. Mean mortality is shown with SEM (n=8) and * indicates significant difference ($p < 0.05$) from controls with no added Cu.

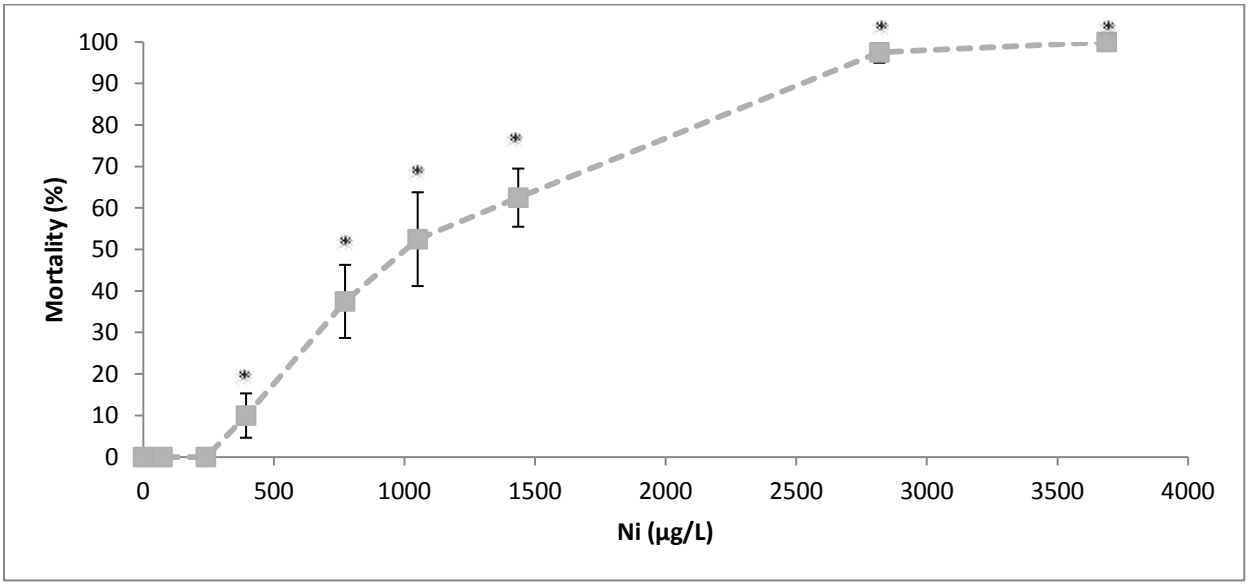


Figure 3.2: 48h acute Ni exposure to *D. pulex-pulicaria*. The 48h LC50 is 995 µg/L (95% CI 877- 1125 µg/L). Error bars represent SEM(n = 8)and * indicates significant difference from unexposed controls ($p < 0.05$).

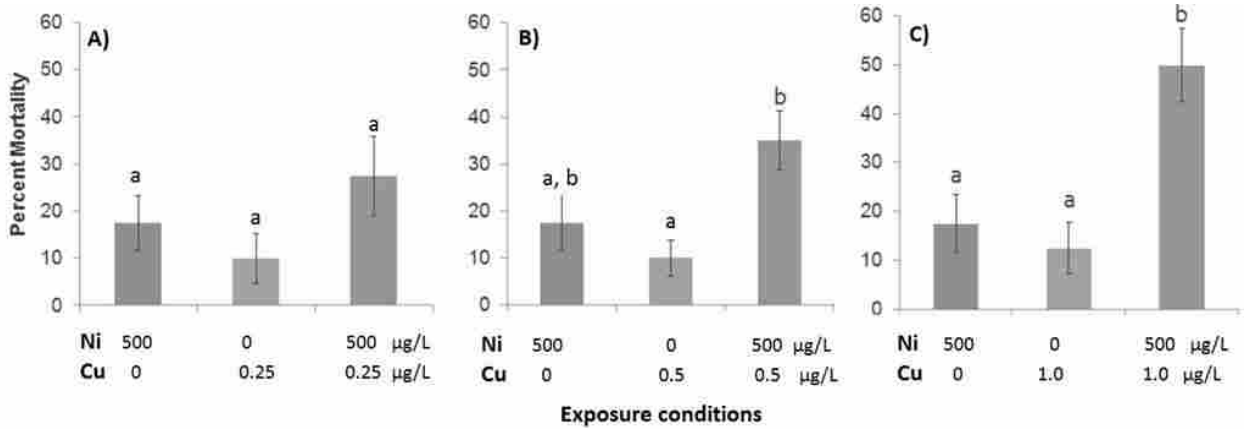


Figure 3.3: 48h acute effect of Ni and Cu mixtures to *D. pulex-pulicaria* in comparison to exposure to the individual metal at high Ni concentrations. From left to right, panels show increased Cu exposure. Error bars represent SEM. Within each graph, bars labelled with the same letter are not significantly different ($p < 0.05$).

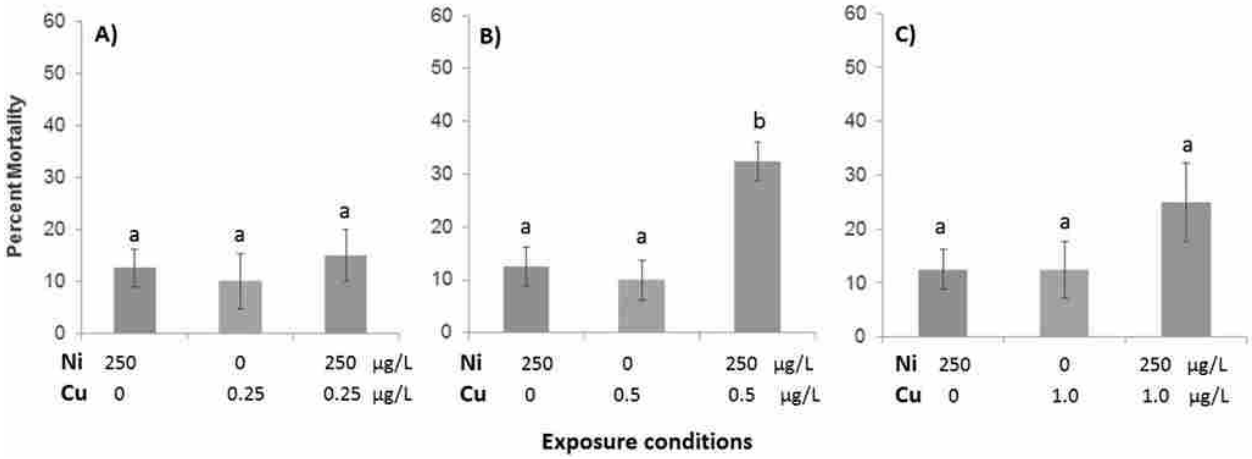


Figure 3.4: 48h acute effect of Ni and Cu mixtures to *D. pulex-pulicaria* in comparison to exposure to the individual metal at low Ni concentration. From left to right, panels show increased Cu exposure. Error bars represent SEM. Within each graph, bars labelled with the same letter are not significantly different ($p < 0.05$).

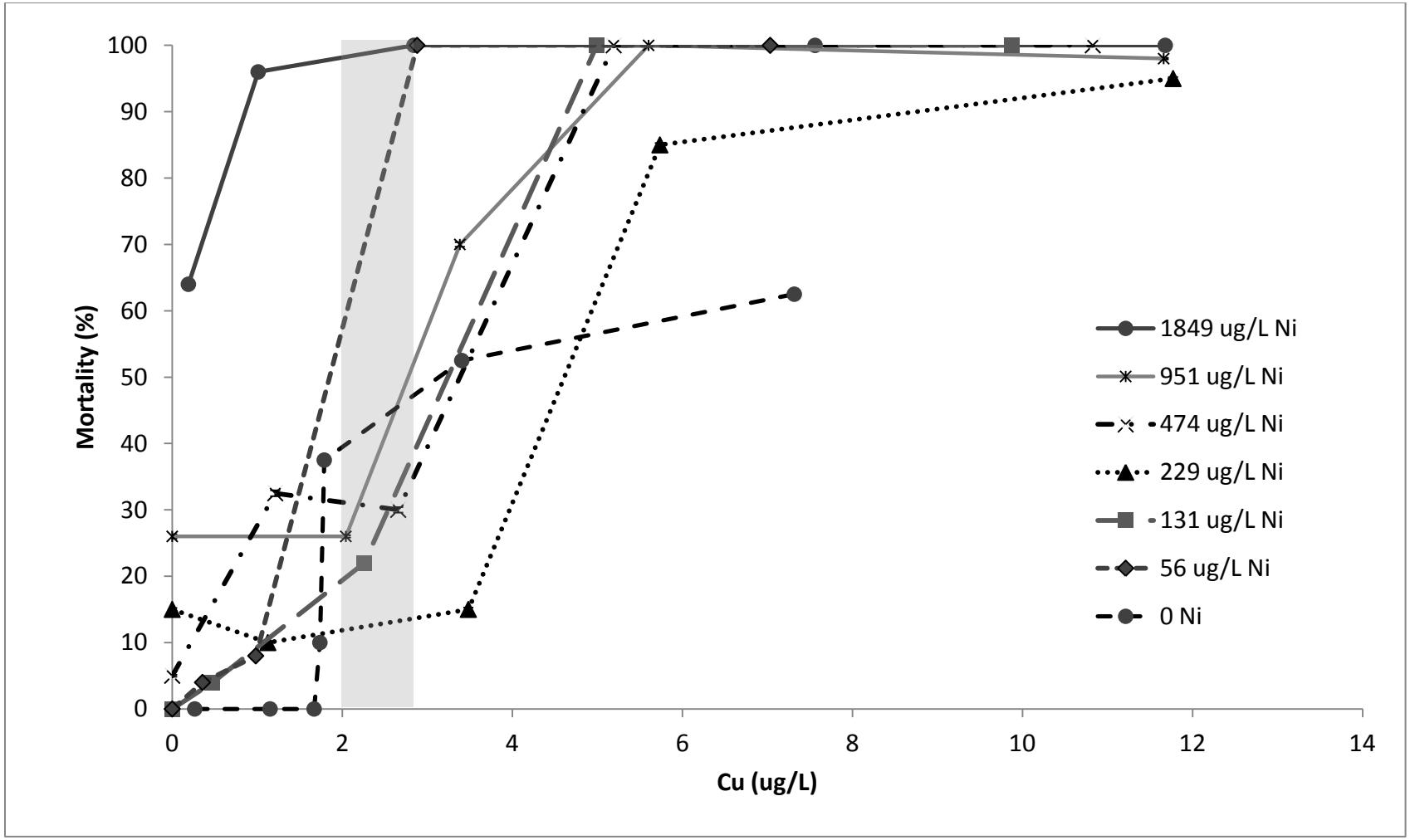


Figure 3.5: Mean mortality over 48 h of exposure to Ni-Cu mixtures. Means are shown with n=8 for each Ni-Cu combination and error bars have been left off for clarity. The grey box provides the range of LC50 values for single-metal Cu only exposures.

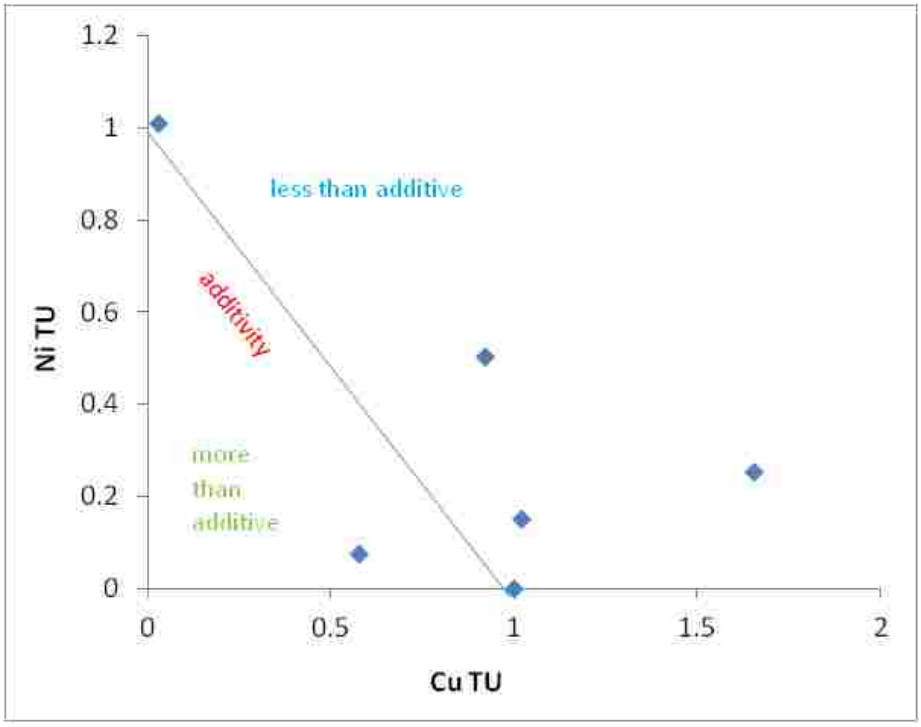


Figure 3.6: Toxic units plotted for each Cu-Ni mixture pair. Points that fall on the blue line indicate an additive response. Points that fall to the left of the blue line are greater than additive, and to the right are less than additive.

