INFLUENCE OF DISSOLVED ORGANIC CARBON ON THE SPECIATION, BIOAVAILABILITY AND TOXICITY OF METALS TO AQUATIC BIOTA IN SOFT WATER LAKES

by

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ABSTRACT

INFLUENCE OF DISSOLVED ORGANIC CARBON ON THE SPECIATION, BIOAVAILABILITY AND TOXICITY OF METALS TO AQUATIC BIOTA IN SOFT WATER LAKES

Cu and Cd are extremely toxic to aquatic biota in low alkalinity water. In these calcium poor (< 5 $mg \cdot L^{-1}$), moderately acidic (pH < 7.0) waters, the role of organic complexation is important in determining the speciation and bioavailable forms of these metals. The current paradigm of metal-organism interaction, the Free Ion Activity Model, states that the biological response of an organism to metal exposure is a function of the free aqueous metal ion activity and not the total metal concentration. Hence, metal toxicity should be proportional to the free metal ion activity (a measure of the reactive metal ion concentration). The usefulness of this model for predicting metal bioavailability in natural lake waters with natural dissolved organic carbon (DOC) is unclear.

The effect of DOC on the acute toxicity of Cu and Cd to *Hyalella azteca* and *Pimephales promelas* was studied in water from St. Mary's Lake, a soft water Precambrian Shield lake. DOC was a significant modifier of Cu toxicity but not of Cd toxicity. Mean 96-h Cu LC50s

ranged from 6.5 to 53.8 μg^{-1} and mean 96-h Cd LC50s ranged from 2.7 to 23.9 μg^{-1}. *H. azteca* had similar sensitivity as *P. promelas* to Cu but was more than an order of magnitude more sensitive to Cd exposure than *P. promelas*.

The chronic toxicity of Cu and Cd to larval fathead minnows (*P. promelas*) was examined in water from two low alkalinity lakes with DOC concentrations of 2.3 to 6.7 $mg \cdot L^{-1}$ respectively. Both metals were toxic at low ppb ($\mu g \cdot L^{-1}$) levels. Metal speciation was determined by dialysis and with Cu and Cd ion specific electrodes. Differences in the observed toxic effects of Cu exposure between the two lakes when expressed as total Cu, were minimized when expressed as the dialysis fraction. When effects from the Cu exposure were expressed as a function of the free Cu ion concentration, the toxicity was roughly similar between the two lakes. The opposite pattern was observed for Cd toxicity. Differences in the observed toxic effects between the two lakes were minimized when expressed as total Cd. When expressed as the dialysis fraction, the effects of Cd exposure were more pronounced in Dickie Lake than in Halls Lake. Estimates of free Cd ion activity also suggest that the effects of Cd exposure were more pronounced in Dickie Lake than in Halls Lake.

These results indicate that while Cu toxicity appears to be a function of the free Cu ion concentration, Cd toxicity may not be a function of the free Cd ion concentration. More precise free Cd ion concentration determinations in natural waters are required in order to conclude that the free Cd ion concentration was not proportional to the observed toxicity.

However, it is clear that the observed Cd toxicity was not due to the free Cd ion concentration only. Measured ligand-bound Cd complexes are clearly bioavailable to larval fathead minnow, as indicated by similar toxicity observed in the fish as a function of total Cd concentration, even though the amount of ligand-bound Cd is different in the two lakes.

These results suggest that the FIAM may not be applicable to all divalent metals. With natural DOC, the results validated the model for Cu (a metal that forms strong covalent bonds with organic ligands) but were ambiguous for Cd (a metal that forms weak electrostatic bonds with organic ligands). Calculated site-specific conditional stability constants (Log K') for the lake water DOC were compared to published fathead minnow gill Log K's for both metals. Both fish gills and lake water DOC had similar mean stability constants for Cu (between 7.4 - 8). However, fish gills had stability constants for Cd 2-3 orders of magnitude higher than lake water DOC (8.6 verses between 5.3 - 6). The enhanced toxicity of Cd in the presence of DOC appears to be due to a kinetically controlled dissociation reaction between Cd bound to organic ligands in the vicinity of the fish gill and not to direct toxicity of Cd-DOC complexes.

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1.0 Cu and Cd toxicity to biota in soft water lakes.

1.1 Trace metals in soft water lakes

Trace metal toxicity in soft water lakes is an important regional concern in North America. Approximately 70% of lakes in Ontario are considered soft water (conductivity < 50 $\mu S \cdot cm^{-1}$) (Neary et al. 1990)[1]. In addition to low conductivity, soft water lakes are characterized by low alkalinity (< 10 $mg \cdot L^{-1}$ as $CaCO_3$), calcium (< 5 $mg \cdot L^{-1}$), hardness (< 20 $mg \cdot L^{-1}$ as $CaCO_3$) and moderately acidic pH (5.5 to 7.0) (Neary et al. 1990). If one considers other Precambrian Shield lakes and mountain headwater lakes, it is probable that the majority of lakes in Canada and the United States are soft water.

Lakes in the Muskoka-Haliburton region of south-central Ontario are underlain by silicate bedrock of low solubility and have little buffering capacity (Dillon et al. 1978). Due to the low buffering capacity, these lakes are very sensitive to the effects of acidification and as a result are subject to increased mobilization of metals from the watershed (Malmer 1976) and sediments (Schindler et al. 1980), increased metal solubility in the water column (Campbell and Stokes 1985) and subsequent increased metal bioavailability and toxicity to aquatic biota (Baker 1981).

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[1] Soft water is an ambiguous term to describe low conductivity water since it also describes low alkalinity and low hardness water as well. Essentially, it is a lack of hardness or divalent cations in solution. Soft water is used many times in this thesis and is defined as water with a conductivity < 50 $\mu S \cdot cm^{-1}$. Since this information is not always available from the literature, for comparison purposes, soft water is also defined as water with a hardness of < 20 $mg \cdot L^{-1}$ as $CaCO_3$. Water with a hardness of between 20 and 60 $mg \cdot L^{-1}$ as $CaCO_3$ is often described as soft water as well, but here it will be referred to as moderately soft water.

Metals can also increase in lakes and surrounding watersheds as a result of atmospheric deposition. This long range transport of metals is an important source of anthropogenic metals in south central Ontario (Evans et al. 1983; Jeffries and Snyder 1980). Estimates of atmospheric deposition of Cu and Cd in lakes Superior, Erie and Ontario range from 330 to 1470 $\mu g \cdot m^{-2} \cdot yr^{-1}$ for Cu and from 148 to 170 $\mu g \cdot m^{-2} \cdot yr^{-1}$ for Cd (Nriagu et al. 1996). The fate of these metals once dissolved in the water column is complex and includes adsorption and precipitation reactions with various inorganic and organic ligands and uptake by biota; in general, Cu tends to be exported from lakes while Cd is retained (Nriagu et al. 1996). LaZerte et al. (1991) observed similar Cu and Cd dynamics in a small Precambrian Shield lake and suggested that Cu complexation with DOC was primarily responsible for the mobility of Cu into and out of the lake while some other mechanism, possibly acid dissolution, was primarily responsible for the Cd dynamics in the lake.

Metal concentrations in natural surface waters have historically been overestimated due to sample contamination (Nriagu et al. 1993; Windom et al. 1991). Since reliable data on trace metal concentrations in lakes is generally lacking (Nriagu et al. 1996), it is difficult to assess the risk of low level Cu and Cd exposure to biota in these environments. From the limited information generated using clean techniques, the concentration of Cu in soft water lakes is quite low, usually $< 1\ \mu g \cdot L^{-1}$ in inflow streams (LaZerte 1991) and $< 2\ \mu g \cdot L^{-1}$ in surficial lake water (Welsh et al. 1996). Estimates of Cd concentrations in soft water lakes are also quite low, ranging from 0.02 to 0.3 $\mu g \cdot L^{-1}$ in inflow streams (LaZerte 1991). Recent

estimates for Cu and Cd in the Great Lakes range from 0.704 to 1.060 $\mu g \cdot L^{-1}$ and from 0.0028 to 0.0045 $\mu g \cdot L^{-1}$, respectively (Nriagu et al. 1996).

1.2 Toxicity of H^+ and metals to biota.

Lake acidification has been associated with the loss of sports fish at pH 5.0 to 5.5 (Kelso et al. 1987) and forage fish, such as cyprinids, at pH 5.5 to 6.0 (Matusek et al. 1990). This loss of sensitive fish populations may be due to recruitment failure, primarily through mortality of the egg and fry stages (Gunn and Keller 1984; Mills et al. 1987). Mortality of eggs and fry for fish species that spawn in the spring are likely due to spring pulses of low pH and elevated Al (Gunn and Keller 1984). However, for summer spawning fish, fry and egg mortality may be a result of interaction between metals in addition to Al, especially Cu and Zn (Hickie et al. 1993; Hutchinson and Sprague 1986). Recent evidence suggests that for acid sensitive fish species (Hickie et al 1993) and zooplankton (Yan et al. 1996a), Cu may predominantly contribute to the trace metal impacts observed in field populations. The cyprinid fathead minnow (*Pimephales promelas*), is sensitive to low Cu concentrations at pH levels < 7.0 in soft water lakes. Acute median lethal concentrations (96-h Cu LC50s) for total Cu to *P. promelas* ranged from 2 to 182 $\mu g \cdot L^{-1}$ in soft water (Welsh et al. 1993).

Metal toxicity studies in soft and moderately soft water lakes have typically focused on the influence of acidification on the speciation and toxicity of key trace metals; notably Al

(Gunn and Belzile 1994; Hutchinson et al. 1989; McCormick et al. 1989) and Cu (Campbell and Stokes 1985). Most of these metal toxicity studies have been done with short term acute exposures and often in water with hardness levels 3 to 4 times the hardness of typical soft water Precambrian Shield lakes (hardness values of 40-50 $mg \cdot L^{-1}$ as $CaCO_3$ verses < 20 $mg \cdot L^{-1}$ as $CaCO_3$). In addition, few metal toxicity studies have examined the influence of natural dissolved organic carbon (DOC) on metal toxicity (Borgmann and Ralph 1984; Giesy et al. 1983; Nelson et al. 1986), especially in soft water (Hutchinson and Sprague 1987; Welsh et al. 1993, 1996).

1.3 Modifying factors of metal exposure.

Several factors are known to modify the toxicity of metals to biota (Wang 1987). These include biotic factors (life stage, species, nutrition) and abiotic factors (alkalinity, hardness, and inorganic and organic complexation). Only abiotic modifying factors of metal toxicity will be discussed here, since metal speciation is predominantly determined by various abiotic chemical reactions.

1.3.1 Alkalinity, hardness and pH.

It is well established that hardness and alkalinity reduce metal toxicity in aquatic organisms during acute metal exposure (Calamari et al. 1980; Chakoumakos et al. 1979; Howarth and

Sprague 1978). Hardness is a measure of all divalent cations (mostly Ca and Mg) but can also be an indirect measure of carbonate ions (Wetzel 1975). Calcium, believed to be the key component of hardness with respect to protective effects, both competes with metals for active sites on the gill surface, and is important in regulating the permeability of the gill membrane to metal ions (McDonald et al. 1989; Varanasi and Gmur 1978). Alkalinity, the ability of a water sample to neutralize acid (often referred to as buffering capacity), is a measure of the carbonate-bicarbonate concentration in the water and is an indicator of a water's ability to reduce metal toxicity by inorganic complexation of the metal ion. Since soft water lakes are moderately acidic, the inorganic complexation of metals by hydroxide and carbonate ions is minimized in these lakes (Campbell and Stokes 1985).

The low calcium levels and alkalinity in soft water lakes, and hence their low inorganic metal complexing capacity, make organisms appear to be more sensitive to the effects of metal exposure; a larger proportion of the total metal ions are available to react with the active binding sites on the organism. The organisms may in fact be more physiologically metal sensitive. The low Ca levels in the lake waters may have resulted in the development of hyper-efficient Ca uptake mechanisms, since Ca is essential for structural, biochemical and physiological processes (Hunn 1985). This enhanced uptake mechanism could be utilized as an entry route for other divalent ions.

In addition to competition by other divalent cations (hardness) or inorganic complexation (alkalinity), the toxicity of metals to biota is modified by pH and the concentration and

binding strengths of organic ligands (Wang 1987). These factors tend to decrease metal toxicity by either reducing the concentration of the free metal ion in solution, or by competing for surface active binding sites on the gill tissue (Pagenkopf 1983; Campbell and Stokes 1985).

1.3.2 Organic Complexation

Numerous studies, mostly with Cu, Cd and Zn, have demonstrated that organic compounds reduce the toxicity of metals to aquatic biota and that toxicity appears to be a function of the free metal ion concentration (Allen et al. 1980). The organic ligands studied range from synthetic organic compounds (Borgmann and Ralph 1984; Poldoski 1979) to humic acids (Poldoski 1979; Stackhouse and Benson 1988; Winner 1986). Usually, the synthetic compounds are chelating agents (e.g., EDTA, ethylene diamine tetraacetic acid; Tris, tris-hydroxymethyl-aminomethane; or NTA, nitrilo triacetic acid) and often the humic acids are from a purified soil extract (Aldrich humic acid). While these experiments are invaluable in determining the mechanisms and effects of organic complexation on metal bioavailability and toxicity, they are not necessarily good models of naturally occurring DOC and hence may not be appropriate to use to make predictions about metal speciation in natural water.

It is difficult to extrapolate results from these studies to the environment, since naturally occurring DOC is composed of a population of organic compounds, each of which have different metal binding capacities. EDTA may be an especially poor ligand to mimic DOC

since there is no evidence of a strong chelator in naturally occurring DOC and using EDTA may result in limitations in other trace metals (such as Zn; Anderson et al. 1978). The use of soil-derived DOC is not appropriate in aquatic systems because aquatic sources of DOC are typically dominated by low molecular weight (MW) fulvic acids (up to 90%) while soil derived DOC are dominated by high MW compounds (Marley et al. 1992). In fact, Garvey et al. (1991) demonstrated significant differences between aquatic fulvic acid, aquatic humic acid and soil humic acid on the Cu toxicity to the green algae, *Chlamydomonas reinhardtii*. In general, examining the impact of isolated organic compounds on metal toxicity removes the variability associated with naturally occurring DOC and may be flawed since purified organic compounds are poor models of naturally occurring DOC.

Both organic complexation of trace metal ions by DOC and competition with H^+ and Ca with trace metal ions are important factors regulating metal toxicity. Organic complexation modifies Cu or Cd toxicity by binding to and reducing the concentration of the free metal ion (Morel 1983). Cu forms strong, inner sphere covalent bonds with S, N, and O containing functional groups (Stumm and Morgan 1981) and will bind to these functional groups in organic ligands forming Cu-bound organic ligands. These Cu-bound organic ligands generally are not reactive with biological membranes; they are not bioavailable (Morel 1983; Campbell 1995). Cd is similar to Cu except it forms weak electrostatic bonds with organic ligands (Stumm and Morgan 1981). Metal-bound organic ligands can, however, act as a reservoir and provide free metal ion upon dissociation of the complex. H^+ and Ca modify metal toxicity by competing with the free metal ion for surface active binding sites

on organic ligands and biological membranes (Campbell and Stokes 1985). At high H^+ concentrations (pH < 5) excessive protonation of the gill membrane will result in a net positive charge that will repel metal ions (Campbell and Stokes 1985).

While both organic complexation and cation competition can influence Cu and Cd speciation, it is important to recognize that the various binding sites on the gill membrane and on organic ligands can react with either H^+, Ca, Cu, Cd or other ions in solution, and that these ions will compete with one another for these sites. This competition between various ions is dynamic and will depend on the concentration of the ion in solution as well as on the binding strength of the ligand for the various ions (Playle et al. 1992 and 1993).

1.3.3 Bioavailability and Toxicity of Metal Ions

The toxicity of metals to aquatic biota depends on the bioavailable fraction of the metals in the environment. Although the free metal ion activity (a measure of the reactive free metal ion concentration) is believed to be largely responsible for the toxic effects of total metal to aquatic biota (Morel 1983), some metal-hydroxides (Cu: Chakoumakos et al. 1979; Howarth and Sprague 1978) and metal-organic ligand complexes (Cu: Borgmann and Charlton 1984; Winner 1985; Cd: Giesy et al. 1977; Poldoski 1979; Winner 1986) may contribute to toxicity in certain circumstances. These metal complexes that are toxic are usually hydrophobic (Campbell 1995), resulting in rapid diffusion through biological membranes (Florence 1986). Since "it is well established that intestinal absorption, blood plasma

reactions, passage through the blood brain barrier, and renal and biliary excretion, all involve low molecular weight transition metal ion complexes" (Florence and Batley 1988), it is surprising that more metal ligand complexes are not toxic.

1.4 The Free Ion Activity Model

The free ion activity model (FIAM) of metal-organism interaction predicts the effect of metal exposure on organisms based on the free ion activity (Morel 1983). The model is based largely on experiments with synthetic organic ligands and has not been adequately tested with naturally occurring DOC (Campbell 1995). The FIAM makes the following assumptions with respect to the reaction between metal ions and an organism (Campbell 1995): (1) the primary site for metal binding is the plasma membrane; (2) metal ions bind to the plasma membrane by a surface complexation reaction forming [M-X-Cell] complexes (where M is the free-metal ion and X-Cell represent the physiologically active sites or ligands on the plasma membrane); (3) the transport of metal ion in solution to the active site on the cell occurs rapidly such that the metal species in bulk solution are in "pseudo-equilibrium" with the biological surface; (4) the concentration of free cell ligand sites [X-Cell] on the plasma membrane remains virtually constant; (5) the biological response is strictly dependent on the [M-X-Cell] complex; and (6) variations in [M-X-Cell] are proportional to the free metal ion activity in solution. The assumption that the free metal ion activity is proportional to the [M-X-Cell] complex is made due to the difficulty of

measuring the [M-X-Cell] complex directly and is invalid if ternary surface complexes are formed (Campbell 1995). These ternary surface compounds, [L-M-X-Cell] (where L is a water soluble ligand), occur if ligand bound metal ion also bind to the ligands on the cell surface. The FIAM does not state that the free metal ion activity is the only toxic or bioactive metal species; instead, it states that the biological response is proportional to the free metal ion activity.

1.5 Natural DOC

The majority of dissolved organic carbon in rivers and lakes is composed of humic and fulvic acids, tannins and lignin (Crompton 1985). While fulvic acids tend to have MWs < 10,000 and humic acids have MWs between 10,000 and 300,000, these compounds are defined based on their acid/base solubilities: humic acids are soluble in basic solutions but insoluble in acidic solutions while fulvic acids are soluble in both acidic and basic solutions (Crompton 1985). Tannins and lignins are also high MW compounds and are highly resistant to chemical or biological degradation. In soft water lakes in the Muskoka-Haliburton region, the DOC is dominated by low MW (1200 - 2200) fulvic acids (Evans 1989). In general, the majority of dissolved metals in freshwater are bound to humic acid colloids or iron oxide particles coated with humic acid (Florence 1982). In soft water lakes, both the fraction of metal that is bound, and the bioavailability of this bound fraction, are not clear.

1.6 Objective of Study

DOC plays a central role as a modifying factor of acute Cu toxicity in poorly buffered soft water lakes (Welsh et al. 1993, 1996). In these lakes, Cu toxicity appears to be a function of the free metal ion concentration where Cu-bound organic complexes are not toxic. Because of the paucity of studies examining metal toxicity with natural DOC, the role of DOC on the chronic toxicity of Cu in soft water lakes is not clear. The protective effect of organic complexation may be time dependent, where Cu exposure and toxicity is merely delayed instead of reduced, or Cu organic ligands may themselves be toxic. To assess the role of organic complexation on metal toxicity, both Cu and Cd toxicity will be examined. These metals are typical of two types of binding characteristics between metals and organic ligands: formation of a strong covalent bond and formation of a weak electrostatic bond.

In this study, the influence of dissolved organic carbon on metal bioavailability and toxicity was determined in soft water. The effect of DOC on the acute toxicity of Cu and Cd to the amphipod *Hyalella azteca* and the cyprinid *Pimephales promelas* was examined in charcoal filtered (low DOC) and unfiltered (high DOC) St. Mary's Lake water. Both of these organisms are common in soft water lakes, but occupy different trophic levels. Based on the acute toxicity observed, the chronic toxicity of Cu and Cd was examined with *P. promelas* in water collected from two soft water, moderately acidic lakes at two DOC concentrations (approximately 2 and 6 $mg \cdot L^{-1}$). Metal speciation in the exposure water was quantified by dialysis and ion specific electrodes to provide estimates of the bioavailable metal species

and to test the FIAM. By comparing the effect of DOC on Cu and Cd bioavailability and toxicity, information generated will provide insight into the effect of these metals in soft water and will contribute to the development of site specific water quality objectives for metals in poorly buffered systems.

2.0 Influence of dissolved organic carbon on the acute toxicity of Cu and Cd to the amphipod *Hyalella azteca* and the cyprinid *Pimephales promelas* in soft water.

2.1 Abstract

The effect of dissolved organic carbon (DOC) on the acute toxicity of Cu and Cd to *Hyalella azteca* and *Pimephales promelas* was studied in water from St. Mary's Lake, a soft water Precambrian Shield lake. Acute 96-h Cu and Cd exposures were performed in charcoal filtered lake water (DOC = 0.3 $mg \cdot L^{-1}$) or natural lake water (DOC = 3.2 $mg \cdot L^{-1}$) at pH 7. DOC was a significant modifier of Cu toxicity but not of Cd toxicity. Over a ten-fold change in DOC concentration, the mean 96-h Cu LC50s ranged from 6.5 to 53.8 $\mu g \cdot L^{-1}$ for *P. promelas* and 9.1 to 24.1 $\mu g \cdot L^{-1}$ for *H. azteca*. However, the mean 96-h Cd LC50s only ranged from 23.3 to 23.9 $\mu g \cdot L^{-1}$ for *P. promelas* and 2.7 to 4.0 $\mu g \cdot L^{-1}$ for *H. azteca*. *H. azteca* had similar sensitivity to Cu exposure as *P. promelas* at 3.2 $mg \cdot L^{-1}$ and 0.3 $mg \cdot L^{-1}$ DOC, but was more sensitive to Cd exposure at both DOC levels .

2.2 Introduction

Soft water lakes in south central Ontario are often moderately acidic (Neary et al. 1990; Spry and Weiner 1991) and since inorganic complexation is minimized in these waters

(Campbell and Stokes 1985), the bioavailability of metals is dependent upon the pH, hardness (or calcium concentration) and organic complexation of metal ions (Welsh et al. 1996). These factors tend to decrease metal toxicity by either reducing the concentration of free metal ions in solution, or by competing for surface active binding sites on biological membranes (Campbell and Stokes 1985; Pagenkopf 1983).

Organic complexation of trace metals is important in determining trace metal bioavailability and toxicity because complexation acts to reduce the free metal ion activity by binding to metal ions in solution (Stumm and Morgan 1981). The free metal ion activity (a measure of the reactive metal ion concentration) is proportional to the bioavailable metal fraction (Campbell 1995; Morel 1983). A common assumption is that only the free ion activity or concentration is bioavailable and that metal-organic complexes are not. Several researchers have demonstrated that this assumption is in error. These researchers have observed toxicity to fish, invertebrates and algae with some organic-Cd complexes (Giesy et al. 1977; Poldoski 1979; Winner 1986) and organic-Cu complexes (Borgmann and Charlton 1984; Marr et al. 1995; Winner 1985).

Few studies have examined the role of organic complexation of metals on metal toxicity in soft or moderately soft water. In general, the trend of reduced toxicity at higher DOC levels has been observed with Cd, to the Daphnidae *Simocephalus serrulatus* (Giesy et al. 1983), with Al, Cu, Zn mixtures to larval American flagfish, *Jordanella americanus* (Hutchinson and Sprague 1987) and with Cu to larval fathead minnow, *Pimephales promelas* (Erickson

et al. 1996, Welsh et al. 1993). In these soft water systems, DOC as well as H^+ and calcium concentrations, are important variables in predicting Cu lethality to larval fathead minnow (Welsh et al. 1996). However, the role of DOC in modifying metal toxicity needs to be further evaluated.

Here, the relationship between naturally occurring DOC and acute Cu and Cd toxicity in soft water lakes is examined in two aquatic organisms *(P. promelas* and *Hyalella azteca*) to generate more site specific data on metal sensitivity to soft water biota and to provide information on setting exposure conditions for chronic metal exposures in a future study (see chapter 4). The cyprinid *P. promelas* and the amphipod *H. azteca* are common soft water organisms that are sensitive to pH (Grapentine and Rosenberg 1992; Welsh et al. 1993) and metals: *H. azteca* is sensitive to Cd (Borgmann et al. 1991); *P. promelas* is sensitive to Cu (Welsh et al. 1993). These two organisms are commonly used in toxicity tests but are not restricted to soft water habitats only. The metals Cu and Cd are studied because both are highly toxic to aquatic organisms but differ in binding affinity to organic ligands; Cu forms relatively strong inner sphere complexes to organic ligands while Cd forms relatively weak outer sphere complexes (LaZerte 1991).

2.3 Materials and Methods

2.3.1 Test Organisms

Wild fathead minnows were collected in 1990 from a small soft water lake near Dorset, Ontario, Canada, and reared in the laboratory. A breeding stock was established by rearing the offspring of these wild fish to maturity and breeding them. Acute toxicity tests were done with offspring from both wild stock and first generation fish. *H. azteca* were collected from Harp Lake, Ontario, Canada in 1991 and were reared in the laboratory through many successive generations.

Fathead minnows were cultured according to Denny (1987). Adult fish were held in St. Mary's Lake water at 22 °C in a 16 h light, 8 h dark, photoperiod cycle. Fish were cultured in St Mary's Lake water after it was passed through a sand filter, a 20 µm filter and a UV sterilizer. The lights were connected in 2 banks and by using automatic timers, were set to turn on and off 15 minutes apart to simulate dawn and dusk conditions. Thirty-four breeding pairs were held in 22-L aquaria, 2 breeding pairs per tank. Adult fish were fed thawed San Francisco Bay® frozen brine shrimp (*Artemia sp.*) (Crude protein min. 5.02%; crude fat min. 0.24%; crude fiber max. 0.29%) 2 to 3 times daily.

Deposition of egg masses on spawning substrate were checked daily, usually before noon. Spawning substrates were either 10-cm segments of clay pipe, clay flower pots or polyethylene pipe. If eggs had been laid during the previous 24 h, the spawning substrate was removed and replaced with a new one. Eggs still attached to the spawning substrate were placed in a 2-L Nalgene beaker and gently aerated. The water was replaced daily and the eggs were checked for signs of fungus daily. Larval fish hatched from the eggs after 5 to 7 days.

H. azteca were cultured in St. Mary's Lake water according to Borgmann et al. (1989). Approximately 20 adult animals were reared in each of 20, 1-L glass beakers with a 4 x 4 cm piece of cotton gauze as substrate. Amphipods were fed crushed tetramin fish food three times per week. Once a week, animals were filtered through a 250-µm filter; adults were returned to fresh St. Mary's Lake water while young, were counted and removed. When adult numbers fell below 15, additional adults were added from a stock breeding culture.

2.3.2 Acute Toxicity Tests

Static 96-h toxicity tests were conducted with either < 24 h old fish or < 1 wk old amphipods. The animals were not fed. Sand filtered, St. Mary's Lake water was obtained in 20 L batches for use in toxicity tests. For some tests, the DOC was removed by continuously filtering the water through a conditioned 0.5 µm charcoal filter (Ametek Model

CBC-10 powdered activated carbon briquette taste/odour sediment cartridge) for 24 to 48 h prior to the initiation of the test.

The charcoal filter was conditioned by pumping reverse osmosis (RO) water once through the filter at 1-2 $L \cdot min^{-1}$ until the conductivity of filtered water was similar to original water. The filtered RO water was chemically characterized after 5, 15, 30, 60, 180 and 300 minutes of filtering. This water initially had high levels of Al, Ca, alkalinity, conductivity and pH. Over time, however, these levels declined (Figure 2.1). Concentrations of Na, Mg, K, Cl, Cu, Cd, and DOC did not change and were at or below detection levels in the original and filtered reverse osmosis water (detection levels provided in Table 2.1). Sulphate and total phosphorus increased in the RO water after 5 minutes of filtering to 0.15 $mg \cdot L^{-1}$ and 1.8 $\mu g \cdot L^{-1}$, respectively, but quickly returned to below detection levels. Levels of Pb and Zn decreased after filtering from 0.144 to 0.003 $\mu g\ Pb \cdot L^{-1}$ and from 0.737 to 0.107 μg $Zn \cdot L^{-1}$. The decrease in sulphate, total phosphorus, Pb and Zn levels was due to these compounds being flushed from the charcoal filter over time.