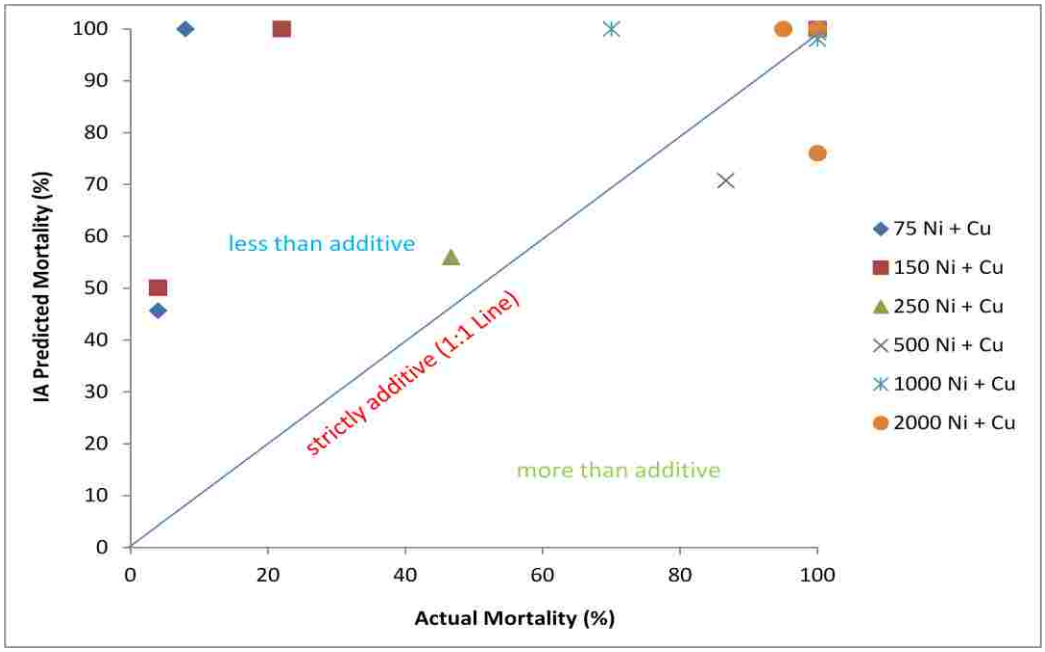


Figure 3.7: Independent Action model predicts the mortality based on the fraction of each metal in the mixture. This was compared to the actual mortality observed in toxicity tests. All mixture pairs to the left of the line are less than additive, and to the right are greater than additive.

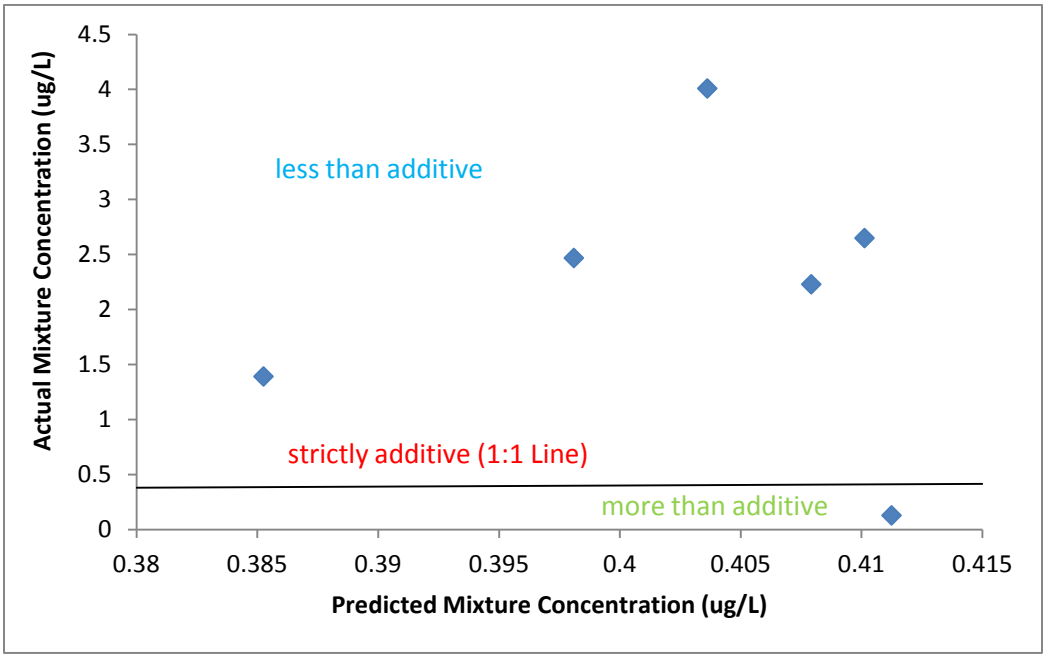


Figure 3.8: Concentration Addition model predicts the concentration at which the effect occurs based on the fraction of each metal in the mixture divided by the concentration at which it exerts this effect. This was compared to the actual LC50s from toxicity tests. All mixture pairs above the black line are less than additive, and below are more than additive.

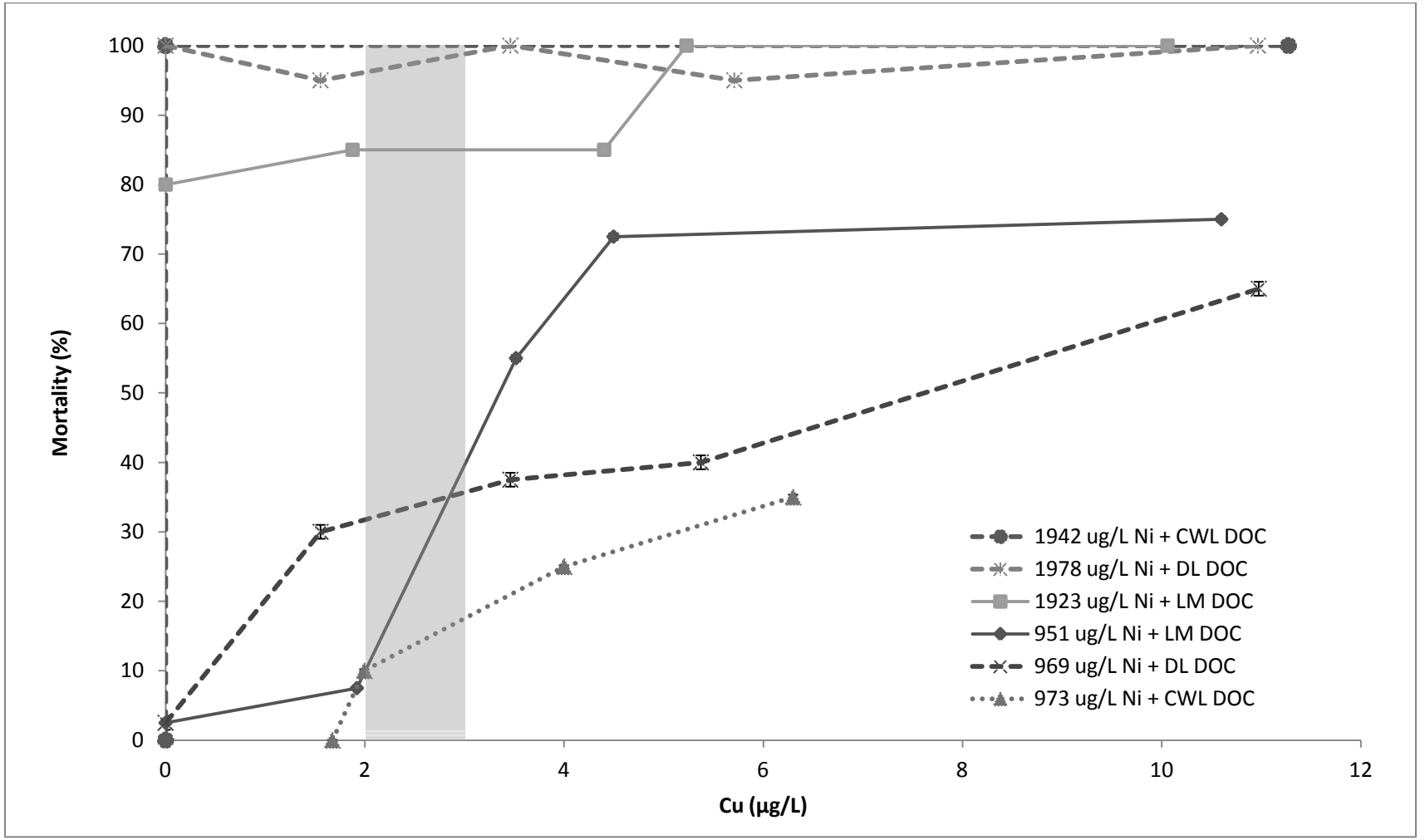
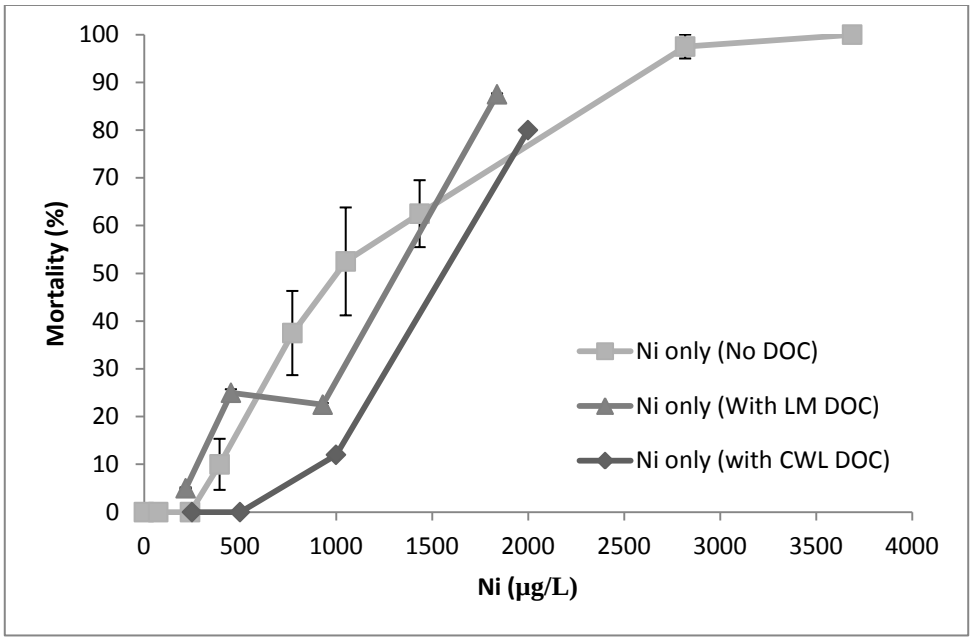


Figure 3.9: DOC from Luther Marsh (LM), Clearwater Lake (CWL), and Daisy Lake (DL) in solution with metal mixtures at 1000 and 2000 µg/L Ni. The grey box indicates the Cu LC50 range without added DOC.