Organisms were exposed to a geometric series of five contiguous metal concentrations ranging between 0.56, 1.0, 1.8, 3.2, 5.6, 10, 18, 32, 56, 100 $\mu g \cdot L^{-1}$ and one control for 96-h in 200 mL of St. Mary's Lake water. Cu was added from $CuSO_4 \cdot 5H_20$ stock solutions. Mortality was typically recorded at 0, 1, 3, 18, 24, 48, 72, 96 h after the start of the test. Temperature and pH were measured daily in each exposure beaker with an alcohol based thermometer and a pH electrode (Radiometer GK2401C). All tests were conducted at room

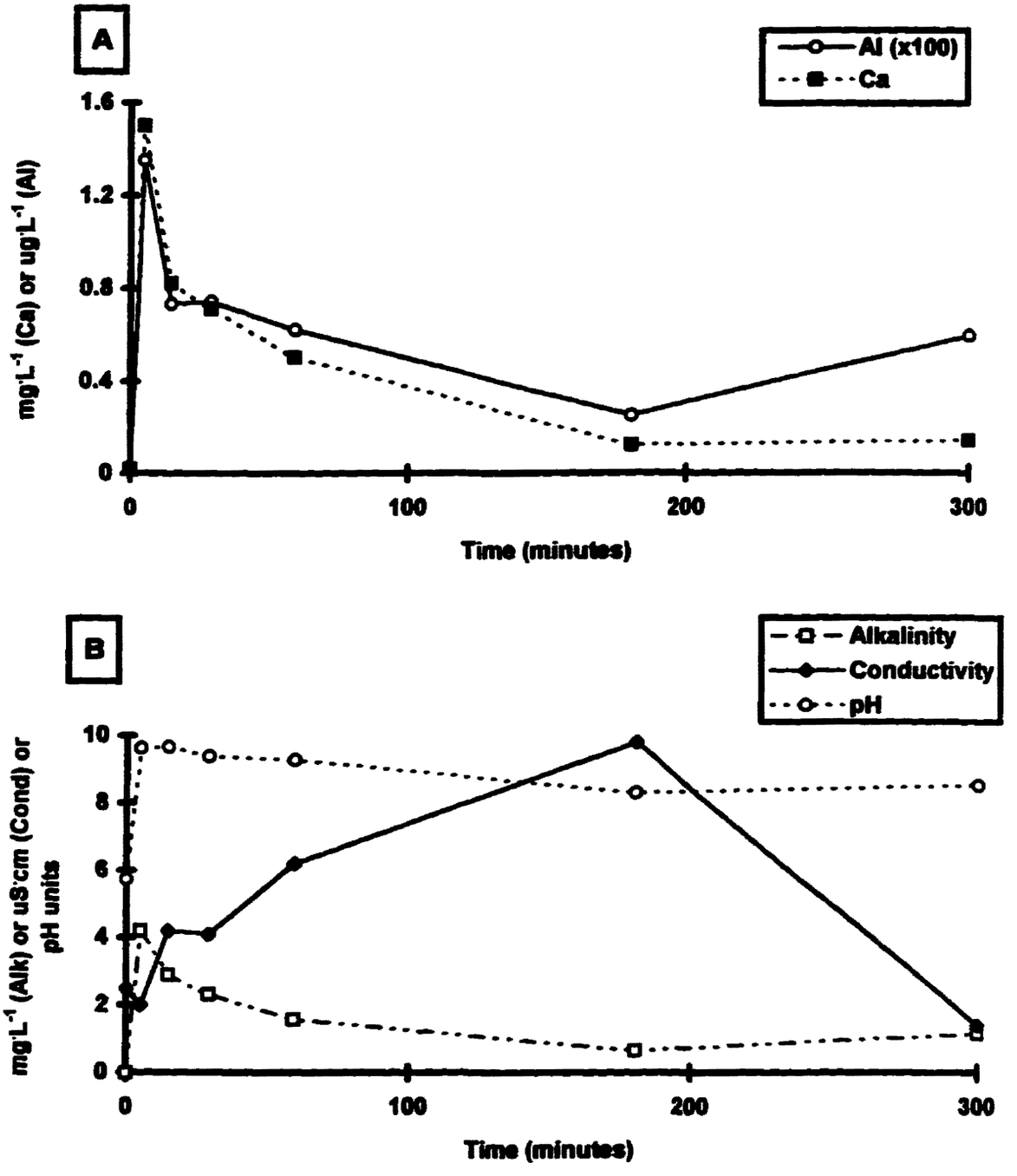


Figure 2.1 Water characteristics of deionized reverse osmosis water during charcoal filter conditioning (see text for details).

temperature (20 to 22 °C). The pH meter was calibrated daily with pH 4 and 7 buffer solutions and pH levels were adjusted by addition of dilute nitric acid or sodium hydroxide if the pH deviated by more than 0.1 pH units from nominal. Mean pH values for each test were calculated as a grand mean by averaging the H^+ ion concentration determined from all pH measurements (including pH measurements before and after adjustments) in all of the exposure containers.

If any individual fish or amphipod died between the start of the test and the 1 h mortality check, it was assumed that death was a result of handling stress and the organism was replaced. In 16 toxicity tests, no fish and 5 amphipods were replaced as a result of presumed handling stress. Occasionally, some *H. azteca* could not be found in the exposure beaker; either the animals had dissolved or were eaten by others after they died. Missing amphipods were treated as dead. A total of 12 of 464 *H. azteca* were missing and presumed dead in 5 out of 8 toxicity tests. Due to the difficulty in observing the neonate *H. azteca*, two beakers were used for each concentration instead of one (5 organisms were placed in each beaker instead of 10) and the data was combined at the end of the test. The patterns of mortality were similar in both series.

Data presented for Cu toxicity in *P. promelas* were derived from Welsh et al. (1993). The methods used were similar to those discussed above except fish were exposed in 2 L of test water instead of 200 mL. The test water quality characteristics for both charcoal filtered and unfiltered water are similar to those used here (Welsh et al. 1993).

2.3.3 Chemical Analysis

Test water was sampled for chemical analyses after pH adjustment to 7.0 and charcoal filtration (if necessary) prior to the start of the tests. Samples were chemically characterized by the Laboratory Services Branch, Ontario Ministry of Environment and Energy (MOEE) according to established protocols (MOE 1981; Janhurst 1993). Dissolved Inorganic Carbon (DIC) concentration ($mg \cdot L^{-1}$) was determined by measuring CO_2 gas liberated from the supernatant of a settled acidified sample. The CO_2 gas diffused through a gas-permeable membrane into a weakly-buffered alkaline phenolphthalein solution and was measured colorimetrically through a 5-cm light path at 550 nm. Dissolved Organic Carbon (DOC) concentration ($mg \cdot L^{-1}$) was measured in a similar fashion to DIC, except the acidified supernatant sample was first flushed with N_2 gas to remove inorganic carbon and the organic carbon was then photo oxidized into CO_2 gas by ultraviolet irradiation in an acid-persulphate media. Alkalinity ($mg \cdot L^{-1}$ as $CaCO_3$) was measured by titration with 0.01M H_2SO_4 to pH < 3.7. Conductivity ($\mu S \cdot cm^{-1}$) was measured at 25 °C with a conductivity meter equipped with a water jacketed conductivity cell. Chloride (Cl) was measured colorimetrically at 480 nm based on the production of ferric thiocyanate from ferric ions and thiocyanate released from the reaction between Cl ions and mercuric thiocyanate. SO_4 was determined by ion chromatography. Ca, K, Mg and Na concentrations ($mg \cdot L^{-1}$) were measured with a flame atomic absorption spectrophotometer. Cd, Cu, Pb and Zn concentrations ($\mu g \cdot L^{-1}$) were measured with a graphite furnace atomic absorption spectrophotometer (GFAAS). Total Al ($\mu g \cdot L^{-1}$) was determined colorimetrically based on the

formation of an Al-catecol violet complex at pH 6.2 after acidified samples were oxidized by UV digestion.

2.3.4 Metal Exposure Concentrations

Five mL water samples were collected in 15 mL polyethylene vials for metal analysis from each exposure beaker at the start and the end of each test and acidified to 0.1% by addition of concentrated ultrapure nitric acid. In the *H. azteca* experiments, 2.5 mL was sampled from each replicate container and combined.

2.3.5 Statistics

Median lethal concentrations (LC50s) were calculated with mortality data and measured total metal concentrations by the trimmed Spearman-Karber method (Hamilton et al. 1977). Control mortality was never greater than 10% (one organism). Differences in the metal toxicity between the DOC concentrations and the two species was determined by a two-way ANOVA. Differences in water characteristics between filtered and unfiltered St. Mary's Lake water was determined by a Student's t-test. All statistical analysis were completed using SYSTAT (Wilkinson 1989).

2.4 Results

2.4.1 Charcoal filtration of exposure water.

Filtering the test water through the conditioned charcoal filter reduced the DOC and the Cu concentration and with the exception of Al, did not change the other water quality characteristics (Table 2.1). Some Al leached from the charcoal filter during filtration, but while the Al concentration was elevated (from 6.6 to 20.5 $\mu g \cdot L^{-1}$), it was also more variable in the charcoal filtered water.

2.4.2 Acute Toxicity Tests

In charcoal filtered and unfiltered St Mary's Lake water, both organisms were sensitive to Cu and Cd exposure at pH 7.0. Mean 96-h Cu LC50s ranged from 6.5 to 53.8 $\mu g \cdot L^{-1}$ in *P. promelas* and 9.1 to 24.1 $\mu g \cdot L^{-1}$ in *H. azteca* (Table 2.2; Fig 2.2). DOC was an important modifier of Cu toxicity in both species (Table 2.3). Although no differences in Cu toxicity were detected between the two species, the DOC-species interaction was significant. Interpretation of this interaction is confounded by the different DOC concentrations in the unfiltered St. Mary's Lake test water (2.8 verses 3.3 $mg \cdot L^{-1}$ DOC; Figure 2.2). For comparison, acute Cu toxicity in *P. promelas* was predicted for a DOC concentrations of 2.8 $mg \cdot L^{-1}$ using the model Log LC50 = 1.033 + 0.999 Log DOC (Welsh et al. 1993). This model was generated from acute Cu 96-h LC50 data with larval fathead minnow in St.

Table 2.1 **Water quality characteristics for St. Mary's Lake water and charcoal filtered St. Mary's Lake water. Mean values (± S.D.) include data from this study (n=4) and from Welsh et al. (1993) (n=2). Significant differences ($p<0.05$) between the mean values denoted in bold type.**

Water Quality Characteristic	St. Mary's Lake		p-value	Detection Limit
	Unfiltered	Charcoal Filtered		
Alkalinity ($mg \cdot L^{-1}$ as $CaCO_3$)	6.7 ± 0.5	11.0 ± 7.5	0.197	N.A.
Conductivity ($\mu S \cdot cm^{-1}$)	79.6 ± 21.1	79.4 ± 16.0	0.989	0.2
DIC ($mg \cdot L^{-1}$)	1.7 ± 0.2	2.9 ± 1.8	0.137	0.02
DOC ($mg \cdot L^{-1}$)	3.2 ± 0.2	0.3 ± 0.2	**<0.001**	0.1
pH (pH units)	7.0 ● 0.1	7.1 ± 0.3	0.494	N.A.
Al ($\mu g \cdot L^{-1}$)	6.6 ± 8.1	20.5 ± 20.1	0.183	2
Cd ($\mu g \cdot L^{-1}$)	0.02 ± 0.02	0.01 ± 0.01	0.233	0.001
Cu ($\mu g \cdot L^{-1}$)	2.7 ± 1.1	0.4 ± 0.4	**<0.001**	0.003
Pb ($\mu g \cdot L^{-1}$)	0.2 ± 0.2	0.5 ± 1.0	0.607	0.003
Zn ($\mu g \cdot L^{-1}$)	8.0 ± 7.5	5.5 ± 3.9	0.493	0.001
Ca ($mg \cdot L^{-1}$)	5.1 ± 0.9	5.3 ± 0.8	0.782	0.02
K ($mg \cdot L^{-1}$)	0.8 ● 0.2	0.8 ± 0.2	0.610	0.005
Mg ($mg \cdot L^{-1}$)	1.9 ● 0.3	1.8 ± 0.3	0.871	0.005
Na ($mg \cdot L^{-1}$)	5.3 ± 2.4	5.5 ± 2.0	0.865	0.005
Cl ($mg \cdot L^{-1}$)	10.0 ± 4.7	9.7 ± 2.7	0.915	0.01
SO4 ($mg \cdot L^{-1}$)	9.3 ± 1.0	7.0 ± 4.9	0.292	0.05

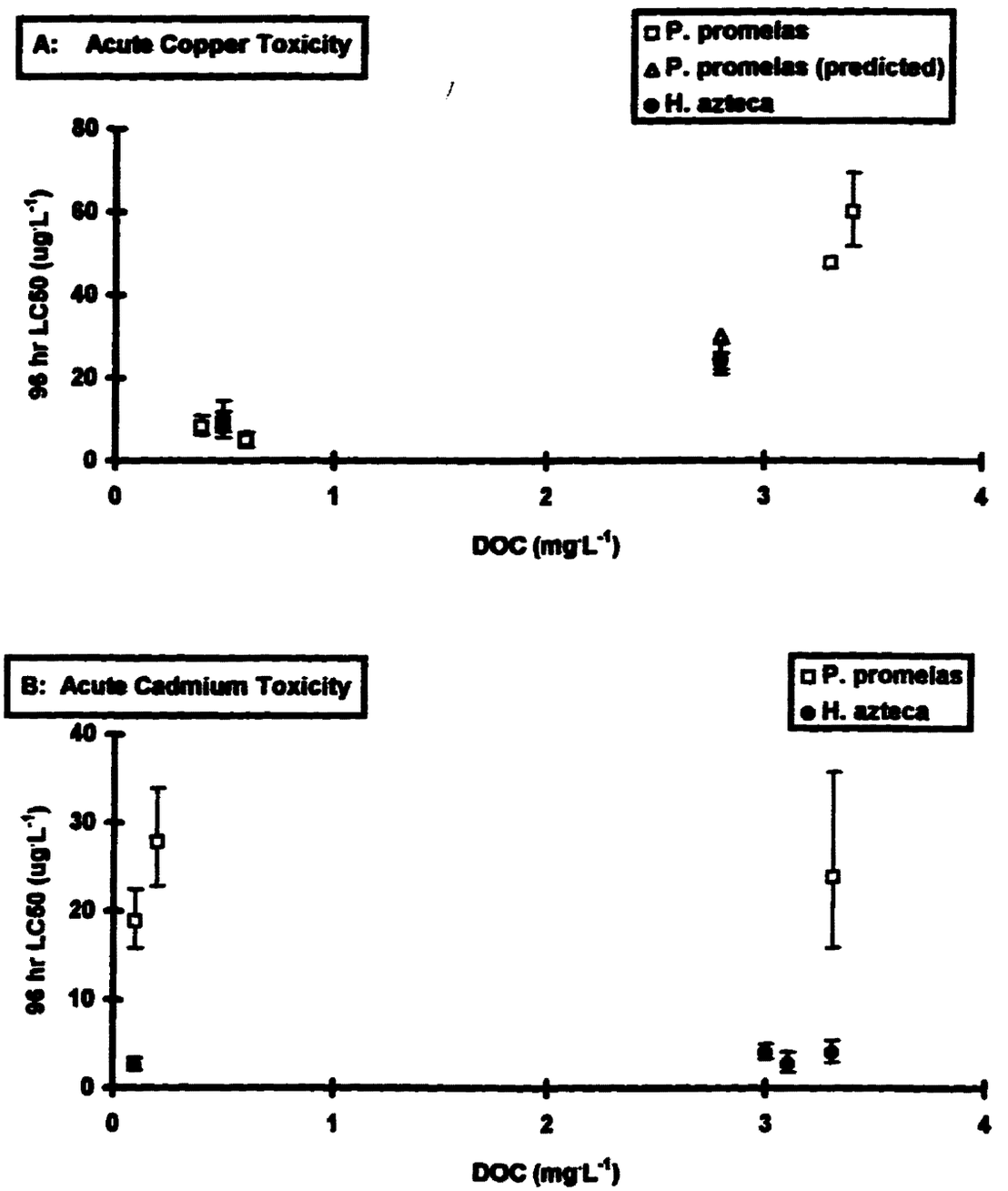


Figure 2.2 Median lethal metal concentrations (96-h LC50s) for *Pimephales promelas* and *Hyalella azteca* in charcoal filtered and unfiltered St. Mary's Lake water. *P. promelas* predicted Cu LC50 calculated at 2.8 mg·L^{-1} DOC from the model: Log Cu LC50 = 1.033 + 0.999*Log DOC (Welsh et al. 1993).

Table 2.2 Acute Cu and Cd toxicity (96-h LC50s) to *Pimephales promelas* and *Hyalella azteca* in charcoal filtered and unfiltered St. Mary's Lake water.

Organism	Metal	pH	DOC ($mg \cdot L^{-1}$)	96-h LC50 ($\mu g \cdot L^{-1}$)	95 % fiducial limits
P. Promelas	Cu	7.15	3.4	59.8	51.7 - 69.3
		7.10	3.3	47.7	----
		7.25	0.4	8.2	6.3 - 10.8
		7.06	0.6	4.8	3.4 - 6.8
	Cd	6.88	3.3	23.9	15.9 - 35.8
		7.07	0.1	18.8	15.8 - 22.4
		6.85	0.2	27.8	22.8 - 33.9
H. azteca	Cu	7.15	2.8	24.3	20.9 - 28.2
		7.15	2.8	23.8	21.9 - 25.8
		7.14	0.5	8.2	5.6 - 11.8
		7.14	0.5	10	6.9 - 14.4
	Cd	6.84	3.3	4.0	2.9 - 5.4
		6.88	3.0	4.0	3.3 - 5.0
		6.89	3.1	2.7	1.8 - 4.1
		6.95	0.1	2.6	2.0 - 3.4

Table 2.3 Analysis of Variance of Log 96-h LC50 values for two DOC levels (0.3 and 3.2 $mg \cdot L^{-1}$) and two species (*Pimephales promelas* and *Hyalella azteca*) in St. Mary's Lake water. LC50 Data was log transformed to improve normality.

Metal	Source	SS	df	MS	F-Ratio	p-value
Copper	DOC	4.862	1	4.862	102.979	0.001
	Species	0.093	1	0.093	1.966	0.233
	DOC*Species	0.678	1	0.678	14.370	0.019
	Error	0.189	4	0.047		
Cadmium	DOC	0.042	1	0.042	0.699	0.465
	Species	5.911	1	5.911	98.798	0.002
	DOC*Species	0.023	1	0.023	0.385	0.579
	Error	0.180	3	0.060		

Mary's Lake water and Brandy Lake water (both soft water lakes) at pH 7. The predicted 96-h Cu LC50 was 30.2 $\mu g \cdot L^{-1}$; a value similar to the Cu toxicity of 24.1 $\mu g \cdot L^{-1}$ in *H. azteca*. It appears that the significant interaction observed between species and DOC concentration for Cu toxicity would probably not occur once the 96-h Cu LC50 was standardized to 2.8 $mg \cdot L^{-1}$ DOC.

In contrast, Cd toxicity was different between the two species; *H. azteca* was significantly more sensitive to Cd exposure than *P. promelas*. Mean 96-h Cd LC50s ranged from 2.7 to 4.0 $\mu g \cdot L^{-1}$ for *H. azteca* and 23.3 to 23.9 $\mu g \cdot L^{-1}$ for *P. promelas* (Table 2.2; Fig 2.2). DOC was not an important modifier of Cd toxicity in either species (Table 2.3).

2.5 Discussion

Dissolved organic carbon concentration proved to be an important modifier of acute Cu, but not Cd, toxicity to the cyprinid *Pimephales promelas* and the amphipod *Hyalella azteca*. In soft water, both organisms were sensitive to Cu and Cd exposure. Cu toxicity was similar between the two organisms in both charcoal filtered, low DOC water and unfiltered, high DOC water. In contrast, *H. azteca* was more sensitive than *P. promelas* to Cd exposure.

Cu is very toxic to fish in soft water (Marr et al 1995; Welsh et al. 1993, 1996). The results presented here are consistent with previously observed 96-h acute Cu LC50s (between 5.3

and 31.9 $\mu g \cdot L^{-1}$) in 17 soft water lakes with DOC < 12 $mg \cdot L^{-1}$ (Welsh et al. 1996). Over a 14 month period, 96-h Cu LC50s to *P. promelas* ranged from 32 to 105 $\mu g \cdot L^{-1}$ in Lake Superior water (hardness 45 $mg \cdot L^{-1}$ as $CaCO_3$; Nelson et al. 1986). The low Cu toxicity observed in the charcoal filtered lake water is consistent with Cu toxicity in "DOC free" reconstituted water (Marr et al. 1995; MacRae 1992). In similar soft water as tested here (hardness 9.2 $mg \cdot L^{-1}$ as $CaCO_3$), Cusimano et al. (1985) measured acute 168-h Cu LC50s between 2.3 $\mu g \cdot L^{-1}$ at pH 7 to 3.1 $\mu g \cdot L^{-1}$ at pH 5.7 to steelhead trout fry. Similarly, Chapman (1978) measured acute 200-h LC50s for Cu to steelhead trout of 17 $\mu g \cdot L^{-1}$ and to chinook salmon of 19 $\mu g \cdot L^{-1}$ in waters at pH 7 (6.1 $mg \cdot L^{-1}$ Ca and 1.4 $mg \cdot L^{-1}$ organic carbon concentration).

Measured acute Cu toxicity to *H. azteca* is also similar to published values. Borgmann et al. (1993) observed reduced survival of *H. azteca* after 6 and 10 weeks exposure to 25 $\mu g \cdot L^{-1}$ Cu in Lake Ontario dechlorinated tap water (hardness 130 $mg \cdot L^{-1}$ as $CaCO_3$, pH 7.9-8.6). Similarly, West et al. (1993) measured 10-d Cu LC50 of 31 $\mu g \cdot L^{-1}$ in *H. azteca* in Lake Superior water (hardness 44 $mg \cdot L^{-1}$ as $CaCO_3$, pH 7, DOC approximately 2 $mg \cdot L^{-1}$).

The acute Cd toxicity to larval fathead minnow presented here (18.8 - 27.8 $\mu g \cdot L^{-1}$), are comparable to reported 96-h Cd LC50s in 30 day old fathead minnow of 13.2 $\mu g \cdot L^{-1}$ in Lake Superior water (Spehar and Fiandt 1986). In soft water, fathead minnow are more resistant to acute Cd exposure than other fish, especially trout (Sprague 1987). Acute 96-h Cd LC50s to juvenile rainbow trout (*Oncorhynchus mykiss*), coho salmon (*O. kisutch*) and arctic

grayling (*Thymallus arcticus*) were 1.5, 3.4 and 4.0 $\mu g \cdot L^{-1}$, respectively, in moderately soft (hardness 41 $mg \cdot L^{-1}$ as $CaCO_3$) circumneutral (pH 7.1 to 8) water (Buhl and Hamilton 1991). Similarly, Cusimano et al. (1985) measured acute 168-h Cd LC50s to steelhead trout fry (*O. mykiss*) between < 0.5 $\mu g \cdot L^{-1}$ at pH 7 to 0.7 $\mu g \cdot L^{-1}$ at pH 5.7 in soft water (hardness 9.2 $mg \cdot L^{-1}$ as $CaCO_3$).

Nebeker et al (1986) measured similar Cd toxicity to *H. azteca* in moderately soft water as this study (96-h LC50 was 8 $\mu g \cdot L^{-1}$ Cd, 10-d static LC50 was approximately 4.4 $\mu g \cdot L^{-1}$ Cd; hardness 34 $mg \cdot L^{-1}$ as $CaCO_3$) while Borgmann (1991) measured 6 week Cd-EC50s at 0.53 $\mu g \cdot L^{-1}$ to *H. azteca* in dechlorinated Lake Ontario tap water (Ca 40 $mg \cdot L^{-1}$, pH 7.9 - 8.7, DOC 2 $mg \cdot L^{-1}$).

An interesting finding from this study was the lack of influence of DOC on the Cd toxicity to either species. This was unexpected; other researchers have found relationships between Cd and DOC. Both Winner (1986) and Giesy et al. (1977) observed an increase in toxicity of some Cd-DOC complexes, while others have observed reduced Cd toxicity in the presence of DOC (Buckley et al. 1985; Giesy et al. 1983). However, in a review on Cd toxicity to fish, Sprague (1987) concludes that the effect of organic complexation on reducing Cd toxicity is minimal since the majority of Cd in lake water, even in eutrophic lakes, is as the inorganic species.

The speciation of metals is important in determining metal toxicity to aquatic organisms. The current paradigm of trace metal-organism interaction for most divalent metals is that the free metal ion activity is proportional to the biological response observed in organisms exposed to metals in solution (Campbell 1995). Without direct measurements of the free metal ion concentration, and assuming that the free ion concentration is the bioavailable metal species, our data suggests that DOC reduces the free Cu ion concentration, but not the free Cd ion concentration, primarily by complexation reactions (Stumm and Morgan 1981). Hence, complexation reactions between the metals and the naturally occurring DOC ligands only occur with Cu and not with Cd. An alternate hypothesis is that both Cu and Cd-organic ligand complexes are formed but that Cd-organic ligand complexes are bioavailable while Cu-organic ligand complexes are not.

In order to properly assess the impacts of naturally occurring DOC on trace metal bioavailability and toxicity to these soft water organisms, detailed information on metal speciation at the low total metal concentrations where toxicity is observed is required. In the following chapters, a metal speciation scheme will be described and measured concentrations of metal species will be compared to observed chronic toxicity of Cu and Cd to *P. Promelas* in two soft water lakes.

3.0 Metal speciation and metal DOC interactions

3.1 Abstract

Cu and Cd speciation was measured by both dialysis and metal ion specific electrodes in natural lake water at environmentally realistic concentrations (low $\mu g \cdot L^{-1}$). The dialysis fraction represents the combined concentrations of free metal ion, as well as membrane permeable (1000 molecular weight cut-off) inorganic and low molecular weight organic metal species; 72% and 39% of Cu and 91% and 60% of Cd was membrane permeable in Halls Lake (DOC = 2.3 $mg \cdot L^{-1}$) and Dickie Lake (DOC = 6.7 $mg \cdot L^{-1}$) water, respectively.

The metal ion specific electrodes (ISE), which measure the free metal ion activity, behaved in a linear fashion down to 10 $\mu g\ Cu \cdot L^{-1}$ and 30 $\mu g\ Cd \cdot L^{-1}$. More free Cu and Cd ion were present in Halls Lake than in Dickie Lake water. Comparisons between the amount of free Cu verses free Cd concentrations at a given DOC concentration within a lake were inappropriate since the free Cd ion estimates were less precise than free Cu ion estimates. In addition, the free Cd ion estimates were biased due to confounding effects of interfering substances. However, comparisons of free Cd ion between lake waters are appropriate as the relative bias in the Cd ion estimates are similar between the two lake waters tested.

Cu and Cd binding characteristics of the lake water DOC was determined by metal ion titrations. The average conditional stability constants calculated using the metal speciation

model FITEQL, for lake water DOC were 7.47 and 7.99 for Cu and 5.31 and 6.04 for Cd in Halls Lake and Dickie Lake water, respectively. Total metal binding capacity was higher in Dickie Lake than in Halls Lake water for both Cu and Cd. Based on Scatchard analysis, two dominant ligands were present in the lake water for Cu and one or two dominant ligands were present for Cd. Lake water DOC had Cu binding characteristics typical of fulvic acids. Very large complexation capacity estimates for Cd (3.3 to 29.6 μmole Cd·mg DOC^{-1}), but not for Cu (0.04 to 0.16 μmole Cu·mg DOC^{-1}), are indicative of biased free Cd ion estimates.

The metal speciation scheme used here determines the free metal ion directly (via ISEs) and the metal ion fraction bound to large molecular weight organic compounds (via size exclusion equilibrium dialysis). Metal associated with small molecular weight compounds can be estimated from the difference between the total metal concentration and both the metal fraction bound to large MW organic compounds and the free metal ion concentration.

More free Cu ion concentration and Cu associated with membrane permeable complexes were present in Halls Lake water (DOC = 2.3 mg $\cdot L^{-1}$) than in Dickie Lake water (DOC = 6.7 mg $\cdot L^{-1}$). A similar pattern was observed for Cd, where more free Cd ion and Cd associated with membrane permeable complexes were present in Halls Lake water (DOC = 2.3 mg $\cdot L^{-1}$) than in Dickie Lake water (DOC = 6.7 mg $\cdot L^{-1}$). However, based on dialysis, less Cd was complexed to large molecular weight complexes (membrane impermeable complexes) than Cu. Hence, organic complexation reduces both the free Cu and Cd ion

activity but more Cu than Cd is bound to naturally occurring fulvic and humic ligands in the lake water DOC (i.e., large molecular weight complexes).

3.2 Introduction

The degree to which metal exposure affects aquatic biota is a function of the bioavailable fraction of the total metal concentration. This bioavailable fraction is related to the concentration of the various individual physico-chemical forms of the metal (Florence 1982). In order to assess the effect of metal speciation on toxicity, it is necessary to estimate the concentration of the toxic metal fraction. The current paradigm of metal-organism interaction is that the free metal ion activity is proportional to the biological response. It is often assumed that the free metal ion concentration is the only bioavailable metal fraction and any bound metal complexes (inorganic or organic) are not bioavailable. Direct measurement of the free metal ion and various bound fractions are essential to test if this paradigm and common assumption are valid.

Measuring the free metal ion concentration is not a straight forward process at environmentally relevant Cu and Cd concentrations. In soft water shield lakes, the concentrations of Cu and Cd that have deleterious effects are in the low $\mu g \cdot L^{-1}$ range (Welsh et al. 1996; Chapter 2). At these low metal levels (< 5 to 50 $\mu g \cdot L^{-1}$), the concentration of the various metal forms are near the detection limit for most analytical methods and the risk of sample contamination and erroneous results is high.

Metal speciation is often predicted based on known thermodynamic information for ions in a fully characterized water sample using geochemical speciation models (e.g., Mineql$^+$, Schecher and McAvoy 1994). Unfortunately, these models are only useful for fully defined systems and, in most surface waters, DOC will interact with metal ions and alter the metal speciation. DOC is a general term that describes a large number of unknown organic ligands, each with different metal ion binding-site affinities and binding-site concentrations. At the present time, DOC has not been properly modelled such that confident estimates of metal binding to these mixtures of unknown organic ligands can be made. In order to properly define the nature of metal-DOC interactions on a site specific basis, it is first necessary to measure the free metal ion concentration directly. With information on the amount of metal bound and free, metal binding capacity and conditional stability constants of dominant ligands of the DOC can be determined.

One objective of this study was to measure the bioavailable metal form, presumably the free Cu and Cd ion concentration, in soft lake water samples with natural DOC present at low total metal concentrations (< 100 $\mu g \cdot L^{-1}$). An approach that determined free Cu or Cd ion concentrations using two methods was employed, since there is no standard method to measure free metal ion (or metal speciation in general) at low total metal concentrations in natural waters. The approach included size separation via equilibrium dialysis (500 and 1000 molecular weight cut-off (MWCO) membranes) and measurement of the free aquo metal ion with Cu or Cd ion-specific electrodes (ISE). Equilibrium dialysis is a common method to separate metal species based on size (Hutchinson and Sprague 1987; Truitt and Weber

1981). With an appropriate dialysis membrane the inorganic and organic metal fractions can be separated. In practice, however, only metal ions bound to large molecular weight compounds are excluded by the dialysis membrane; free metal ion plus metal bound to small molecular weight compounds (organic and inorganic) are measured in the dialysate (LaZerte 1984). As a result dialysis tends to provide an overestimate of the free metal ion. Dialysis membranes with 500 and 1000 MWCO were used as these sizes should exclude fulvic and humic acids in the naturally occurring DOC. Hence, any metal bound to the large organic ligands should not be measured in the membrane permeable fraction.

Metal ISE's measure the free aquo ion of interest. The electrodes behave in a Nernstian fashion where the electrical potential is proportional to the free ion activity. The metal ISE potential is determined by the surface concentration of metal ion absorbed onto the electrode; this surface concentration is in equilibrium with the bulk metal ions in solution (Blaedel and Dinwiddie 1974). At low metal concentrations ($< 10^{-7}$ M Cu), longer equilibrium times are required between the surface concentration and the bulk metal ions in solution, resulting in an non-Nernstian response and long stabilization times (Blaedel and Dinwiddie 1974). Hence, the usefulness of ISEs for measuring metal ion concentration at low total metal concentrations is limited because of the slow equilibrium time between the bulk metal ion concentration and the surface adsorption equilibrium.