A)



B)

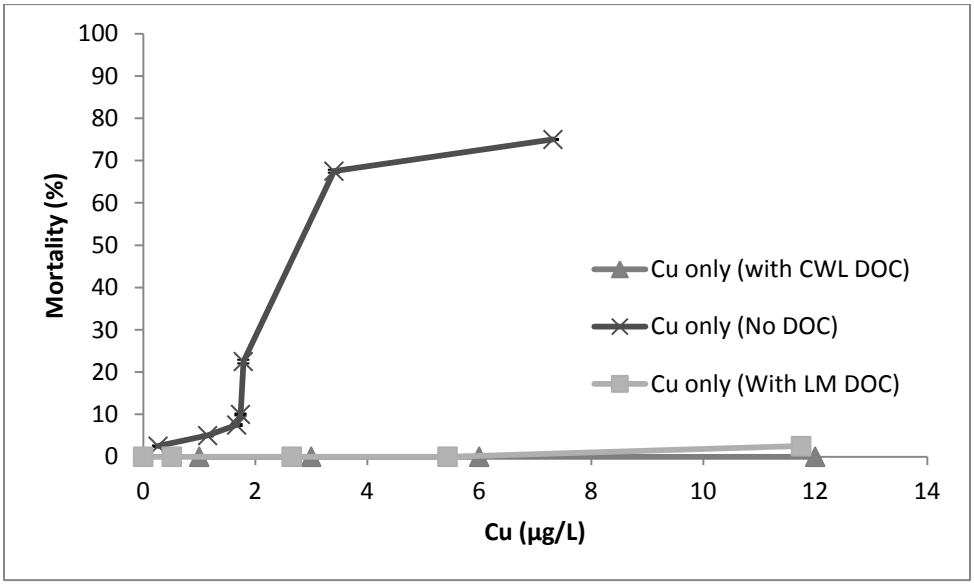
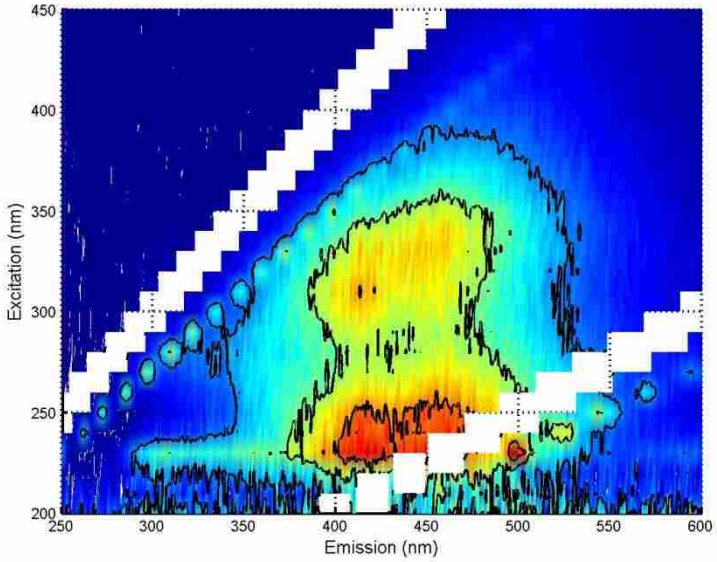
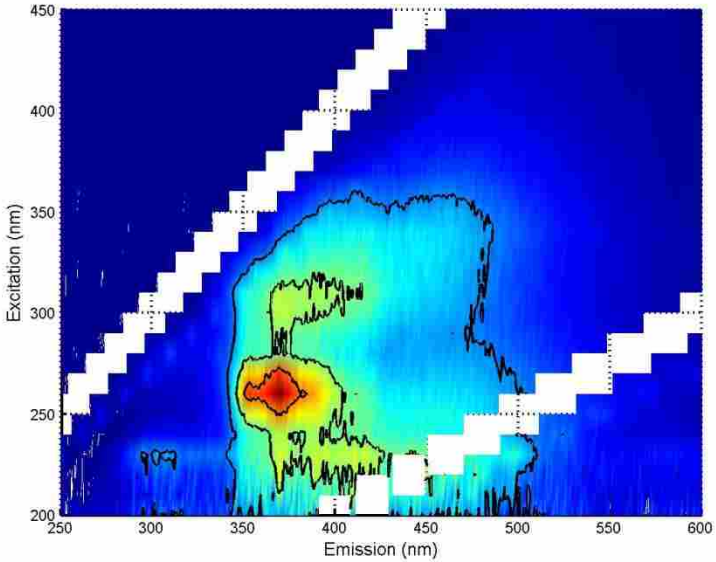


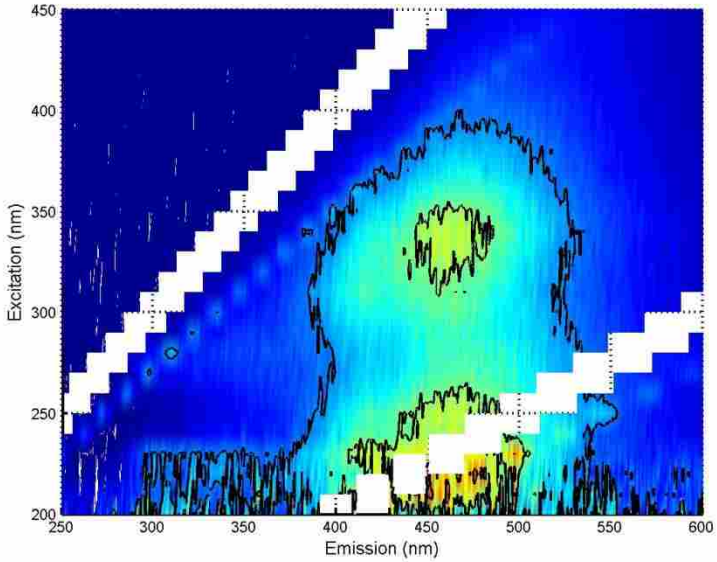
Figure 3.10 : Effect of adding 4 mg/L DOC from Luther Marsh to a solution with A) only Ni, and B) only Cu. There is a significant effect of Ni on daphnid mortality, with and without DOC ($p < 0.05$). There is a significant effect of Cu on daphnid mortality ($p < 0.05$) only when there is no DOC present. Cu does not have a significant effect on mortality in the presence of DOC ($p > 0.05$).



(A) Clearwater



(B) Daisy Lake



(C) Luther Marsh

Figure 3.11: Spectral contour plots of fluorescence intensities from excitation-emission matrices for the NOM isolates from three different sources: A) Clearwater Lake, B) Daisy Lake, C) Luther Marsh.

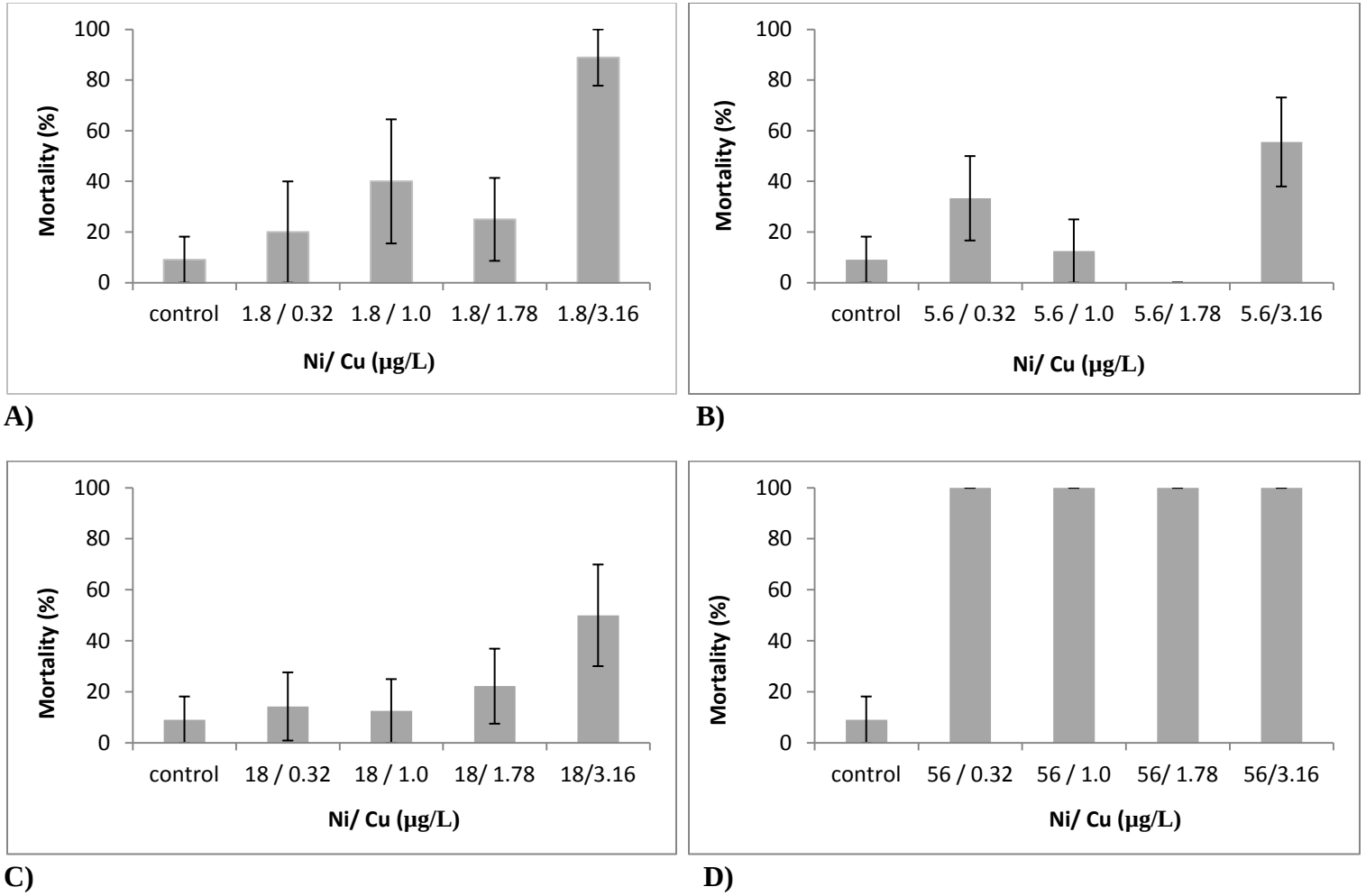
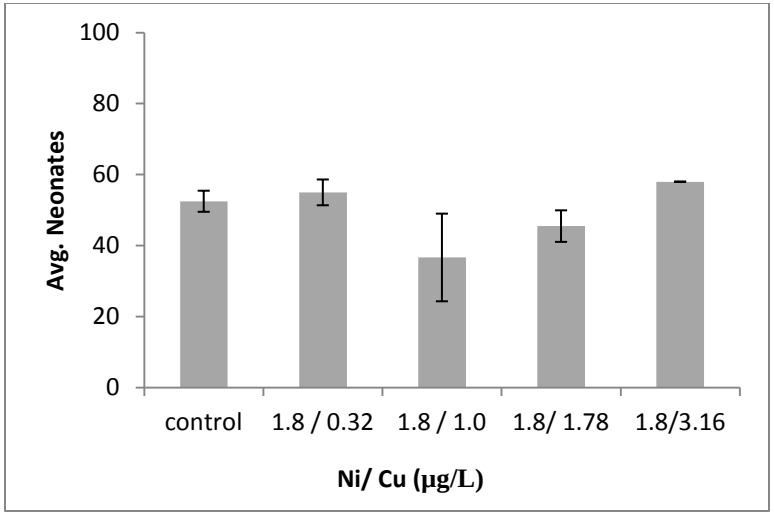
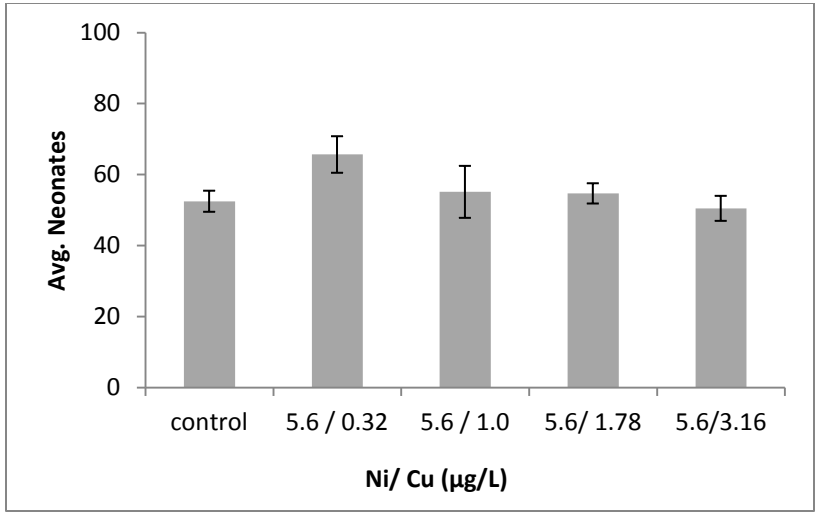


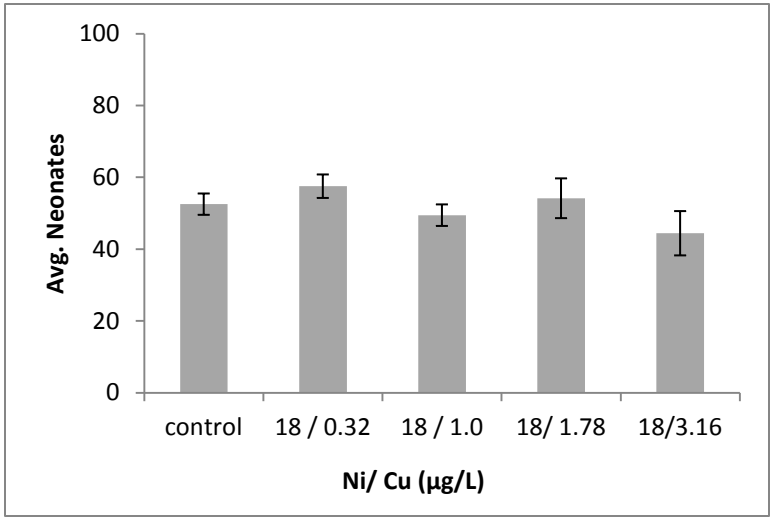
Figure 3.12: 21-day chronic effects of Ni and Cu mixtures on *D. pulex-pulicaria*. Each of the four panels show the response across a gradient of Cu exposure at different Ni concentrations and each bar is the mean for n = 10 daphnids. Error bars indicate the SEM for mortality. All Cu treatments were significantly different from controls for Ni concentrations of 1.8, 5.6 and 56 µg/L. A) $F(4, 33) = 5.51$, $p < 0.05$; B) $F(4, 39) = 2.79$, $p < 0.05$, C) $F(4, 40) = 1.61$, $p > 0.05$; D) $F(4, 41) = 77.99$, $p < 0.05$.



A)



B)



C)

Figure 3.13: Average neonates produced by daphnids surviving after 21 days of chronic Ni-Cu exposure. Error bars indicate SEM for number of neonates produced. All Cu treatments from each of the three Ni were not significantly different from controls ($p > 0.05$). $F(4, 19) = 1.69$ for 1.8 µg/L Ni, $F(4, 30) = 0.61$ for 5.6 µg/L Ni, and $F(4, 30) = 1.17$ for 18 µg/L Ni.

7. Tables

Table 2.1: Chemical composition of the FLAMES medium (Celis et al., 2008).

Compound Name	**Formula**	**Concentration (g/L)**
Calcium sulfate dihydrate	$CaSO_4.2H_2O$	0.547
Ferric chloride hexahydrate	$FeCl_3.6H_2O$	0.082
Borid Acid	H_3BO_3	0.715
Sodium metasilicate nonahydrate	$Na_2SiO_2.9H_2O$	4.573
Potassium Chloride	KCl	0.705
Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	7.6
Potassium phosphate monobasic	KH_2PO_4	0.044
Sodium nitrate	$NaNO_3$	0.082
Disodium EDTA	Na_2EDTA	1.00
Biotin	From Lynch et al. 1986	0.100
Animate	See Table 6 in Celis et al. 2008	--
Vitamin Mix	See Table 1 in Lynch et al. 1986	--

Table 2.2: Location coordinates for DOM sampling sources.

Lake	Sampling Location	GPS Coordinates
Luther Marsh	43°54'16.4"N 80°24'34.1"W	43.904621, -80.409556
Daisy Lake	46°26'37.4"N 80°54'14.8"W	46.443922, -80.904342
Clearwater Lake	46°22'14.8"N 81°03'12.1"W	46.370766, -81.053368

Table 3.1: Ni and Cu single-metal acute test LC_{50}'s over time. LC50 values were calculated in SPSS. Cu measured via Graphite furnace and Ni via Flame-AAS.

Nominal		Total		Dissolved						
Ni (µg/L)	Cu (µg/L)	Ni (µg/L)	Cu (µg/L)	Ni (µg/L)	Cu (µg/L)	n	Avg Mortality (%)	Probit LC50	Lower CI	Upper CI
0	0	0	0		0	8	0	2.65	2.15	3.2
0	2	0	1.008		0.873		15			
0	4	0	3.339		2.893		42.5			
0	8	0	6.823		5.144		100			
0	16	0	14.19		14.024		100			
0	32	0	31.6		30.224		100			
0	0	0	0.12		0.09	10	2	2.968	2.536	3.452
0	1	0	1.05		0.782		18			
0	2	0	2.25		1.985		30			
0	3	0	2.89		2.114		38			
0	4	0	4.27		3.121		54			
0	6	0	6.44		5.823		90			
0	12	0	11.62		10.982		100			
0	0	0	0.265		0.102	10	3.33	2.425	2.145	2.823
0	1	0	1.151		0.927		6.67			
0	2	0	1.669		1.362		10			

0 0 0 0	3 4 6 12	0 0 0 0	1.737 1.788 3.406 7.317		1.625 1.657 2.996 6.852		13.33 30 90 100			
0 500 1000 2000 4000 8000 16000	0 0 0 0 0 0 0	0 486 1140 2560 3948 9655 14785	0 0 0 0 0 0 0	455 1129 2381 3192 8768 12478		10	0 0 17.5 37.5 55 97.5 100	4.68	3.86	5.74
0 500 1000 2000 4000 8000 16000	0 0 0 0 0 0 0	0 486 1210 2340 4110 8790 15030	0 0 0 0 0 0 0	477 1082 2084 4022 7972 14268		8	0 16 52 58 86 100 100	1.37	1.115	1.64
0 100 250 500 1000 1500 2000	0 0 0 0 0 0 0	0 74 240 394 773 1051 1436	0 0 0 0 0 0 0	68 256 391 704 989 1274		8	0 0 0 10 37.5 52.5 62.5	0.995	0.877	1.125

3000	0	2819	0	2608			97.5			
4000	0	3689	0	3216			100			

Table 3.2: 48h acute LC50 values for Ni-Cu mixture combinations with no added DOC. Cu concentrations are 0, 1, 3, 6, and 12 µg/L. Probit LC50 values were calculated in SPSS.

Sample ID	**LC50**	**Upper CI**	**Lower CI**	**Ni TU**	**Cu TU**	**Sum of TU**
75 µg/L Ni + Cu	1.392	1.199	1.639	0.076	0.57	0.65
150 µg/L Ni + Cu	2.469	2.074	2.884	0.15	1.02	1.17
250 µg/L Ni + Cu	4.01	3.35	4.78	0.25	1.65	1.91
500 µg/L Ni + Cu	2.23	1.84	2.65	0.51	0.92	1.42
1000 µg/L Ni + Cu	2.65	2.32	2.96	1.01	0.029	1.04
2000 µg/L Ni + Cu	0.129	0.06	0.191	2.02	0.0062	2.03

Table 3.3: 48h acute LC50 values calculated for mixtures with DOC from 3 different sources: LM, CWL, DL. The Probit value for CWL + 2 mg Ni and DL + 2 mg Ni could not be calculated since the mortality was greater than 50% in all treatments.

Sample ID	**LC50**	**Lower CI**	**Upper CI**
LM DOC + 1 mg Ni	3.97	3.206	4.852
LM DOC + 2 mg Ni	0.569	0.005	1.273
CWL DOC + 1 mg Ni	8.3	5.5	38.6
DL DOC + 1 mg Ni	6.3	4.05	16.52
DL DOC + 2 mg Ni	NA	NA	NA
CWL DOC + 2 mg Ni	NA	NA	NA

Table 3.4: Cu free ion concentrations in solutions containing DOC from either Clearwater Lake (CWL) or Luther Marsh (LM). DOC concentrations were kept constant throughout the treatments. Free ions were measured using the Cu ISE. Actual Cu and Ni concentrations were measured using the Spectra AA Flame and Graphite Furnace.

Number	**Source ID**	**Ni (µg/L)**	**DOC (mg/L)**	**Cu (µg/L)**	**Cu Free ions (ng/L)**	**Cu free ions (%)**	**Cu Free Ions (logCuT)**	**SD**	**Mortality**
1	CWL + Cu + Ni	1800	5.1	5	134	2.7	- 8.68	0.23	100
2	CWL + Cu	1800	5.1	2.23	41	1.8	- 9.19	0.08	0
3	CWL + Ni	1800	5.1	4.0	114	2.9	- 8.75	0.26	80
4	LM + Cu + Ni	1800	4.9	3.1	1423	45.9	-7.653	1.56	85
5	LM + Cu	1800	4.9	3.0	42	1.4	- 9.187	0.08	0
6	LM + Ni	1800	4.9	0.32	2	0.6	-10.42	0.31	90

Table 3.5: Absorbance for DOC solutions corresponding to the Cu ISE test solutions in Table 3.2. Absorbance was measured using a spectrophotometer. DOC was measured using TOC-L. SAC_{340} coefficients were calculated using equation 3.

Sample ID	**Absorbance**	**DOC (mg/L)**	**SAC_{340}**
LM + 3.7 Cu	0.074	4.8	35.50
LM + 1 Ni	0.082	4.8	39.34
LM + Mixture	0.087	4.8	41.74
CWL + 3.7 Cu	0.032	4.8	15.35
CWL + 1 Ni	0.034	4.8	16.31
CWL + Mixture	0.034	4.8	16.31

Table 3.6: Fluorescence Indices (FI) for the three sources of DOC: LM, CWL, and DL. Excitation intensities at 370 nm are reported for the emission intensity wavelengths of 450 and 500 nm, which were used for calculating the FI value (equation 4). Predicted composition is based on FEEM optical characterization plots.

DOC Source	**Wavelength (nm)**	**Emission Intensity (a.u.)**	**FI**	**Predicted Origin**	**Predicted Composition**
LM	450	23.53	1.03	Allochthonous	Fulvic
	500	22.94			
CWL	450	18.44	1.22	Allochthonous and/or Autochthonous	Fulvic, Humic, Tryptophan, Tyrosine
	500	15.08			
DL	450	33.52	1.41	Autochthonous	Humic, Tryptophan, Tyrosine
	500	23.79			

Table 4.1: Data published on the toxicity of Ni and Cu to *G. pulex* and *Daphnia* species.