An additional objective of this study was to obtain relevant information about the metal binding characteristics of naturally occurring DOC. Understanding the role of DOC in metal

speciation requires information on its binding characteristics. Since DOC is a complex mixture of hundreds or thousands of organic compounds, information on the individual components is difficult to obtain. As such, DOC is often described according to various operationally defined properties. DOC can be separated into humic acid, fulvic acid or humin based on acid/base solubilities or classified according to hydrophillic/hydrophobic properties (Bourbonnière 1989). DOC can also be fractionated by functional group, (carboxylic, phenolic or amine groups) or analyzed for total mineral content (Schnitzer and Khan 1972). These various DOC classification schemes were not attempted in this study. Instead, biologically relevant aspects of the DOC were determined in regard to metal exposure and toxicity; namely, metal specific binding site concentration and the associated conditional stability constants of dominant generalized ligands.

3.3 Materials and Methods

3.3.1 Trace metals collection and analysis

All sample handling and analysis for this study was completed in a class-100 clean laboratory. Unless otherwise noted, plasticware used to store water samples for metal determination were either nonreactive polyethylene or Teflon (Batley and Gardner 1977). Containers were soaked in a 1% Contrad-70® soap solution (an anionic detergent) for a minimum of 24 h, rinsed, and soaked in either 1% sulphuric acid or 5 % nitric acid for at

least 24 h before use. Containers were subsequently rinsed 7 to 10 times with high purity water (demineralized reverse osmosis water pumped through a Modulab® Modupure Plus™ Reagent Grade water system: resistivity > 16.7 megohm·cm^{-1}). Water samples for total metal analysis were acidified with Suprapure® nitric acid to 0.1% and stored at room temperature until analyzed. The water used for the determination of metal speciation was either high purity water or 5-μm filtered lake water. Samples were usually analyzed for total metal concentration within 4 months. No problem with adsorption losses were expected to occur during this time period (Florence 1982).

Water samples were analyzed for Cu or Cd by Graphite Furnace Atomic Absorption Spectroscopy (GFAAS). Samples were analyzed in duplicate and results were rejected if the relative standard deviation exceeded 5%. Other quality control measures included regular analysis of standard water samples from the National Research Council of Canada (SLRS-2), long term blanks, dilution water blanks and in-run standards. In addition, standard water samples were added to the sample load and triplicate analyses were performed on random samples in several analytical runs. Cu analysis was performed at two levels: low, range < 4 μg·L^{-1} and high, between 4 and 20 μg·L^{-1}. An internal calibration standard (AAS-15), made up to 15 μg·L^{-1} nominal Cu, was used in the high Cu analysis.

3.3.2 Dialysis

3.3.2.1 Dialysis membrane preparation and procedure

Cellulose acetate dialysis membrane tubing (Spectrapor® 500 and 1000 nominal MWCO; diameter, 2.5 cm) were used to separate metal bound to fulvic and humic acids from free aquo metal ion and metal bound to small inorganic and organic ligands. The membranes were cut into 7 to 10 cm lengths and thoroughly rinsed of sodium azide preservative. Cut dialysis membranes were then acid washed (and stored) in 1% nitric acid. For long term storage the membranes were immersed in a solution containing 1% nitric acid and 5% hydrogen peroxide. Immediately prior to use, tubing was successively soaked in two 5 L batches of high purity water for 24 h to remove any remaining acid and thoroughly rinsed (LaZerte 1984).

Dialysis membranes were clipped at one end with Spectrapor® dialysis clips and filled with high purity water. The dialysis tubing was then gently closed at the top (with no air bubbles present in the inner solution) and added to 450 mL of sample in a 500 mL PET (polyethylene terephthalate) container. A 4.5 mL aliquot of 1 M KCl was added to each solution (final concentration, 0.01M KCl) to act as an ionic strength adjuster (ISA): maximizing the effective pore size of the membrane and minimizing charge effects within the cellulose acetate membrane. In all experiments where KCl was added, the rinse water and the inner filling solution also contained similar KCl solution. Sample containers were

capped and solutions were allowed to equilibrate for 48 h. The time for equilibration was determined from a method development experiment (see below). After equilibrium was reached, samples of the dialysate (membrane permeable solution, inside the dialysis membrane) and retentate (membrane impermeable solution, outside the dialysis membrane) were collected for determination of DOC and metal concentration.

3.3.2.2 Measuring DOC in dialysate

DOC could not be measured directly in the dialysate sample because of low total volumes (< 15 mL). Instead, DOC in the dialysate sample was estimated by measuring the absorbance in phosphate buffered samples (pH 6.88) at 250 nm (A_{250}) (Lawrence 1980). A_{250} is a good measure of dissolved organic matter in lakes (DeHaan et al. 1982) and provides a measure of the amount of DOC compounds migrating through the dialysis membrane. A standard curve was constructed using purified Laurentian fulvic acid (Ecolinc Inc., Roxboro, Quebec) as a surrogate for fulvic acids in naturally occurring DOC. Infiltration of the fulvic acid standard through the dialysis membrane was determined over time by examining A_{250} in the dialysate in a 2 $mg \cdot L^{-1}$ fulvic acid solution containing 100 $\mu g \cdot L^{-1}$ nominal Cu and 0.01 M KCl. This infiltration of the standard fulvic acid was used to determine appropriate equilibrium dialysis times.

3.3.2.3 Dialysis method development.

The effect of dialysis time and sample agitation was tested to determine the time to equilibrium for both 500 MWCO and 1000 MWCO dialysis membranes. Samples of high purity water containing 100 $\mu g \cdot L^{-1}$ nominal Cu and 0.01M KCl were dialysed for 2, 24, 48, or 96 h under conditions of either gentle stirring or no stirring. The effect of membrane size was further examined by comparing the time to reach equilibrium with Dickie Lake water containing 100 $\mu g \cdot L^{-1}$ nominal Cu using 500 MWCO membranes with KCl added, to 1000 MWCO membranes without KCl added. The effect of KCl addition on the time to reach equilibrium was tested by comparing the amount of Cu in the dialysate at 48 and 96 h in a sample of Dickie Lake water containing 100 $\mu g \cdot L^{-1}$ nominal Cu using 1000 MWCO membranes with no KCl added to the expected Cu concentration in the dialysate at equilibrium with 1000 MWCO membranes with KCl added. No experiments were done with 500 MWCO dialysis membranes without KCl added.

3.3.2.4 Bioassay Lake Water Samples

Samples of test water for dialysis, collected from the metal exposure tanks (see chapter 4) between 72 and 120 h after the start of an experiment, were stored unacidified in the dark at 4 °C until used within 10 days of collection. For these samples, only 1000 MWCO dialysis membranes were used since equilibrium could not be reached with 48 or 96 h with the 500 MWCO dialysis membranes.

3.3.3 Metal Ion Specific Electrodes

3.3.3.1 Electrode Operation

Cu and Cd ion activities were determined with metal ISE's. These electrodes are sensitive to various interfering substances (temperature fluctuations, degree of illumination, interfering ions; Orion Research Inc., Cambridge, Massachusetts, 1979). Extra care was required when working with ISE's at low metal concentrations ($\mu g \cdot L^{-1}$) during cleaning and handling since both can affect baseline conditions. In general, the free metal ion is measured with a metal ion specific ISE and a double junction reference electrode. The metal ion of interest, interacts with the ISE by surface adsorption and changes the electrical potential. This change in potential is measured against a constant reference potential and is proportional to the metal ion activity in solution according to the Nernst equation (Nelson et al. 1986):

$$E = E_o + S \cdot Log\{Me^{2+}\} \qquad (1)$$

where: E = measured electrode potential

E_o = reference potential (a constant)

S = electrode slope

$\{Me^{2+}\}$ = free metal ion activity in solution

At constant ionic strength (0.01 M), the activity coefficient is constant and the metal ion activity $\{Me^{2+}\}$ is directly proportional to the free metal ion concentration, (Radiometer Cd ISE Instruction Manual, 1989). The metal ion activity is related to the free metal ion concentration based on the activity coefficient (γ):

$$\{Me^{2+}\}=\gamma \cdot Me^{2+} \tag{2}$$

This activity coefficient is a function of the ionic strength of a solution (I) and is calculated from the Davies equation (Stumm and Morgan 1981):

$$\gamma = 10^{\left(-0.5 z_x^2 \left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.2I\right)\right)} \tag{3}$$

where: z_x = charge of ion x

and

$$I = \frac{1}{2}\sum C_x \cdot z_x^2 \tag{4}$$

where: C_x = concentration of ion x

Σ = sum of all the types of ions in solution.

A work station was built for measuring free metal ion concentration that minimized the effect of interference. Water was heated in a hot water bath and recirculated through a sample chamber (final temperature 26 ● 0.5°C). The sample chamber consisted of a 2-L polypropylene Nalgene beaker placed on a stir plate at a fixed low stirring rate. Sample solutions were held in a 500 mL PET jar with a teflon stir bar and placed in the heated sample chamber. The sample, stir plate, the double junction reference electrode (Orion, Model 90-02) and the cupric ion electrode (Orion, Model 94-29) or cadmium ion electrode (Radiometer, FK1502Cd) were all enclosed in a PVC box to minimize light intensity. Both electrodes were plugged into an Orion Research Microprocessor pH/millivolt meter (Model 811; digital read-out, 0.1 mV). The mV output was connected to a linear chart recorder and continuously monitored. Electrical interference from other electrical devices and electrical surges were minimized by leaving the stir plate on at a fixed rate, limiting the electrical circuit to devices used in measuring metal ion activity and by having the mV meter plugged into a surge protector.

3.3.3.2 Cleaning ISE's and sample preparation

Gloves were worn at all times to minimize sample contamination. The solutions in the double junction electrode were replaced before use (inner solution, 0.5 M KCl saturated with Ag; outer solution, 10% KNO_3). The ISE was conditioned by polishing the base of the electrode in a circular fashion with a prewetted polishing (aluminum oxide) strip for approximately one minute. Trace Cu, Cd and other interfering compounds on the electrodes

were removed by soaking both electrodes in a 500 mL 1% nitric acid solution for 3 to 5 minutes (Blaedel and Dinwiddie 1974). While soaking in the 1% nitric acid, the mV reading would decrease as interfering compounds and trace Cu were stripped off the electrode. Both electrodes were then rinsed well, soaked for 5 minutes in high purity water and rinsed again.

450 mL samples of exposure water were collected in an acid washed 500 mL PET jar and KCl was added (final concentration, 0.01 M). Although early trials used 0.01 M $NaNO_3$ as an ionic strength adjuster, 0.01 M KCl appeared to provide greater sensitivity. The pH was adjusted to 6.3 by adding dilute nitric acid or sodium hydroxide. The samples were then moved to the work station described above. They were preheated in the water bath for 30 min, then transferred to the sample chamber. Samples were stirred at a constant low rate (to avoid swirling) with a Teflon coated stir bar on a stir plate. Conditioned electrodes were placed in the sample solution to a depth of 2 - 4 cm and mV readings were recorded after the output had stabilized as determined visually from a plot of mV reading over time on a linear chart recorder.

3.3.3.3 Standard Curve

A standard curve was generated in a defined medium (high purity water, pH 6.3, 0.01 M KCl) with known metal ion concentrations and free metal ion was measured in an unknown sample by comparison to the standard curve. In these calibration curves, the total metal concentration in the defined medium is proportional to the metal ion activity. Since all of

the measurements were made at constant ionic strength, the free metal ion activity was assumed to equal the total metal concentration added; no conversion from metal activity to metal ion concentration was done. In addition, no correction was made for inorganic CdCl complexes, as the electrode appeared to measure both free Cd and the CdCl complexes (see Section 3.4.3).

The Cu standard curve was generated by sequential additions of concentrated $CuSO_4 \cdot 5H_2O$ stock solutions into 450 mL distilled water plus 0.01 M KCl. The standard curve included nominal Cu concentrations of 0, 0.1, 1, 2, 4, 10, 100, and occasionally 500, 1000, and 10000 $\mu g \cdot L^{-1}$. At 0 and 100 $\mu g \cdot L^{-1}$ total Cu, 4.5 mL of sample was collected for determination of actual Cu concentration by GFAAS. Curves were adjusted to actual total Cu added instead of nominal by correcting for the difference between measured and nominal Cu at 100 $\mu g \cdot L^{-1}$ nominal total Cu. Initial mV readings from the standard curve were zeroed (0.0 mV) at 0 $\mu g \cdot L^{-1}$ nominal total Cu in distilled water after the mV reading had stabilized. This mV reference point was held constant for subsequent titrations with lake water. The Cu standard curve took approximately 5 h to generate due to the long time required for the mV reading to stabilize. To reduce the time required, subsequent standard curves were standardized at 0.0 mV at 0.1 $\mu g \cdot L^{-1}$ Cu instead of 0 $\mu g \cdot L^{-1}$ Cu.

The Cd standard curve was generated using the methods described for the Cu standard curve, with the exception that sequential additions of concentrated $3CdSO_4 \cdot 8H_2O$ stock solutions were added instead of Cu and that samples for total Cd measurement were taken at

0 and 10 μg $Cd \cdot L^{-1}$. The standard curve included nominal Cd concentrations of 0.1, 0.4, 1, 4, 10, 40, 100, 400, and 1000 $\mu g \cdot L^{-1}$.

3.3.4 Characterizing DOC

Lake water samples containing natural DOC were titrated against both Cu and Cd. Free metal ion concentration was determined in these titrations using metal ISEs. Average log conditional stability constant (log K') and binding site concentration (CC) were calculated using FITEQL, a computer program for determining chemical equilibrium constants (Herbelin and Westall 1994). In FITEQL, the estimates for log K' and CC were made simultaneously using nonlinear optimization procedures (Fish et al. 1986). Due to the low number of titration datapoints, FITEQL was unable to calculate individual log K' and CC estimates for dominant (generalized) ligands. To overcome this shortcoming, 22 datapoints between 1 and 1000 $\mu g \cdot L^{-1}$ total metal concentration were generated for total and free Cu or Cd ion concentrations from the titration plots by interpolation. These interpolated datapoints were used in subsequent Scatchard analysis and nonlinear regression analysis (see below) to estimate the metal binding characteristics of each dominant ligand.

Initial estimates of Log K_i' and CC_i were calculated for each dominant ligand (where *i* denotes each individual ligand) using Scatchard plots. In these plots, the ratio of ligand-bound metal and free-metal ion concentration is plotted against the ligand-bound metal concentration and the slope of the individual linear sections of the plot are equal to the

negative stability constant (-log K's) and the x-intercepts are the CCs (Buffle 1988b). A 1-ligand or 2-ligand model for Cu and Cd binding to DOC was assumed based on the literature (McKnight et al. 1983) and the shape of the curve in the Scatchard plot. A one ligand Scatchard plot would produce a straight line, while a two or more ligand Scatchard plot would result in a characteristic concave curve. The 2 dominant ligands estimated by this technique do not exist as a chemical entity but represent an approximation of a series of ligands whose combined characteristic is modeled by each dominant ligand (Fish et al. 1986). It should be noted, however, that the Log K_i's are specific for the pH, ionic strength and competing ion concentration of the sample (Fish and Morel 1983); the results are not transferrable to other sample matrices.

These initial estimates of log K_i' and CC_i from the Scatchard plot were then used as starting parameters for a nonlinear regression analysis that calculated log K_i' and CC_i (assuming two dominant ligands in the DOC) using the model (MacRae et al. 1995 based on Dzombak et al. 1986):

$$[Me\text{-}bound] = \{Me^{2+}\}\left(\left(\frac{K_1 \cdot CC_1}{1 + K_1 \cdot \{Me^{2+}\}}\right) + \left(\frac{K_2 \cdot CC_2}{1 + K_2 \cdot \{Me^{2+}\}}\right)\right) \qquad (5)$$

where: [Me-bound] represents the ligand bound metal concentration

$\{Me^{2+}\}$ represents the free metal ion activity

K_1 and K_2 represent the conditional stability constant for ligand #1 and #2

CC_1 and CC_2 represent the binding site concentration of ligand #1 and #2

At I = 0.0104 M, the ionic strength for the samples used to determine the free metal ion concentration, $\gamma = 0.659$ (for both Cu and Cd) and from equation (2), $\{Me^{2+}\}$ is equal to 0.659 multiplied by the free metal ion concentration.