Author	Organism	Metal	Age	Duration	Water	Measured Effect	Effect Concentration (µg/L)	Ca (mg/L)	pH	DOC (mg/L)
Biesinger and Christensen, 1972	*D. magna*	Ni	< 24h	48 h	Lake Superior	LC50	510	18.045	7.3-7.6	1
Leonard and Wood, 2013	*D. pulex*	Ni	6-8 d	48 h	dechlorinated Hamilton tap water	LC50	750	134.736	7.8–8.0	2.3
Present Study	***D. pulex-pulicaria***	**Ni**	**< 24h**	**48 h**	**FLAMES Media**	**LC50**	**995**	**2.5**	**6.3-6.6**	**1**
Pane et al., 2003	*D. magna*	Ni	< 24h	48 h	Ottawa city tap water	LC50	1,068	18.045	7.3-7.6	3.6
Leonard and Wood, 2013	*D. pulex*	Ni	6-8 d	48 h	dechlorinated Hamilton tap water	LC50	2600	421.451	7.8–8.0	2.3
Charles et al., 2013	*Gammarus pulex*	Ni	Adult >6 mm/male	48h	mineral water Evian®	LC50	466,000	80	7.5±0.02	NA
Present Study	***D. pulex-pulicaria***	**Cu**	**< 24h**	**48 h**	**FLAMES Media**	**LC50**	**2.43**	**2.5**	**6.3-6.6**	**1**
Long et al., 2004	*D. magna*	Cu	< 24h	48 h	laboratory water with salts	LC50	2 ± 1.5	2.8471	5.6	NA
Long et al., 2004	*D. magna*	Cu	< 24h	48 h	laboratory water with salts	LC50	2.0 ± 0.5	8.2606	5.5	NA

Long et al., 2004	*D. magna*	Cu	< 24h	48 h	laboratory water with salts	LC50	2.8 ± 1	2.8471	7	NA
Stoddard and Harper, 2007	*D. magna*	Cu	< 24h	48 h	reconstituted hard water	LC50	4.72	46.115	7.8-8.2	NA
Dave, 1984	*D. magna*	Cu	< 24h (unfed)	48 h	carbon filtered well water	EC50	6.5	48.12	8-8.1	NA
Long et al., 2004	*D. magna*	Cu	< 24h	48 h	laboratory water with salts	LC50	7.4 ± 1.3	8.2606	7	NA
Biesinger and Christensen, 1972	*D. magna*	Cu	< 24h (unfed)	48 h	Lake Superior	LC50	9.8	18.045	7.3-7.6	1
Dave, 1984	*D. magna*	Cu	< 24h (fed)	48 h	carbon filtered well water	LC50	18.5	48.12	8-8.1	NA
Biesinger and Christensen, 1972	*D. magna*	Cu	< 24h (fed)	48 h	Lake Superior	LC50	60	18.045	7.3-7.6	1
Guilhermino et al., 2000	*D. magna*	Cu	< 24h	48 h	ASTM hard water	LC50	82.6		NA	NA
Meyer et al., 2015	*D. magna*	Cu	< 24h	48 h	moderately hard reconstituted water	EC50	103	36.09	7.4-7.8	3
Charles et al., 2013	*Gammarus pulex*	Ni	Adult >6 mm/male	48 h	mineral water Evian®	LC50	249	80	7.5±0.02	NA

8. Appendix

Table 5.1a: 2-way ANOVA was conducted for Ni-Cu mixtures without added DOC, where mortality was the dependent variable.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	531840.968[a]	34	15642.381	99.876	.000
Intercept	882996.252	1	882996.252	5637.891	.000
Ni	80694.301	6	13449.050	85.872	.000
Cu	390506.503	4	97626.626	623.342	.000
Ni * Cu	59474.409	24	2478.100	15.823	.000
Error	43070.000	275	156.618		
Total	1524800.000	310			
Corrected Total	574910.968	309			

a. R Squared = .925 (Adjusted R Squared = .916)

Table 5.1b: Pairwise comparisons from Post-Hoc Tukey test corresponding to the 2-way ANOVA for mixtures without added DOC. The difference between Cu treatments at each Ni treatment are indicated by the letters (A, B, C ...).

Ni	(I) Cu	(J) Cu	Mean Difference (I-J)	Std. Error	Sig.[#]	Significance Comparison	95% Confidence Interval for Difference[#]	
							Lower Bound	Upper Bound
.00	.00	1.00	-3.333	7.225	1.000	A	-23.780	17.113
		3.00	-10.000	7.225	1.000	A	-30.447	10.447
		6.00	-86.667*	7.225	.000	B	-107.113	-66.220
		12.00	-96.667*	7.225	.000	B	-117.113	-76.220
	1.00	.00	3.333	7.225	1.000	A	-17.113	23.780
		3.00	-6.667	7.225	1.000	A	-27.113	13.780
		6.00	-83.333*	7.225	.000	B	-103.780	-62.887
		12.00	-93.333*	7.225	.000	B	-113.780	-72.887
	3.00	.00	10.000	7.225	1.000	A	-10.447	30.447
		1.00	6.667	7.225	1.000	A	-13.780	27.113
		6.00	-76.667*	7.225	.000	B	-97.113	-56.220
		12.00	-86.667*	7.225	.000	B	-107.113	-66.220
	6.00	.00	86.667*	7.225	.000	A	66.220	107.113
		1.00	83.333*	7.225	.000	A	62.887	103.780

		3.00	76.667*	7.225	.000	A	56.220	97.113
		12.00	-10.000	7.225	1.000	B	-30.447	10.447
	12.00	.00	96.667*	7.225	.000	A	76.220	117.113
		1.00	93.333*	7.225	.000	A	72.887	113.780
		3.00	86.667*	7.225	.000	A	66.220	107.113
		6.00	10.000	7.225	1.000	B	-10.447	30.447
55.86	.00	1.00	-4.000	5.597	1.000	A	-19.838	11.838
		3.00	-8.000	5.597	1.000	A	-23.838	7.838
		6.00	-100.000*	5.597	.000	B	-115.838	-84.162
		12.00	-100.000*	5.597	.000	B	-115.838	-84.162
	1.00	.00	4.000	5.597	1.000	A	-11.838	19.838
		3.00	-4.000	5.597	1.000	A	-19.838	11.838
		6.00	-96.000*	5.597	.000	B	-111.838	-80.162
		12.00	-96.000*	5.597	.000	B	-111.838	-80.162
	3.00	.00	8.000	5.597	1.000	A	-7.838	23.838
		1.00	4.000	5.597	1.000	A	-11.838	19.838
		6.00	-92.000*	5.597	.000	B	-107.838	-76.162
		12.00	-92.000*	5.597	.000	B	-107.838	-76.162
	6.00	.00	100.000*	5.597	.000	A	84.162	115.838
		1.00	96.000*	5.597	.000	A	80.162	111.838
		3.00	92.000*	5.597	.000	A	76.162	107.838
		12.00	2.442E-14	5.597	1.000	B	-15.838	15.838
	12.00	.00	100.000*	5.597	.000	A	84.162	115.838
		1.00	96.000*	5.597	.000	A	80.162	111.838
		3.00	92.000*	5.597	.000	A	76.162	107.838
		6.00	-2.442E-14	5.597	1.000	B	-15.838	15.838
131.22	.00	1.00	-4.000	5.597	1.000	A	-19.838	11.838
		3.00	-22.000*	5.597	.001	B	-37.838	-6.162
		6.00	-100.000*	5.597	.000	C	-115.838	-84.162
		12.00	-100.000*	5.597	.000	C	-115.838	-84.162
	1.00	.00	4.000	5.597	1.000	A	-11.838	19.838
		3.00	-18.000*	5.597	.015	B	-33.838	-2.162
		6.00	-96.000*	5.597	.000	C	-111.838	-80.162
		12.00	-96.000*	5.597	.000	C	-111.838	-80.162
	3.00	.00	22.000*	5.597	.001	A	6.162	37.838
		1.00	18.000*	5.597	.015	B	2.162	33.838
		6.00	-78.000*	5.597	.000	C	-93.838	-62.162
		12.00	-78.000*	5.597	.000	C	-93.838	-62.162
	6.00	.00	100.000*	5.597	.000	A	84.162	115.838
		1.00	96.000*	5.597	.000	A	80.162	111.838

		3.00	78.000*	5.597	.000	A	62.162	93.838
		12.00	2.720E-14	5.597	1.000	B	-15.838	15.838
	12.00	.00	100.000*	5.597	.000	A	84.162	115.838
		1.00	96.000*	5.597	.000	A	80.162	111.838
		3.00	78.000*	5.597	.000	A	62.162	93.838
		6.00	-2.720E-14	5.597	1.000	B	-15.838	15.838
	.00	1.00	5.000	6.257	1.000	A	-12.707	22.707
		3.00	-1.421E-14	6.257	1.000	A	-17.707	17.707
		6.00	-70.000*	6.257	.000	B	-87.707	-52.293
		12.00	-80.000*	6.257	.000	B	-97.707	-62.293
	1.00	.00	-5.000	6.257	1.000	A	-22.707	12.707
		3.00	-5.000	6.257	1.000	A	-22.707	12.707
		6.00	-75.000*	6.257	.000	B	-92.707	-57.293
		12.00	-85.000*	6.257	.000	B	-102.707	-67.293
	3.00	.00	1.421E-14	6.257	1.000	A	-17.707	17.707
		1.00	5.000	6.257	1.000	A	-12.707	22.707
228.87		6.00	-70.000*	6.257	.000	B	-87.707	-52.293
		12.00	-80.000*	6.257	.000	B	-97.707	-62.293
	6.00	.00	70.000*	6.257	.000	A	52.293	87.707
		1.00	75.000*	6.257	.000	A	57.293	92.707
		3.00	70.000*	6.257	.000	A	52.293	87.707
		12.00	-10.000	6.257	1.000	B	-27.707	7.707
	12.00	.00	80.000*	6.257	.000	A	62.293	97.707
		1.00	85.000*	6.257	.000	A	67.293	102.707
		3.00	80.000*	6.257	.000	A	62.293	97.707
		6.00	10.000	6.257	1.000	B	-7.707	27.707
	.00	1.00	-27.500*	6.257	.000	A	-45.207	-9.793
		3.00	-25.000*	6.257	.001	B	-42.707	-7.293
		6.00	-95.000*	6.257	.000	C	-112.707	-77.293
		12.00	-95.000*	6.257	.000	C	-112.707	-77.293
	1.00	.00	27.500*	6.257	.000	A	9.793	45.207
		3.00	2.500	6.257	1.000	B	-15.207	20.207
		6.00	-67.500*	6.257	.000	C	-85.207	-49.793
474.37		12.00	-67.500*	6.257	.000	C	-85.207	-49.793
	3.00	.00	25.000*	6.257	.001	A	7.293	42.707
		1.00	-2.500	6.257	1.000	B	-20.207	15.207
		6.00	-70.000*	6.257	.000	C	-87.707	-52.293
		12.00	-70.000*	6.257	.000	C	-87.707	-52.293
	6.00	.00	95.000*	6.257	.000	A	77.293	112.707
		1.00	67.500*	6.257	.000	A	49.793	85.207
		3.00	70.000*	6.257	.000	A	52.293	87.707

		12.00	7.661E-15	6.257	1.000	B	-17.707	17.707
	12.00	.00	95.000*	6.257	.000	A	77.293	112.707
		1.00	67.500*	6.257	.000	A	49.793	85.207
		3.00	70.000*	6.257	.000	A	52.293	87.707
		6.00	-7.661E-15	6.257	1.000	B	-17.707	17.707
950.97	.00	1.00	.000	5.597	1.000	A	-15.838	15.838
		3.00	-44.000*	5.597	.000	B	-59.838	-28.162
		6.00	-74.000*	5.597	.000	B	-89.838	-58.162
		12.00	-72.000*	5.597	.000	B	-87.838	-56.162
	1.00	.00	.000	5.597	1.000	A	-15.838	15.838
		3.00	-44.000*	5.597	.000	B	-59.838	-28.162
		6.00	-74.000*	5.597	.000	B	-89.838	-58.162
		12.00	-72.000*	5.597	.000	B	-87.838	-56.162
	3.00	.00	44.000*	5.597	.000	A	28.162	59.838
		1.00	44.000*	5.597	.000	A	28.162	59.838
		6.00	-30.000*	5.597	.000	A	-45.838	-14.162
		12.00	-28.000*	5.597	.000	A	-43.838	-12.162
	6.00	.00	74.000*	5.597	.000	A	58.162	89.838
		1.00	74.000*	5.597	.000	A	58.162	89.838
		3.00	30.000*	5.597	.000	A	14.162	45.838
		12.00	2.000	5.597	1.000	B	-13.838	17.838
	12.00	.00	72.000*	5.597	.000	A	56.162	87.838
		1.00	72.000*	5.597	.000	A	56.162	87.838
		3.00	28.000*	5.597	.000	A	12.162	43.838
		6.00	-2.000	5.597	1.000	B	-17.838	13.838
1849.05	.00	1.00	-16.000*	5.597	.046	A	-31.838	-.162
		3.00	-32.000*	5.597	.000	B	-47.838	-16.162
		6.00	-36.000*	5.597	.000	B	-51.838	-20.162
		12.00	-36.000*	5.597	.000	B	-51.838	-20.162
	1.00	.00	16.000*	5.597	.046	A	.162	31.838
		3.00	-16.000*	5.597	.046	A	-31.838	-.162
		6.00	-20.000*	5.597	.004	B	-35.838	-4.162
		12.00	-20.000*	5.597	.004	B	-35.838	-4.162
	3.00	.00	32.000*	5.597	.000	A	16.162	47.838
		1.00	16.000*	5.597	.046	B	.162	31.838
		6.00	-4.000	5.597	1.000	A	-19.838	11.838
		12.00	-4.000	5.597	1.000	A	-19.838	11.838
	6.00	.00	36.000*	5.597	.000	A	20.162	51.838
		1.00	20.000*	5.597	.004	B	4.162	35.838

		3.00	4.000	5.597	1.000	A	-11.838	19.838
		12.00	-7.838E-14	5.597	1.000	A	-15.838	15.838
	12.00	.00	36.000*	5.597	.000	A	20.162	51.838
		1.00	20.000*	5.597	.004	B	4.162	35.838
		3.00	4.000	5.597	1.000	A	-11.838	19.838
		6.00	7.838E-14	5.597	1.000	A	-15.838	15.838

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

♯. Adjustment for multiple comparisons: Bonferroni.