For Cd titrations in Halls Lake and Dickie Lake water, a one ligand model similar to the 2-ligand model above was also used:

$$[Me\text{-}bound] = \{Me^{2+}\}\left(\left(\frac{K_1 \cdot CC_1}{1 + K_1 \cdot \{Me^{2+}\}}\right)\right) \qquad (6)$$

The values for log K_i' and CC_i were estimated by non-linear regression using the above models in addition to the Scatchard plots because the Scatchard plots are subject to 2 major sources of error, namely trying to fit a linear line to non-linear data and treating data with variable relative error with equal weight (Fish et al. 1986).

3.3.5 Estimating Free Metal Ion Concentration in Lake Water.

Free metal ion concentrations were estimated at total metal concentrations in the bioassay exposure water. Since the metal ion titrations were performed at I = 0.0104 M, the estimated free metal ion concentrations are specific for that ionic strength. A correction was made to the free metal ion concentration predicted at the total metal concentrations in the bioassay exposure water (at I = 0.0004 M) by multiplying the free metal ion concentration by the ratio of the activity coefficients at both ionic strengths ($\gamma_{I=0.0004} / \gamma_{I=0.0104} = 1.387$). From equation (3), recall that for both Cu and Cd, $\gamma = 0.659$ at I = 0.0104 M, and $\gamma = 0.914$ at I = 0.0004 M.

3.4 Results

3.4.1 Analytical Precision and Variability

The analytical method used to measure total Cu and Cd was accurate and precise (Table 3.1). Measured Cu and Cd in standard water samples were comparable to expected values. The high Cu analysis procedure tended to slightly overestimate the NRC standard. However, the expected value for the NRC standard (2.70 $\mu g \cdot L^{-1}$) was below the lowest concentration in the standard curve (4 $\mu g \cdot L^{-1}$). The calibration standard (AAS-15) was not acidified nor independently verified, so comparisons to expected values were not possible. However, the

Table 3.1 **Accuracy and precision of Cu and Cd analysis. A: Concentration of Cu and Cd in standard water samples (mean ● SD, $\mu g \cdot L^{-1}$) and high purity water (median, $\mu g \cdot L^{-1}$). B: Precision of analytical methods used to measure Cu and Cd by GFAAS based on triplicate analyses of samples with Cu or Cd added (median coefficient of variation, CV = (SD/Mean)*100, %).**

A	Metal	Range ($\mu g \cdot L^{-1}$)	Standard	Expected Value ($\mu g \cdot L^{-1}$)	Measured Value ($\mu g \cdot L^{-1}$)	n	Median Water Conc ($\mu g \cdot L^{-1}$)	n
	Cd	< 1	SLRS-2	0.030±0.002	0.033±0.009	93	0.002	92
	Cu	<4	SLRS-2	2.70±0.20	2.71±0.40	104	0.08	103
		4-20	SLRS-2	2.70±0.20	2.94±0.47	95	0.14	96
			AAS-15		14.12±1.05	86		

B	Metal	Range ($\mu g \cdot L^{-1}$)	Median Triplicate Anal. CV (%)	n
	Cd	< 1	8.4	27
	Cu	<4	11.3	31
		4-20	1.9	24

measured Cu concentration in this standard was relatively constant at 14 $\mu g \cdot L^{-1}$ and did not show any temporal pattern over 26 analytical runs.

Some water samples had to be diluted with high purity water to bring the expected concentration within analytical range. Metal concentrations in high purity water used for dilution were quite low, usually at or near detection (Table 3.1). However, high values due to contamination occurred occasionally. In all cases, the concentration of metal in the dilution water was subtracted from the concentration of metal in the diluted sample before correcting for the dilution. As a result of the contaminated distribution of Cu in the high purity water, the median metal concentration in the dilution water was calculated instead of the mean concentration (Table 3.1). In most cases, the concentration in the dilution water was several orders of magnitude below the concentration in the lake water samples. The median coefficient of variation in triplicate analyses of samples with Cu or Cd added provides an estimate of the repeatability of the analytical method. The low Cu method was the most variable (11.3%) while the high Cu method was the least variable (1.9%) (Table 3.1).

3.4.2 Dialysis

3.4.2.1 Dialysis Method Development

Equilibrium conditions between dialysate and retentate with 0.01 M KCl were reached after 48 h of dialysis with the 1000 MWCO membranes, but were not reached even after 96 h of dialysis with the 500 MWCO membranes (Figure 3.1). Stirring had no effect on the time to reach equilibrium with either membrane, but time and membrane type were important (Table 3.2). The 500 MWCO dialysis membrane appeared to have a pore size too small, or a path length too torturous, for equilibrium to be reached rapidly. No difference was detected in the equilibrium conditions between 48 and 96 h of dialysis with the 1000 MWCO membranes.

1000 MWCO membranes without KCl added behaved in a fashion similar to 500 MWCO membranes with KCl (Figure 3.2). The 1000 MWCO membranes without KCl added appeared to have an effective pore size equivalent to that of the 500 MWCO membranes. The predicted Cu fraction in the dialysate in Dickie lake water if KCl had been added to 1000 MWCO membranes indicates that equilibrium conditions are not met after 48 but were met after 96 h dialysis time in the 1000 MWCO membranes without KCl addition. Overall, KCl addition appeared to maximize effective pore size and minimize time to reach equilibrium.

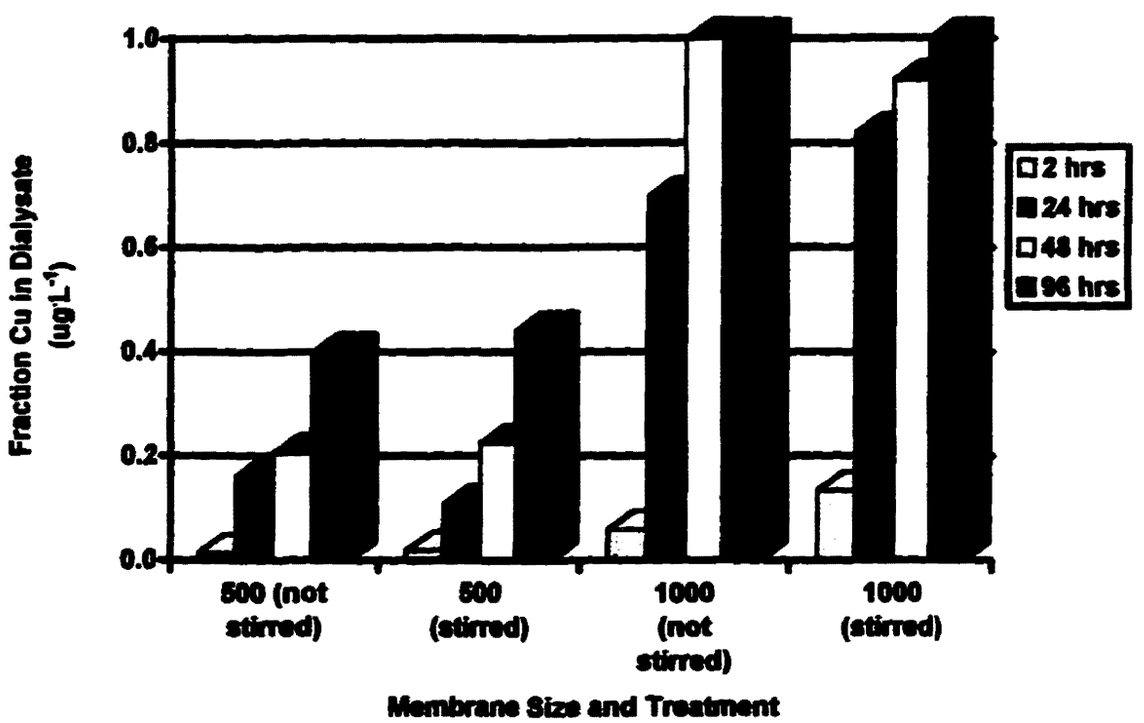


Figure 3.1 The effect of membrane size (500 and 1000 MWCO) and stirring on time to equilibrium in samples of high purity water with 100 ug·L^{-1} nominal Cu and 0.01 M KCl. (Statistics in Table 3.2). Fraction Cu in dialysate is the amount of Cu inside the dialysis membrane divided by the amount of Cu outside (=total) the dialysis membrane.

Table 3.2 **Dialysis method development. ANOVA table describing the effect of stirring, dialysis time (2, 24, 48, 96 h) and membrane type (500 or 1000 MWCO) on the time to reach equilibrium. The fraction Cu in the dialysate verses the retentate was the dependent variable.**

Membrane	Source	SS	df	MS	F-Ratio	p-value
500	Stirred (S)	< 0.001	1	< 0.001	0.050	0.833
	Time (T)	0.268	3	0.089	169.068	< 0.001
	S*T	0.003	3	0.001	1.962	0.238
	Error	0.003	5	0.001		
1000	Stirred (S)	0.002	1	0.002	0.592	0.464
	Time (T)	2.251	3	0.750	263.118	< 0.001
	S*T	0.035	3	0.012	4.063	0.050
	Error	0.023	8	0.003		
Both	Membrane (M)	1.925	1	1.925	621.365	< 0.001
	Time (T)	1.942	3	0.647	209.028	< 0.001
	M*T	0.521	3	0.174	56.103	< 0.001
	Error	0.066	21	0.003		

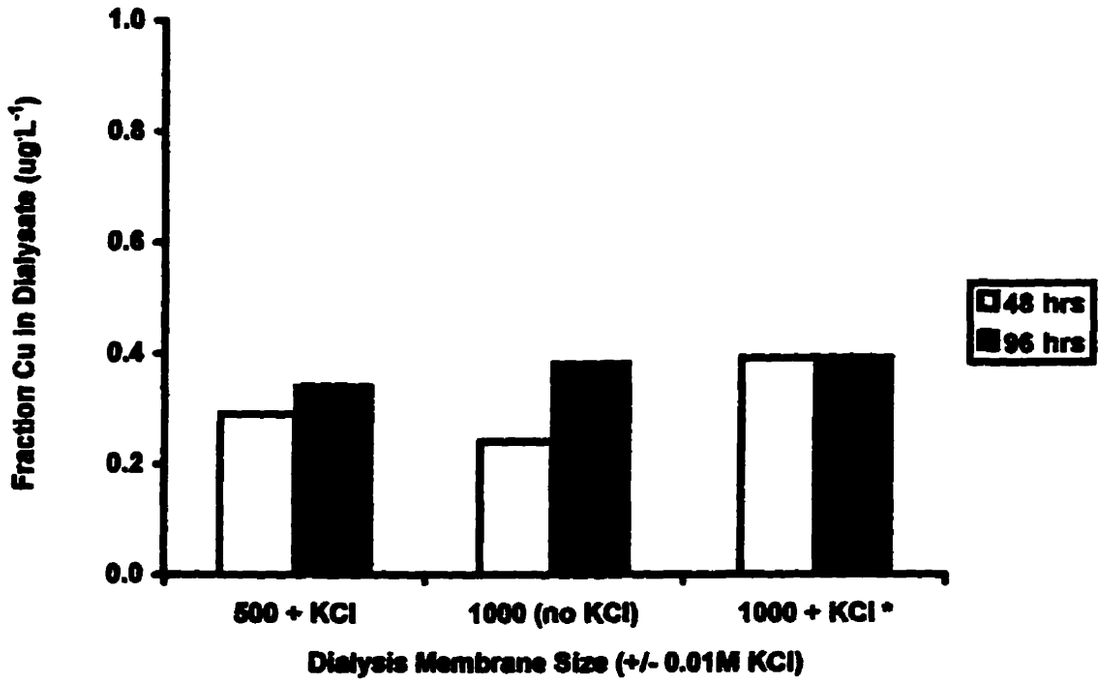


Figure 3.2 The effect of membrane size and ionic strength adjuster (0.01 M KCl) on time to equilibrium in Dickie Lake (DOC = 6.3 $mg \cdot L^{-1}$) with 100 $ug \cdot L^{-1}$ nominal Cu. (*) denotes the expected Cu concentration in dialysate based on the regression: dialysed Cu = 0.29 + (0.39)(Total Cu). (see Figure 3.5 and text for details).

A_{250} nm was linearly related to the DOC concentration of the Laurentian fulvic acid standard and was a good surrogate for the DOC concentration of the fulvic acid standard (Figure 3.3):

$$DOC\ (mg \cdot L^{-1}) = 0.045\ [\pm 0.077] + (19.673\ [\pm 0.670]\ * A_{250});\ r^2 = 0.97,\ p<0.001 \qquad (7)$$

However, measuring A_{250} nm is not a good surrogate for natural lake water DOC since only the uv-absorbing compounds are detected and the non-uv-absorbing aliphatic compounds are not detected (Evans et al. 1989)(Table 3.3). Hence, A_{250} nm is a good measure of the fulvic acid compounds in the natural DOC only. Absorbance at 250 nm will also measure humic acids but in soft water Precambrian Shield lakes, < 5-10% of the DOC is composed of humic acids (Evans et al. 1989). In dialysis experiments with lake water, 68% of Halls Lake and 39% of Dickie Lake fulvic acid fraction of the DOC migrated through the dialysis membrane after 48 hours (Table 3.3). Hence, the concentration of metal ion in the dialysate includes not only the free metal ion but also membrane permeable inorganic and low molecular weight organic metal species.

Fulvic acid infiltration through the 1000 MWCO dialysis membrane (with 0.01 M KCl) increased over time with 10.3 and 17.3% of the fulvic acid migrating through the dialysis membrane after 48 and 96 h, respectively (Figure 3.4).

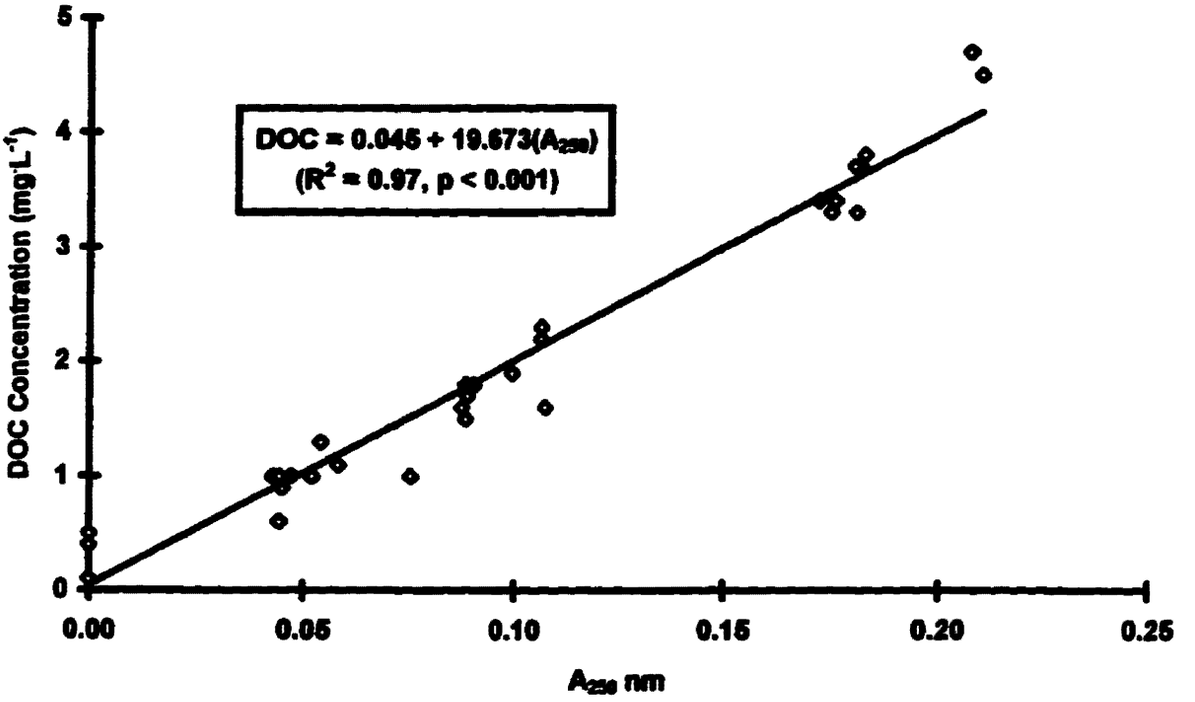


Figure 3.3 The relationship between DOC concentration of Laurentian fulvic acid ($mg \cdot L^{-1}$) and absorbance at 250 nm.