Table 5.1 c: Pairwise comparisons from Post-Hoc Tukey test corresponding to the 2-way ANOVA for mixtures without added DOC in 5.1a. The difference between Ni treatments at each Cu treatment are indicated by the letters (A, B, C ...).

Cu	(I) Ni	(J) Ni	Mean Difference (I-J)	Std. Error	Sig.♯	Significance comparison	95% Confidence Interval for Difference♯	
							Lower Bound	Upper Bound
.00	.00	55.86	3.333	6.463	1.000	A	-16.485	23.151
		131.22	3.333	6.463	1.000	A	-16.485	23.151
		228.87	-11.667	6.759	1.000	A	-32.393	9.059
		474.37	-1.667	6.759	1.000	A	-22.393	19.059
		950.97	-22.667*	6.463	.011	B	-42.485	-2.849
		1849.05	-60.667*	6.463	.000	C	-80.485	-40.849
	55.86	.00	-3.333	6.463	1.000	A	-23.151	16.485
		131.22	.000	5.597	1.000	A	-17.163	17.163
		228.87	-15.000	5.936	.253	B	-33.204	3.204
		474.37	-5.000	5.936	1.000	A	-23.204	13.204
		950.97	-26.000*	5.597	.000	C	-43.163	-8.837
		1849.05	-64.000*	5.597	.000	C	-81.163	-46.837
	131.22	.00	-3.333	6.463	1.000	A	-23.151	16.485
		55.86	.000	5.597	1.000	A	-17.163	17.163
		228.87	-15.000	5.936	.253	B	-33.204	3.204
		474.37	-5.000	5.936	1.000	A	-23.204	13.204
		950.97	-26.000*	5.597	.000	C	-43.163	-8.837
		1849.05	-64.000*	5.597	.000	C	-81.163	-46.837
	228.87	.00	11.667	6.759	1.000	A	-9.059	32.393
		55.86	15.000	5.936	.253	B	-3.204	33.204
		131.22	15.000	5.936	.253	B	-3.204	33.204
		474.37	10.000	6.257	1.000	A	-9.189	29.189
		950.97	-11.000	5.936	1.000	A	-29.204	7.204

		1849.05	-49.000*	5.936	.000	C	-67.204	-30.796
	474.37	.00	1.667	6.759	1.000	A	-19.059	22.393
		55.86	5.000	5.936	1.000	A	-13.204	23.204
		131.22	5.000	5.936	1.000	A	-13.204	23.204
		228.87	-10.000	6.257	1.000	A	-29.189	9.189
		950.97	-21.000*	5.936	.010	B	-39.204	-2.796
		1849.05	-59.000*	5.936	.000	C	-77.204	-40.796
	950.97	.00	22.667*	6.463	.011	A	2.849	42.485
		55.86	26.000*	5.597	.000	B	8.837	43.163
		131.22	26.000*	5.597	.000	B	8.837	43.163
		228.87	11.000	5.936	1.000	C	-7.204	29.204
		474.37	21.000*	5.936	.010	D	2.796	39.204
		1849.05	-38.000*	5.597	.000	B	-55.163	-20.837
	1849.05	.00	60.667*	6.463	.000	A	40.849	80.485
		55.86	64.000*	5.597	.000	A	46.837	81.163
		131.22	64.000*	5.597	.000	A	46.837	81.163
		228.87	49.000*	5.936	.000	A	30.796	67.204
		474.37	59.000*	5.936	.000	A	40.796	77.204
		950.97	38.000*	5.597	.000	A	20.837	55.163
1.00	.00	55.86	2.667	6.463	1.000	A	-17.151	22.485
		131.22	2.667	6.463	1.000	A	-17.151	22.485
		228.87	-3.333	6.759	1.000	A	-24.059	17.393
		474.37	-25.833*	6.759	.003	B	-46.559	-5.107
		950.97	-19.333	6.463	.064	C	-39.151	.485
		1849.05	-73.333*	6.463	.000	D	-93.151	-53.515
	55.86	.00	-2.667	6.463	1.000	A	-22.485	17.151
		131.22	-4.263E-14	5.597	1.000	A	-17.163	17.163
		228.87	-6.000	5.936	1.000	A	-24.204	12.204
		474.37	-28.500*	5.936	.000	B	-46.704	-10.296
		950.97	-22.000*	5.597	.002	C	-39.163	-4.837
		1849.05	-76.000*	5.597	.000	B	-93.163	-58.837
	131.22	.00	-2.667	6.463	1.000	A	-22.485	17.151
		55.86	4.263E-14	5.597	1.000	A	-17.163	17.163
		228.87	-6.000	5.936	1.000	A	-24.204	12.204
		474.37	-28.500*	5.936	.000	B	-46.704	-10.296
		950.97	-22.000*	5.597	.002	C	-39.163	-4.837
		1849.05	-76.000*	5.597	.000	B	-93.163	-58.837
	228.87	.00	3.333	6.759	1.000	A	-17.393	24.059
		55.86	6.000	5.936	1.000	A	-12.204	24.204

		131.22	6.000	5.936	1.000	A	-12.204	24.204
		474.37	-22.500*	6.257	.008	B	-41.689	-3.311
		950.97	-16.000	5.936	.157	C	-34.204	2.204
		1849.05	-70.000*	5.936	.000	D	-88.204	-51.796
	474.37	.00	25.833*	6.759	.003	A	5.107	46.559
		55.86	28.500*	5.936	.000	B	10.296	46.704
		131.22	28.500*	5.936	.000	B	10.296	46.704
		228.87	22.500*	6.257	.008	C	3.311	41.689
		950.97	6.500	5.936	1.000	D	-11.704	24.704
		1849.05	-47.500*	5.936	.000	B	-65.704	-29.296
	950.97	.00	19.333	6.463	.064	A	-.485	39.151
		55.86	22.000*	5.597	.002	B	4.837	39.163
		131.22	22.000*	5.597	.002	B	4.837	39.163
		228.87	16.000	5.936	.157	C	-2.204	34.204
		474.37	-6.500	5.936	1.000	D	-24.704	11.704
		1849.05	-54.000*	5.597	.000	E	-71.163	-36.837
	1849.05	.00	73.333*	6.463	.000	A	53.515	93.151
		55.86	76.000*	5.597	.000	A	58.837	93.163
		131.22	76.000*	5.597	.000	A	58.837	93.163
		228.87	70.000*	5.936	.000	A	51.796	88.204
		474.37	47.500*	5.936	.000	A	29.296	65.704
		950.97	54.000*	5.597	.000	A	36.837	71.163
3.00	.00	55.86	5.333	6.463	1.000	A	-14.485	25.151
		131.22	-8.667	6.463	1.000	A	-28.485	11.151
		228.87	-1.667	6.759	1.000	A	-22.393	19.059
		474.37	-16.667	6.759	.300	B	-37.393	4.059
		950.97	-56.667*	6.463	.000	C	-76.485	-36.849
		1849.05	-82.667*	6.463	.000	C	-102.485	-62.849
	55.86	.00	-5.333	6.463	1.000	A	-25.151	14.485
		131.22	-14.000	5.597	.272	B	-31.163	3.163
		228.87	-7.000	5.936	1.000	A	-25.204	11.204
		474.37	-22.000*	5.936	.005	C	-40.204	-3.796
		950.97	-62.000*	5.597	.000	D	-79.163	-44.837
		1849.05	-88.000*	5.597	.000	D	-105.163	-70.837
	131.22	.00	8.667	6.463	1.000	A	-11.151	28.485
		55.86	14.000	5.597	.272	B	-3.163	31.163
		228.87	7.000	5.936	1.000	A	-11.204	25.204
		474.37	-8.000	5.936	1.000	A	-26.204	10.204
		950.97	-48.000*	5.597	.000	C	-65.163	-30.837
		1849.05	-74.000*	5.597	.000	C	-91.163	-56.837
	228.87	.00	1.667	6.759	1.000	A	-19.059	22.393

		55.86	7.000	5.936	1.000	A	-11.204	25.204
		131.22	-7.000	5.936	1.000	A	-25.204	11.204
		474.37	-15.000	6.257	.361	B	-34.189	4.189
		950.97	-55.000*	5.936	.000	C	-73.204	-36.796
		1849.05	-81.000*	5.936	.000	C	-99.204	-62.796
	474.37	.00	16.667	6.759	.300	A	-4.059	37.393
		55.86	22.000*	5.936	.005	B	3.796	40.204
		131.22	8.000	5.936	1.000	C	-10.204	26.204
		228.87	15.000	6.257	.361	D	-4.189	34.189
		950.97	-40.000*	5.936	.000	E	-58.204	-21.796
		1849.05	-66.000*	5.936	.000	E	-84.204	-47.796
	950.97	.00	56.667*	6.463	.000	A	36.849	76.485
		55.86	62.000*	5.597	.000	A	44.837	79.163
		131.22	48.000*	5.597	.000	A	30.837	65.163
		228.87	55.000*	5.936	.000	A	36.796	73.204
		474.37	40.000*	5.936	.000	A	21.796	58.204
		1849.05	-26.000*	5.597	.000	A	-43.163	-8.837
	1849.05	.00	82.667*	6.463	.000	A	62.849	102.485
		55.86	88.000*	5.597	.000	A	70.837	105.163
		131.22	74.000*	5.597	.000	A	56.837	91.163
		228.87	81.000*	5.936	.000	A	62.796	99.204
		474.37	66.000*	5.936	.000	A	47.796	84.204
		950.97	26.000*	5.597	.000	A	8.837	43.163
6.00	.00	55.86	-10.000	6.463	1.000	A	-29.818	9.818
		131.22	-10.000	6.463	1.000	A	-29.818	9.818
		228.87	5.000	6.759	1.000	A	-15.726	25.726
		474.37	-10.000	6.759	1.000	A	-30.726	10.726
		950.97	-10.000	6.463	1.000	A	-29.818	9.818
		1849.05	-10.000	6.463	1.000	A	-29.818	9.818
	55.86	.00	10.000	6.463	1.000	A	-9.818	29.818
		131.22	-1.110E-14	5.597	1.000	A	-17.163	17.163
		228.87	15.000	5.936	.253	B	-3.204	33.204
		474.37	4.774E-15	5.936	1.000	A	-18.204	18.204
		950.97	1.710E-14	5.597	1.000	A	-17.163	17.163
		1849.05	1.055E-13	5.597	1.000	A	-17.163	17.163
	131.22	.00	10.000	6.463	1.000	A	-9.818	29.818
		55.86	1.110E-14	5.597	1.000	A	-17.163	17.163
		228.87	15.000	5.936	.253	B	-3.204	33.204
		474.37	1.588E-14	5.936	1.000	A	-18.204	18.204
		950.97	2.842E-14	5.597	1.000	A	-17.163	17.163
		1849.05	1.166E-13	5.597	1.000	A	-17.163	17.163