Table 3.3 Estimated mean (± SD) fulvic acid concentration in dialysate and retentate in Halls and Dickie water dialysis experiments. Fulvic acid concentration was calculated using the formula: FA = 0.045 + 19.673*Absorbance at 250 nm (Figure 3.3). [a] Measured mean DOC concentration in lake water (see chapter 4). (b) Percent of the lake water DOC that is composed of fulvic acids = (fulvic acid concentration based on A_{250} nm in the retentate/lake water DOC concentration)*100.

	Estimated Fulvic Acid concentration ($mg \cdot L^{-1}$)				
Lake water	Dialysate	Retentate	Percent Fulvic Acid in Dialysate (%)	DOC Conc. ($mg \cdot L^{-1}$)[a]	Amount of DOC composed of Fulvic acids (%)[b]
Halls	0.65 ± 0.08	0.96 ± 0.07	67.7	2.3	41.7
Dickie	1.93 ± 0.27	4.91 ± 0.60	39.3	6.7	73.3

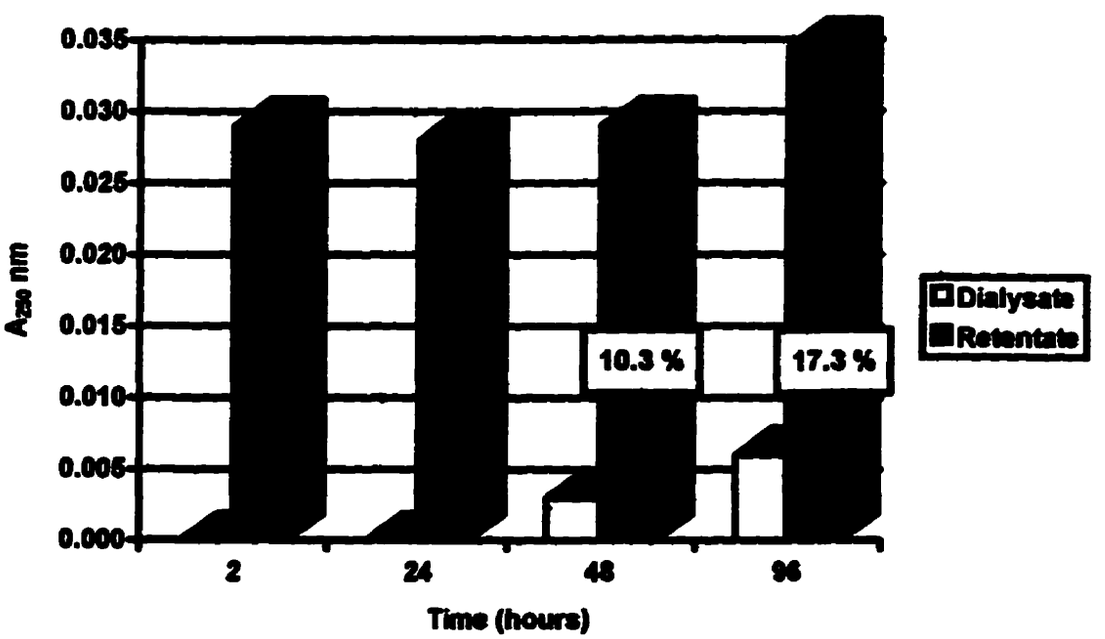


Figure 3.4 Infiltration of fulvic acid through 1000 MWCO dialysis membranes over time as determined by A_{250} nm. Outer solution contains 2 $mg \cdot L^{-1}$ Laurentian fulvic acid (DOC = 0.7 $mg \cdot L^{-1}$) plus 0.01 M KCl. Percent A_{250} nm in the dialysate compared to the retentate is illustrated for 48 and 96 h.

3.4.2.2 Dialysis Method

From the dialysis method development experiments, dialysis conditions were chosen to minimize the time to reach equilibrium conditions. These included 1000 MWCO dialysis membranes, 0.01M KCl, and 48 h of dialysis time. No further experiments with 500 MWCO dialysis membranes were done since equilibrium conditions were not met with these membranes within 96 h.

In the lake water samples, Cu and Cd concentrations in the dialysate (dialysed, membrane permeable, metal) were linearly related to total metal concentration (Figure 3.5 and 3.6). The relationship between dialysed Cu ($\mu g \cdot L^{-1}$) and total Cu ($\mu g \cdot L^{-1}$) in Halls Lake (DOC = 2.3 $mg \cdot L^{-1}$) and Dickie Lake (DOC = 6.7 $mg \cdot L^{-1}$) were:

$$\text{Dialysed Cu (Halls)} = -0.254\ [\pm 0.351] + (0.717\ [\pm 0.065] \bullet \text{Total Cu}) \quad (8)$$

$$(r^2 = 0.91,\ p < 0.001)$$

$$\text{Dialysed Cu (Dickie)} = 0.293\ [\pm 0.227] + (0.390\ [\pm 0.020] * \text{Total Cu}) \quad (9)$$

$$(r^2 = 0.94,\ p < 0.001)$$

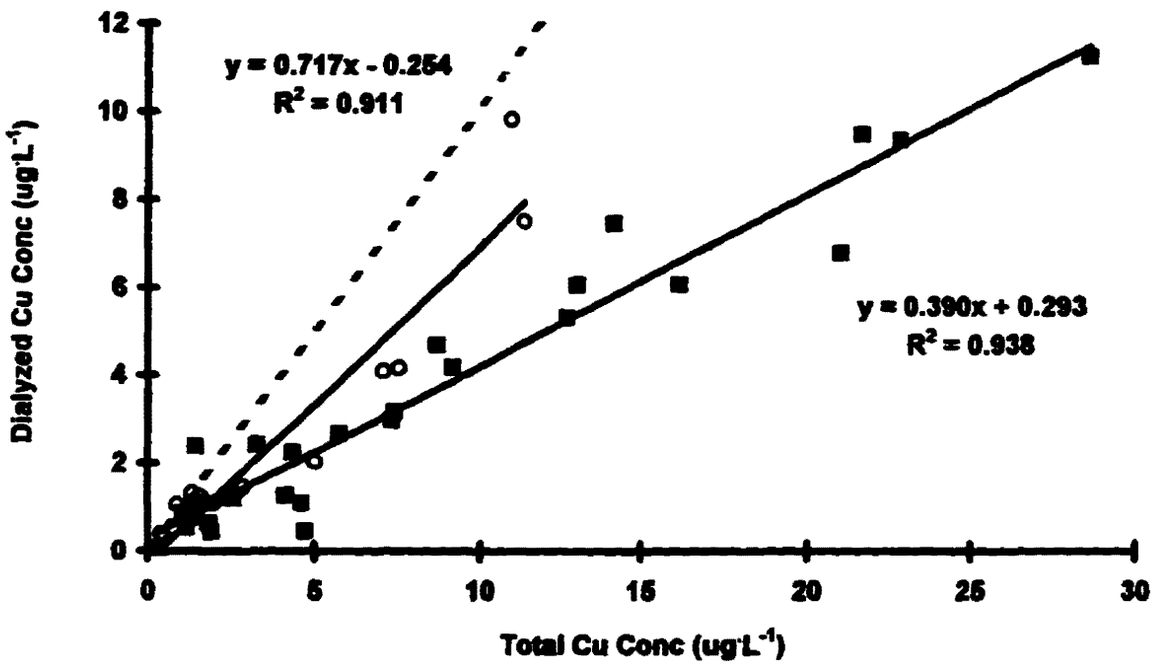


Figure 3.5 Cu Dialysis of Halls and Dickie lake water; (o) denotes Halls Lake (DOC = 2.3 $mg \cdot L^{-1}$); (■) denotes Dickie Lake (DOC = 6.7 $mg \cdot L^{-1}$). Both regression equations are significant ($p<0.001$). The dotted line indicates the 1:1 line.

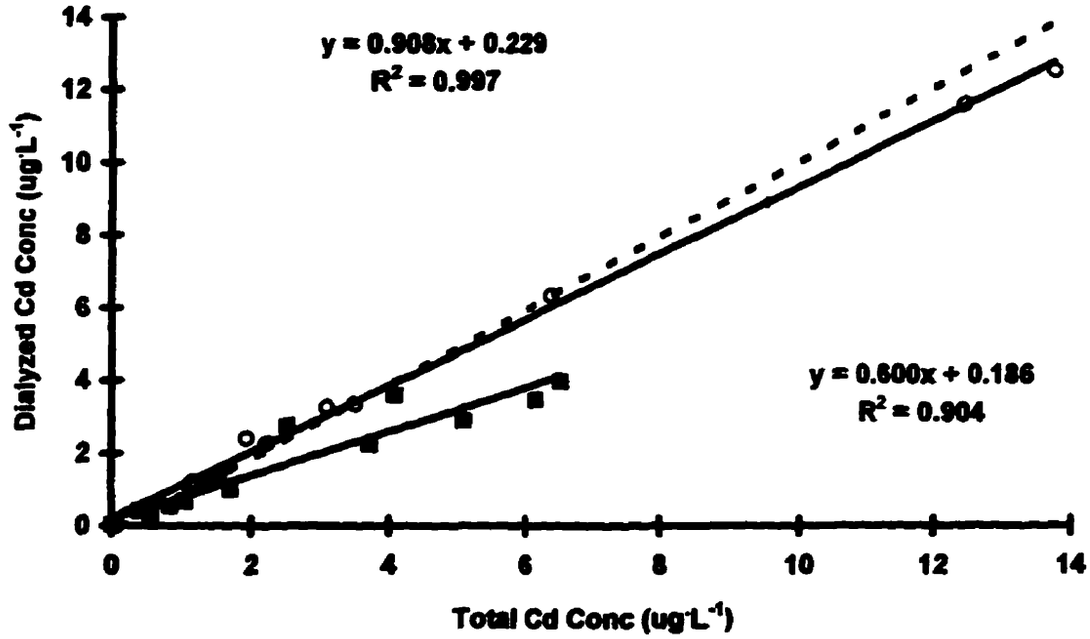


Figure 3.6 Cd Dialysis of Halls and Dickie lake water. (o) denotes Halls Lake (DOC = 2.3 $mg \cdot L^{-1}$); (■) denotes Dickie Lake (DOC = 6.7 $mg \cdot L^{-1}$). Both regression equations are significant ($p<0.001$). The dottted line indicates the 1:1 line.

The relationship between dialysed Cd ($\mu g \cdot L^{-1}$) and total Cd ($\mu g \cdot L^{-1}$) in Halls Lake (DOC, 2.3 $mg \cdot L^{-1}$) and Dickie Lake (DOC, 6.7 $mg \cdot L^{-1}$) were:

Dialysed Cd (Halls) = 0.229 [±0.094] + (0.908 [±0.015] *Total Cd) (10)

($r^2 = 0.99$, $p < 0.001$)

Dialysed Cd (Dickie) = 0.186 [±0.186] + (0.600 [±0.056] * Total Cd) (11)

($r^2 = 0.90$, $p < 0.001$)

Based on the slope of the regression equations, at any given total metal concentration, 72% of the Cu and 91% of the Cd was dialysable in Halls Lake, while only 39% of the Cu and 60% of the Cd was dialysable in Dickie Lake. Hence at any given total Cu or Cd concentration, more Cu or Cd was bound to large MW organic compounds in Dickie Lake than in Halls Lake.

3.4.3 ISE

3.4.3.1 ISE Standard Curves

The Cu ISE exhibited Nernstian behaviour where the slope between mV and total Cu added was within the calculated 29.5 ± 1.5 mV at 25 °C for divalent cations (Buffle 1988a), although the slope was > 30 mV at total Cu concentrations < 10 $\mu g \cdot L^{-1}$ (Figure 3.7). The Cd

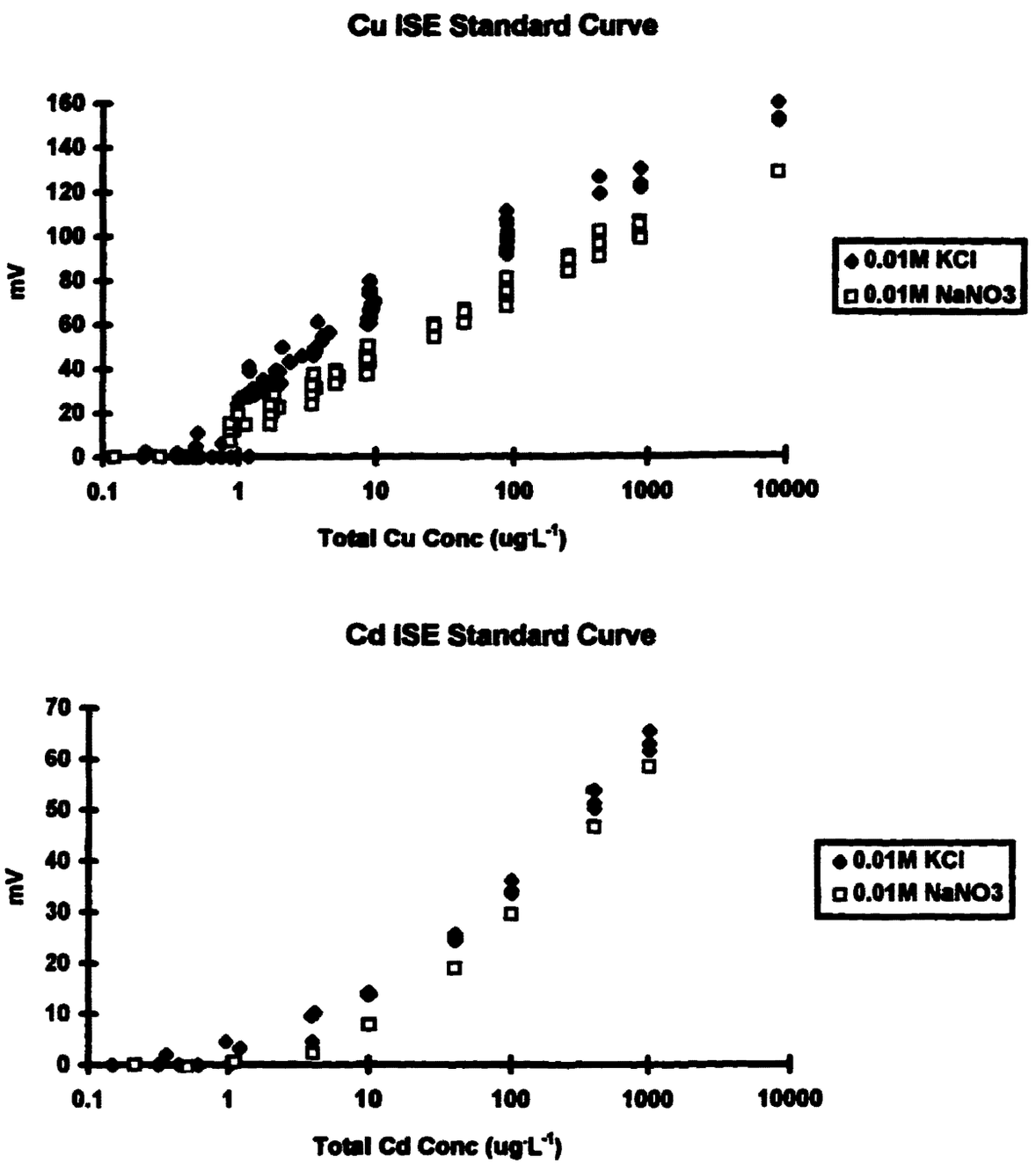


Figure 3.7 Cu and Cd titrations with high purity water and either 0.01 M KCl or 0.01 M $NaNO_3$ added as an ionic strength aduster. The number of separate titrations for the Cu ISE was 10 for 0.01 M $NaNO_3$ and 15 for 0.01 M KCl and with the Cd ISE was 1 for 0.01 M $NaNO_3$ and 4 for 0.01 M KCl.