	228.87	.00	-5.000	6.759	1.000	A	-25.726	15.726
		55.86	-15.000	5.936	.253	B	-33.204	3.204
		131.22	-15.000	5.936	.253	B	-33.204	3.204
		474.37	-15.000	6.257	.361	C	-34.189	4.189
		950.97	-15.000	5.936	.253	B	-33.204	3.204
		1849.05	-15.000	5.936	.253	B	-33.204	3.204
	474.37	.00	10.000	6.759	1.000	A	-10.726	30.726
		55.86	-4.774E-15	5.936	1.000	A	-18.204	18.204
		131.22	-1.588E-14	5.936	1.000	A	-18.204	18.204
		228.87	15.000	6.257	.361	B	-4.189	34.189
		950.97	1.243E-14	5.936	1.000	A	-18.204	18.204
		1849.05	1.007E-13	5.936	1.000	A	-18.204	18.204
	950.97	.00	10.000	6.463	1.000	A	-9.818	29.818
		55.86	-1.710E-14	5.597	1.000	A	-17.163	17.163
		131.22	-2.842E-14	5.597	1.000	A	-17.163	17.163
		228.87	15.000	5.936	.253	B	-3.204	33.204
		474.37	-1.243E-14	5.936	1.000	A	-18.204	18.204
		1849.05	8.837E-14	5.597	1.000	A	-17.163	17.163
	1849.05	.00	10.000	6.463	1.000	A	-9.818	29.818
		55.86	-1.055E-13	5.597	1.000	A	-17.163	17.163
		131.22	-1.166E-13	5.597	1.000	A	-17.163	17.163
		228.87	15.000	5.936	.253	B	-3.204	33.204
		474.37	-1.007E-13	5.936	1.000	A	-18.204	18.204
		950.97	-8.837E-14	5.597	1.000	A	-17.163	17.163
12.00	.00	55.86	4.852E-14	6.463	1.000	A	-19.818	19.818
		131.22	4.019E-14	6.463	1.000	A	-19.818	19.818
		228.87	5.000	6.759	1.000	A	-15.726	25.726
		474.37	3.653E-14	6.759	1.000	A	-20.726	20.726
		950.97	2.000	6.463	1.000	A	-17.818	21.818
		1849.05	5.118E-14	6.463	1.000	A	-19.818	19.818
	55.86	.00	-4.852E-14	6.463	1.000	A	-19.818	19.818
		131.22	-8.327E-15	5.597	1.000	A	-17.163	17.163
		228.87	5.000	5.936	1.000	A	-13.204	23.204
		474.37	-1.199E-14	5.936	1.000	A	-18.204	18.204
		950.97	2.000	5.597	1.000	A	-15.163	19.163
		1849.05	2.665E-15	5.597	1.000	A	-17.163	17.163
	131.22	.00	-4.019E-14	6.463	1.000	A	-19.818	19.818
		55.86	8.327E-15	5.597	1.000	A	-17.163	17.163
		228.87	5.000	5.936	1.000	A	-13.204	23.204

	474.37	-3.664E-15	5.936	1.000	A	-18.204	18.204
	950.97	2.000	5.597	1.000	A	-15.163	19.163
	1849.05	1.099E-14	5.597	1.000	A	-17.163	17.163
228.87	.00	-5.000	6.759	1.000	A	-25.726	15.726
	55.86	-5.000	5.936	1.000	A	-23.204	13.204
	131.22	-5.000	5.936	1.000	A	-23.204	13.204
	474.37	-5.000	6.257	1.000	A	-24.189	14.189
	950.97	-3.000	5.936	1.000	A	-21.204	15.204
	1849.05	-5.000	5.936	1.000	A	-23.204	13.204
474.37	.00	-3.653E-14	6.759	1.000	A	-20.726	20.726
	55.86	1.199E-14	5.936	1.000	A	-18.204	18.204
	131.22	3.664E-15	5.936	1.000	A	-18.204	18.204
	228.87	5.000	6.257	1.000	A	-14.189	24.189
	950.97	2.000	5.936	1.000	A	-16.204	20.204
	1849.05	1.465E-14	5.936	1.000	A	-18.204	18.204
950.97	.00	-2.000	6.463	1.000	A	-21.818	17.818
	55.86	-2.000	5.597	1.000	A	-19.163	15.163
	131.22	-2.000	5.597	1.000	A	-19.163	15.163
	228.87	3.000	5.936	1.000	A	-15.204	21.204
	474.37	-2.000	5.936	1.000	A	-20.204	16.204
	1849.05	-2.000	5.597	1.000	A	-19.163	15.163
1849.05	.00	-5.118E-14	6.463	1.000	A	-19.818	19.818
	55.86	-2.665E-15	5.597	1.000	A	-17.163	17.163
	131.22	-1.099E-14	5.597	1.000	A	-17.163	17.163
	228.87	5.000	5.936	1.000	A	-13.204	23.204
	474.37	-1.465E-14	5.936	1.000	A	-18.204	18.204
	950.97	2.000	5.597	1.000	A	-15.163	19.163

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

≠. Adjustment for multiple comparisons: Bonferroni.

Table 5.2: Predicted mortality calculations for the IA model. Actual mortalities are taken from toxicity tests (Fig 3.5).

Ni (µg/L)	Cu (µg/L)	Ni LC50	Cu LC50	R_{Ni} Ni Fraction	R_{Cu} Cu Fraction	Y	Actual Mortality	Predicted Mortality
75	1	995	2.425	0.075	0.412	45.67	4	45.66648
75	3	995	2.425	0.075	1.237	121.92	8	100
75	6	995	2.425	0.075	2.474	236.32	100	100
75	12	995	2.425	0.075	4.948	465.084	100	100
150	1	995	2.425	0.151	0.412	50.096	4	50.09584
150	3	995	2.425	0.151	1.237	120.137	22	100
150	6	995	2.425	0.151	2.474	225.197	100	100
150	12	995	2.425	0.151	4.948	435.321	100	100
250	1	995	2.425	0.251	0.412	56.002	46.66667	56.00166
250	3	995	2.425	0.251	1.237	117.754	100	100
250	6	995	2.425	0.251	2.474	210.382	100	100
250	12	995	2.425	0.251	4.948	395.638	100	100
500	1	995	2.425	0.503	0.412	70.766	86.66667	70.7662
500	3	995	2.425	0.503	1.237	111.796	100	100
500	6	995	2.425	0.503	2.474	173.341	100	100
500	12	995	2.425	0.503	4.948	296.431	100	100
1000	1	995	2.425	1.00	0.412	100.293	70	100
1000	3	995	2.425	1.00	1.237	99.881	100	99.88085
1000	6	995	2.425	1.00	2.474	99.259	100	99.25918
1000	12	995	2.425	1.00	4.948	98.016	100	98.01585
2000	1	995	2.425	2.01	0.412	159.354	95	100
2000	3	995	2.425	2.01	1.237	76.050	100	76.05035
2000	6	995	2.425	2.01	2.474	148.904	100	100
2000	12	995	2.425	2.01	4.948	398.814	100	100

Table 5.3: Predicted mortality calculations for the CA model. Actual probit concentrations were obtained from Table 3.2.

Ni (µg/L)	Cu (µg/L)	Ni + Cu	P_{Ni} Ni Fraction	P_{Cu} Cu Fraction	X_{Ni} Ni LC_{50}	X_{Cu} Cu LC_{50}	X Mixture (µg/L)	Actual Mortality	Predicted conc. (avg of X's)	Actual conc. Probit LC_{50} for mixture
75	1	76	0.987	0.013	995	2.425	0.407	4	0.385	1.392
75	3	78	0.962	0.038	995	2.425	0.397	8		
75	6	81	0.926	0.074	995	2.425	0.382	100		
75	12	87	0.862	0.138	995	2.425	0.356	100		
150	1	151	0.993	0.007	995	2.425	0.410	4	0.398	2.469
150	3	153	0.980	0.020	995	2.425	0.404	22		
150	6	156	0.962	0.038	995	2.425	0.397	100		
150	12	162	0.926	0.074	995	2.425	0.382	100		
250	1	251	0.996	0.004	995	2.425	0.411	46.66667	0.404	4.01
250	3	253	0.988	0.012	995	2.425	0.407	100		
250	6	256	0.977	0.023	995	2.425	0.403	100		
250	12	262	0.954	0.046	995	2.425	0.394	100		
500	1	501	0.998	0.002	995	2.425	0.412	86.66667	0.408	2.23
500	3	503	0.994	0.006	995	2.425	0.410	100		
500	6	506	0.988	0.012	995	2.425	0.407	100		
500	12	512	0.977	0.023	995	2.425	0.403	100		
1000	1	1001	0.999	0.001	995	2.425	0.412	70	0.410	2.65
1000	3	1003	0.997	0.003	995	2.425	0.411	100		
1000	6	1006	0.994	0.006	995	2.425	0.410	100		
1000	12	1012	0.988	0.012	995	2.425	0.407	100		
2000	1	2001	1.000	0.000	995	2.425	0.412	95	0.411	0.129
2000	3	2003	0.999	0.001	995	2.425	0.412	100		
2000	6	2006	0.997	0.003	995	2.425	0.411	100		
2000	12	2012	0.994	0.006	995	2.425	0.410	100		

Table 5.4: Measured Cu values using GF-AAS. Total Cu samples were not filtered, while dissolved Cu samples were filtered using 0.45 µm filter.

Nominal Cu (µg/L)	**Total Cu (µg/L)** n = 10	**Dissolved Cu (µg/L)** n = 10	**Percent Difference**
1	1.47 ± 0.22	1.39 ± 0.56	97.72 ± 3.49
3	2.47 ± 0.31	2.34 ± 0.33	97.54 ± 6.52
6	4.45 ± 0.63	4.34 ± 0.62	97.21 ± 3.76
12	10.37 ± 0.57	10.08 ± 0.54	97.69 ± 2.71

Table 5.5: Measured Ni values taken via Flame-AAS. Total Ni samples were not filtered, while dissolved Ni samples were filtered using 0.45 µm filter.

Nominal Ni (µg/L)	**Total Ni (µg/L)** n = 10	**Dissolved Ni (µg/L)** n = 10	**Percent Difference**
75	56.04 ± 0.19	54.36 ± 1.00	97.02 ± 0.75
150	132.73 ± 1.92	132.73 ± 1.92	98.21 ± 0.82
250	228.34 ± 0.33	227.82 ± 0.35	99.77 ± 0.06
500	474.78 ± 0.51	464.84 ± 2.54	99.49 ± 0.16
1000	956.91 ± 2.51	944.255 ± 2.39	99.73 ± 0.07
2000	1891.65 ± 8.72	1882. 26 ± 8.97	99.51 ± 0.27

Table 5.6: Total and Dissolved measurements for water chemistry cations. Measurements taken via Flame-AAS.

Ion	**Nominal (mg/L)**	**Total (mg/L)**	**Dissolved (mg/L)**	**n**
Ca	2.53	2.84 ± 0.47	2.76 ± 0.23	12
Mg	0.77	0.67 ± 0.07	0.65 ± 0.15	25
Na	0.78	1.09 ± 0.21	0.87 ± 0.34	23

Table 5.7: Raw Data for 48h Acute Mixture tests without DOC added. Replicate mortality is calculated as the number of deaths out of 5 total daphnids per rep.

Nominal		Total		Replicate Mortality (out of 5)														
Ni (µg/L)	Cu (µg/L)	Ni (µg/L)	Cu (µg/L)	1	2	3	4	5	6	7	8	9	10	Total	Average Mortality (%)	SD	pH	n	SEM
0	0	0	0	0	0	0	0	0	0	0	0			0	0	0	6.54		0
0	2	0	1.008	0	0	0	0	1	0	3	2			6	15	1.15	6.55		0.41
0	4	0	3.339	2	1	4	0	3	0	5	2			17	42.5	1.81	6.56	8	0.64
0	8	0	6.823	5	5	5	5	5	5	5	5			40	100	0	6.51		0
0	16	0	14.19	5	5	5	5	5	5	5	5			40	100	0	6.52		0
0	32	0	31.6	5	5	5	5	5	5	5	5			40	100	0	6.58		0
0	0	0	0	0	0	0	0	0	0	0	0			0	0		6.51		
500	0	486	0	0	0	0	0	0	0	0	0			0	0	0	6.49		0
1000	0	1140	0	0	1	0	1	0	0	2	3			7	14	1.13	6.46		0.4
2000	0	2560	0	2	1	3	1	3	2	1	2			15	30	0.83	6.44	8	0.3
4000	0	3948	0	3	3	3	3	2	2	1	5			22	44	1.16	6.49		0.41
8000	0	9655	0	5	5	5	4	5	5	5	5			39	78	0.35	6.51		0.13
16000	0	14785	0	5	5	5	5	5	5	5	5			40	80	0	6.47		0
0	0	0	0	0	0	0	0	0	0	0	0			0	0	0	6.47		0
100	0	74	0	0	0	0	0	0	0	0	0			0	0	0	6.43		0
250	0	240	0	0	0	0	0	0	0	0	0			0	0	0	6.47		0
500	0	394	0	2	0	0	0	0	0	1	1			4	10	15.12	6.48		5.3
1000	0	773	0	3	2	0	0	3	3	2	2			15	37.5	24.93	6.47	8	8.8
1500	0	1051	0	0	5	3	4	2	1	3	3			21	52.5	31.96	6.47		11.3
2000	0	1436	0	4	3	3	4	4	1	3	3			25	62.5	19.82	6.49		7.0
3000	0	2819	0	5	5	5	4	5	5	5	5			39	97.5	7.071	6.45		2.5
4000	0	3689	0	5	5	5	5	5	5	5	5			40	100	0	6.47		0
0	0	0	0.265	1	0	0	0	0	0					1	3.3	0.41	6.52	6	0.17

0	1	0	1.151	0	1	1	0	0	0				2	6.7	0.52	6.55		0.21
0	2	0	1.669	0	0	0	1	1	1				3	10	0.55	6.54		0.22
0	3	0	1.737	0	0	1	1	1	1				4	13.3	0.52	6.57		0.21
0	4	0	1.788	3	1	2	1	0	2				9	30	1.05	6.57		0.43
0	6	0	3.406	5	5	5	5	3	4				27	90	0.84	6.52		0.34
0	12	0	7.317	5	5	5	5	5	5				30	100	0	6.51		0
75	0	55.86	0	0	0	0	0	0	0	0	0	0	0	0	0	6.56		0
75	1	55.86	0.356	0	0	0	0	0	0	0	1	0	1	2	4	0.42	6.54	0.13
75	3	55.86	0.9825 01762	0	1	0	2	0	0	0	0	0	1	4	8	0.69	6.56	10	0.22
75	6	55.86	2.8813 0262	5	5	5	5	5	5	5	5	5	5	50	100	0	6.51		0
75	12	55.86	7.0334 28203	5	5	5	5	5	5	5	5	5	5	50	100	0	6.51		0
150	0	131.2	0	0	0	0	0	0	0	0	0	0	0	0	0	6.57		0
150	1	131.2	0.474	0	1	0	0	0	0	0	0	1	0	2	4	0.42	6.58	0.13
150	3	131.2	2.26	1	1	1	1	0	2	1	1	1	2	11	22	0.57	6.53	10	0.18
150	6	131.2	4.99	5	5	5	5	5	5	5	5	5	5	50	100	0	6.57		0
150	12	131.2	9.88	5	5	5	5	5	5	5	5	5	5	50	100	0	6.53		0
250	0	228.87	0	2	1	1	1	1	0	0	0			6	15	0.71	6.57		0.2
250	1	228.87	1.13	2	0	0	0	1	0	1	0			4	10	0.76	6.57		0.24
250	3	228.87	3.48	2	0	1	2	0	0	0	1			6	15	0.89	6.55	10	0.28
250	6	228.87	5.74	5	3	5	4	5	5	4	3			34	85	0.89	6.55		0.28
250	12	228.87	11.77	3	5	5	5	5	5	5	5			38	95	0.71	6.53		0.22
500	0	474.37	0	1	0	1	0	0	0	0	0			2	5	0.46	6.54		0.15
500	1	474.37	1.21	1	1	0	2	2	0	3	4			13	32.5	1.41	6.57	10	0.44

500	3	474.37	2.66	0	3	1	3	1	1	3	0		12	30	1.31	6.52		0.42
500	6	474.37	5.19	5	5	5	5	5	5	5	5		40	100	0	6.49		0
500	12	474.37	10.83	5	5	5	5	5	5	5	5		40	100	0	6.51		0
1000	0	951	0	2	2	3	2	1	0	1	2	0	0	13	26	0.92	6.46		0.33
1000	1	951	2.04	1	2	1	1	1	1	1	2	2	1	13	26	0.48	6.47		0.17
1000	3	951	3.38	2	4	4	3	4	4	3	5	2	4	35	70	0.97	6.46	10	0.34
1000	6	951	5.60	5	5	5	5	5	5	5	5	5	5	50	100	0	6.48		0
1000	12	951	11.66	5	5	5	4	5	5	5	5	5	5	49	98	0.32	6.48		0.11
2000	0	1849	0.19	4	3	4	4	4	3	3	3	2	2	32	64	0.79	6.51		0.28
2000	1	1849	1.01	5	5	5	5	4	4	2	2	4	4	48	96	0.42	6.52		0.15
2000	3	1849	2.84	4	4	5	5	5	5	5	5	5	5	50	100	0	6.51	10	0
2000	6	1849	7.56	5	5	5	5	5	5	5	5	5	5	50	100	0	6.51		0
2000	12	1849	11.68	5	5	5	5	5	5	5	5	5	5	50	100	0	6.53		0
1000	0	957.5	0	1	2	1								4	26. 7	0.58	6.47		0.2
1000	1	957.5	1.18	2	3	2								7	46. 7	0.58	6.44		0.2
1000	2	957.5	0.424	4	4	3								11	73.3	0.58	6.45		0.2
1000	3	957.5	0.857	5	5	5								15	100	0	6.47	3	0
1000	4	957.5	1.09	5	5	5								15	100	0	6.46		0
1000	6	957.5	1.78	5	5	5								15	100	0	6.48		0
2000	0	1906	0.042	3	4	4								11	73.3	0.58	6.44	3	0.2

2000	1	1906	0.059	5	5	3					13	86.67	1.15	6.46	0.41
2000	2	1906	0.197	4	5	5					14	93.3	0.58	6.48	0.2
2000	3	1906	1.19	5	5	5					15	100	0	6.51	0
2000	4	1906	0.893	5	5	5					15	100	0	6.49	0
2000	6	1906	1.47	5	5	5					15	100	0	6.47	0

Table 5.8: Raw Data for 48h Acute Mixture tests with DOC added. Replicate mortality is calculated as the number of deaths out of 5 total daphnids per rep. DOC from 3 sources was tested: Luther Marsh (LM), Clearwater Lake (CWL), and Daisy Lake (DL).

DOC Source	Nominal Cu (µg/L)	Nominal Ni (µg/L)	DOC	Total Cu (µg/L)	Total Ni (µg/L)	DOC	Replicate Mortality 1	2	3	4	5	6	7	8	To-tal	Avg Mortality (%)	SD	pH	n	SEM
LM + 1 mg Ni	0	1000	4	0	951	5.0	0	0	0	0	0	0	1	0	1	2.5	0.354	6.53	8	0.125
	1	1000	4	1.92	951	5.0	0	1	0	0	1	1	0	0	3	7.5	0.516	6.54		0.183
	3	1000	4	3.52	951	5.0	3	4	2	1	5	2	2	3	22	55	1.282	6.58		0.4533
	6	1000	4	4.49	951	5.0	3	5	2	4	4	5	3	3	29	72.5	1.06	6.61		0.474
	12	1000	4	10.60	951	5.0	4	4	4	4	2	4	5	3	30	75	0.886	6.56		0.396
LM + 2 mg Ni	0	2000	4	0	1923	5.0	5	3	5	3	4	3	5	4	32	80	0.926	6.53	8	0.327
	1	2000	4	1.88	1923	5.0	5	3	5	5	4	3	5	4	34	85	0.886	6.58		0.313
	3	2000	4	4.40	1923	5.0	5	4	5	4	5	3	4	4	34	85	0.707	6.63		0.25
	6	2000	4	5.23	1923	5.0	5	5	5	5	5	5	5	5	40	100	0	6.61		0
	12	2000	4	10.06	1923	5.0	5	5	5	5	5	5	5	5	40	100	0	6.67		0
CWL	0	1000	4	1.67	973	4.6	0	0	0	0					0	0	0	6.52	4	0

+ 1 mg Ni	1	1000	4	1.99	973	4.6	0	1	1	0				2	10	0.577	6.55		0.204
	3	1000	4	4	973	4.6	2	1	1	1				5	25	0.5	6.59		0.177
	6	1000	4	6.3	973	4.6	3	1	1	2				7	35	0.957	6.54		0.334
DL + 1 mg Ni	0	1000	4	0	968	4.9	0	1	0	0	0	0	0	1	2.5	0.354	6.51		0.125
	1	1000	4	1.56	968	4.9	2	0	2	1	2	2	1	12	30	0.756	6.46		0.263
	3	1000	4	3.46	968	4.9	4	2	1	1	2	3	2	15	37.5	1.25	6.54	8	0.441
	6	1000	4	5.37	968	4.9	1	2	2	2	2	2	3	16	40	0.535	6.48		0.239
	12	1000	4	10.98	968	4.9	5	4	1	5	2	3	3	26	65	1.389	6.48		0.621
DL + 2 mg Ni	0	2000	4	0		4.9	5	5	5	5	5	5	5	40	100	0	6.53		0
	1	2000	4	1.56		4.9	4	5	5	5	5	4	5	38	95	0.463	6.57		0.164
	3	2000	4	3.46		4.9	5	5	5	5	5	5	5	40	100	0	6.59	8	0
	6	2000	4	5.71		4.9	5	5	4	5	4	5	5	38	95	0.463	6.55		0.164
	12	2000	4	10.97		4.9	5	5	5	5	5	5	5	40	100	0	6.55		0
CWL + 2 mg Ni	0	2000	0	0	1942	1.2	0	0	0	0	0	0	0	0	0	0	6.50		0
	0	2000	4	0	1942	5.8	0	0	0	0	0	0	0	0	0	0	6.51		0
	6	2000	4	0	1942	5.6	5	5	5	5	5	5	5	40	100	0	6.48		0
	12	2000	4	11	1942	5.6	5	5	5	5	5	5	5	40	100	0	6.47		0
	25	2000	4	21	1942	5.6	5	5	5	5	5	5	5	40	100	0	6.44	8	0
	50	2000	4	52	1942	5.6	5	5	5	5	5	5	5	40	100	0	6.42		0
	100	2000	4	115	1942	5.6	5	5	5	5	5	5	5	40	100	0	6.44		0
	200	2000	4	222	1942	5.6	5	5	5	5	5	5	5	40	100	0	6.47		0
LM + Ni only	0	250	4	0	217	5.1	0	0	1	0	1	0	0	0	2	5	0.463	6.53		0.164
	0	500	4	0	453	5.1	0	0	0	1	0	0	4	5	10	25	2.05	6.52	8	0.7258
	0	1000	4	0	931	5.1	0	1	1	3	1	1	2	0	9	22.5	0.991	6.56		0.3500
	0	2000	4	0	1840	5.1	5	4	4	4	5	4	4	5	35	87.5	0.516	6.52		0.183
LM + Cu	0	0	4	0	0	5.0	0	0	0	0	0	0	0	0	0	0	0	6.51		0
	0	0	4	0	0	5.0	0	0	0	0	0	0	0	0	0	0	0	6.43	8	0
	1	0	4	0.51	0	5.0	0	0	0	0	0	0	0	0	0	0	0	6.47		0

only	3	0	4	2.65	0	5.0	0	0	0	0	0	0	0	0	0	0	0	6.46		0
	6	0	4	5.43	0	5.0	0	0	0	0	0	0	0	0	0	0	0	6.48		0
	12	0	4	11.75	0	5.0	0	0	0	0	0	1	0	0	1	2.5	0.354	6.47		0.125
CWL + Ni only	0	0	4	0	0	5.2	0	0	0	0	0				0	0	0	6.49	5	0
	0	250	4	0	214	5.2	0	0	0	0	0				0	0	0	6.53		0
	0	500	4	0	525	5.2	0	0	0	0	0				0	0	0	6.54		0
	0	1000	4	0	972	5.2	1	0	1	1	0				3	12	0.5	6.52		0.177
	0	2000	4	0	1960	5.2	3	4	4	4	5				20	80	0.5	6.53		0.177
CWL + Cu only	0	0	4	0	0	5.2	0	0	0	0	0	0	0	0	0	0	0	6.47	8	0
	1	0	4	1.03	0	5.2	0	0	0	0	0	0	0	0	0	0	0	6.51		0
	3	0	4	3.54	0	5.2	0	0	0	0	0	0	0	0	0	0	0	6.46		0
	6	0	4	5.76	0	5.2	0	0	0	0	0	0	0	0	0	0	0	6.49		0
	12	0	4	11.09	0	5.2	0	0	0	0	0	0	0	0	0	0	0	6.51		0