ISE also exhibited Nernstian behaviour where the slope between mV and total Cd added was within the calculated 27 ± 2 mV at 25 °C (Radiometer Cd ISE Instruction Manual, 1989) for total Cd concentrations greater than 30 $\mu g \cdot L^{-1}$ (Figure 3.7).

Using 0.01M KCl instead of 0.01 M $NaNO_3$ appeared to improve the sensitivity of both the Cu and Cd electrodes (Figure 3.7). Since the mV output was set to 0 at 0 $\mu g \cdot L^{-1}$ nominal Cu or Cd, a shift in the standard curve upwards was interpreted as indicating an increased sensitivity to the metal ion. When the data was replotted with the mV reference point set at 100 $\mu g \cdot L^{-1}$ Cu instead of 0 $\mu g \cdot L^{-1}$ Cu and a constant added to remove the negative signs, a different plot results (Figure 3.8). With this new plot, the increase in sensitivity by switching to KCl is minimized at Cu concentrations > 10 $\mu g \cdot L^{-1}$. In addition, the Cu titration plot clearly shows the non-linear characteristics (and deviations from the Nernst equation) at Cu concentrations below 10 $\mu g \cdot L^{-1}$.

The Cu ISE standard curves are more variable than the Cd ISE standard curves at low total metal concentrations (Figures 3.7 and 3.8). This apparent variation in the mV reading at any given total Cu concentration is due solely to different standard curves; within any single standard curve, the slope was consistent across the mV range.

The shape of the Cu ISE standard curve at low total Cu concentrations when 0.01 M KCl was used is slightly convex (Figure 3.7 and 3.8). At low total metal concentrations ($\mu g \cdot L^{-1}$) and near the detection limit of the electrode, the shape of the standard curve should be

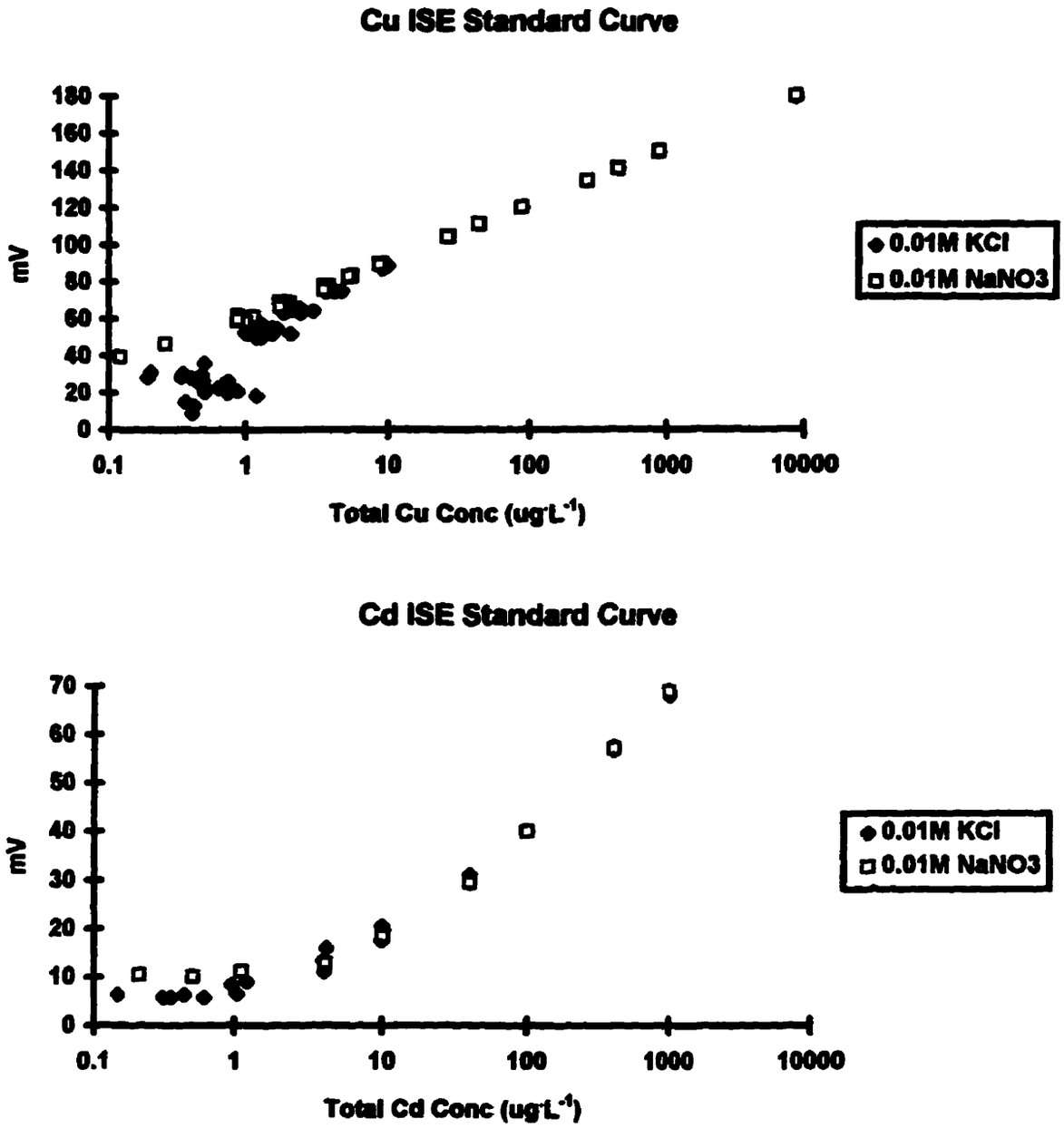


Figure 3.8 Cu and Cd titrations with high purity water calibrated at 100 ug·L^{-1} and either 0.01 M KCl or 0.01 M $NaNO_3$ added as an ionic strength adjuster. The number of titrations for the Cu ISE and the Cd ISE are the same as for Figure 3.7. Calibration curve reference points were set at 120mV at 100 ug·L^{-1} Cu and 40 mV at 100 ug·L^{-1} Cd to remove negative signs.

concave (as seen with the Cd ISE standard curve, Figure 3.7 and 3.8). A concave standard curve indicates that a minimum (threshold) free metal ion concentration must be present for a electrical potential to be recorded and that the change in potential increases as the free metal ion concentration increases. With a convex standard curve, it appears that the potential increases at a constant total Cu concentration; a violation of the Nernst equation (1). These elevated mV responses at low total Cu levels are likely a function of Cl ion interferences with the electrode . The wide variation in the starting values (between 0.2 $\mu g \cdot L^{-1}$ to 1.0 $\mu g \cdot L^{-1}$ Cu) disappear at total Cu concentrations > 10 $\mu g \cdot L^{-1}$, indicating that the electrode is apparently only affected by Cl interference at low total Cu levels. Alternatively, the high Cu results for the initial water samples may be a result of handling or analytical contamination of the sample after it was collected from the bulk solution.

Because of the perceived increased sensitivity at low total Cu concentrations, KCl was used as an ionic strength adjuster instead of $NaNO_3$ for all lake water titrations. At 0.01 M KCl in high purity water at pH 6.3, MINEQL$^+$, a geochemical speciation model (Schecher and McAvoy 1994), predicted that approximately 50% of the Cd, but only 2% of the Cu, ion is complexed with the Cl ion, (Table 3.4). This is in contrast to the models prediction with 0.01 M $NaNO_3$. With $NaNO_3$, only 2.4% of Cd and no Cu was complexed with the NO_3 ion (Table 3.4). This complexation should have resulted in a reduced electrical potential at the electrode surface at any given total Cd concentration due to a reduction in the free Cd ion concentration. However, this was not apparent, as the Cd ISE was slightly more responsive to total Cd when 0.01M KCl was used as opposed to 0.01M $NaNO_3$ (Figure 3.7 and 3.8).

Table 3.4 **Metal speciation in ISE standard curve solutions of high purity water with $8.9 \cdot 10^{-8}$ M Cd and $1.57 \cdot 10^{-7}$ M Cu (both = 10 $\mu g \cdot L^{-1}$), and either 0.01 M KCl or 0.01 M $NaNO_3$ added as an ISA (pH 6.3). Metal speciation was calculated using the geochemical speciation program MINEQL$^+$ (Schecher and McAvoy 1994).**

		Metal Fraction (%)	
Metal Species	Log K	0.01 M KCl	0.01 M $NaNO_3$
$Cd^{(2+)}$	----	50.1	97.5
CdCl	1.98	47.8	----
$CdCl_2$	2.60	2.0	----
$CdNO_3$	0.40	----	2.4
$Cu^{(2+)}$	----	88.5	90.7
CuOH	-8.0	1.8	1.8
$CuOH_2$	-10.4	7.4	7.5
CuCl	0.43	2.4	----

As a result, no correction was made for the Cl ion binding to Cd as the electrode appeared to measure both free Cd ion and the CdCl complexes.

3.4.3.2 Cu ISE

At very low total Cu concentrations (< 2 $\mu g \cdot L^{-1}$), it took up to 2 h for the electrode response to stabilize. The response of the electrode was linear from as low as 0.3 $\mu g \cdot L^{-1}$ and 1.0 $\mu g \cdot L^{-1}$ (depending on the standard curve) to > 1000 $\mu g \cdot L^{-1}$ (Figure 3.7 and 3.8). Placing the electrodes in natural water samples resulted in a decrease in the "zero" mV reading (Figure 3.9). This decrease appears to be due to compounds in the lake water reacting with the electrode, possibly by removing trace Cu or other interfering substances from the electrode surface by complexation or organic carbon molecules binding to the electrode surface and altering the electrode response (Bhat et al. 1981). We measured an increase in the mV reading when Cu was added to these lake water samples but in the negative mV range (Figure 3.9). The free Cu ion concentration was estimated in this negative mV range by extending the standard curve. Extending the linear portion of the standard curve has also been proposed by Erickson et al. (1996) since a linear response has been observed at much lower Cu concentration with metal ion buffers.

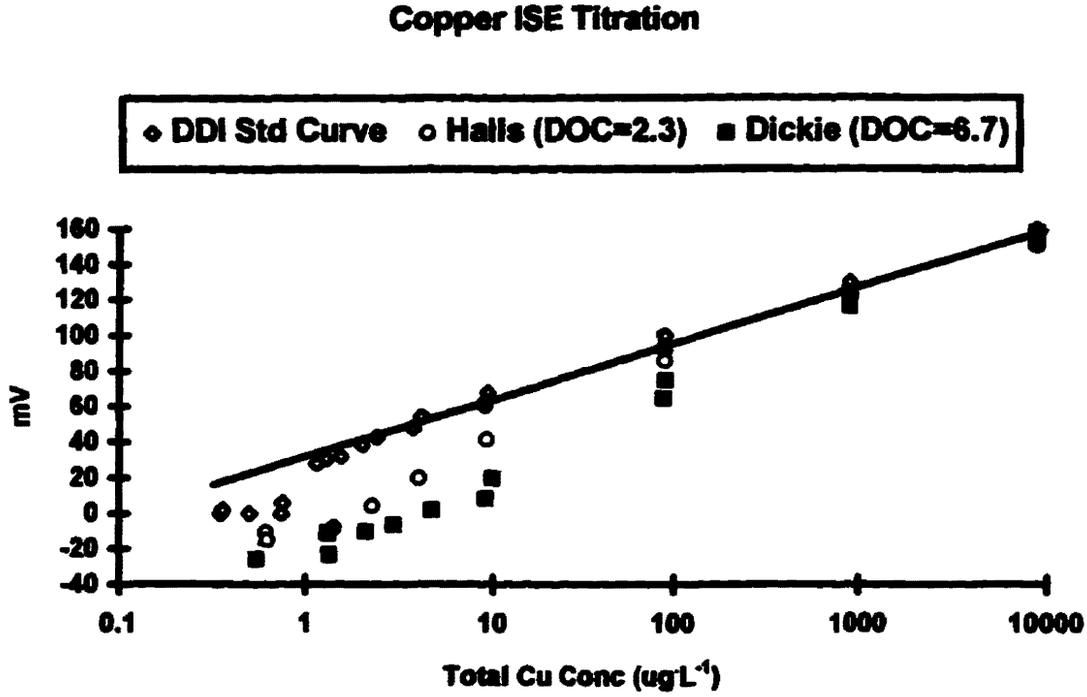


Figure 3.9 Cu ion titrations with Halls Lake and Dickie Lake water (0.01 M KCl). DDI Std Curve denotes standard curves (n = 3) with high purity water determined the same days as Cu titrations in lake water. Dickie Lake data includes data from 2 separate titrations, one with a sample size of 9, the other with a sample size of 6.

Cu ion concentration was calculated from measured mV readings using the regression equation:

$$\text{Log Free Cu} = -0.933\ [\pm 0.062] + (0.031\ [\pm 0.001] \bullet \text{mV})\ (r^2=0.99;\ p<0.001) \quad (12)$$

This regression was calculated from the high purity water standard curves prepared on the same days as the Cu titrations in Dickie and Halls Lake water.

3.4.3.3 Cd ISE

The Cd ISE behaved in a linear fashion down to 30 $\mu g \cdot L^{-1}$ (Figure 3.7 and 3.8). The electrode response was very fast and did not suffer from the slow equilibration times observed with the Cu ISE. Placing the electrodes in natural water samples results in a increase in the "zero" mV reading. The mV reading in Halls Lake water was +32.8 and in Dickie Lake water was +34.6 with no Cd added (Figure 3.10). As with the Cu ion electrode, the Cd ion electrode appears to be reacting with some component of the lake water sample. Instead of a complexation reaction as hypothesized with the Cu electrode where metal ion may be removed from the electrode surface, the increased mV reading appears to indicate that some interfering ion is binding to the electrode surface or that organic carbon molecules are binding to and altering the electrode response. We measured an increase in the mV reading when Cd was added to these lake water samples but at mV readings above the standard curve (Figure 3.10). As a result of this effect, the mV readings for water from

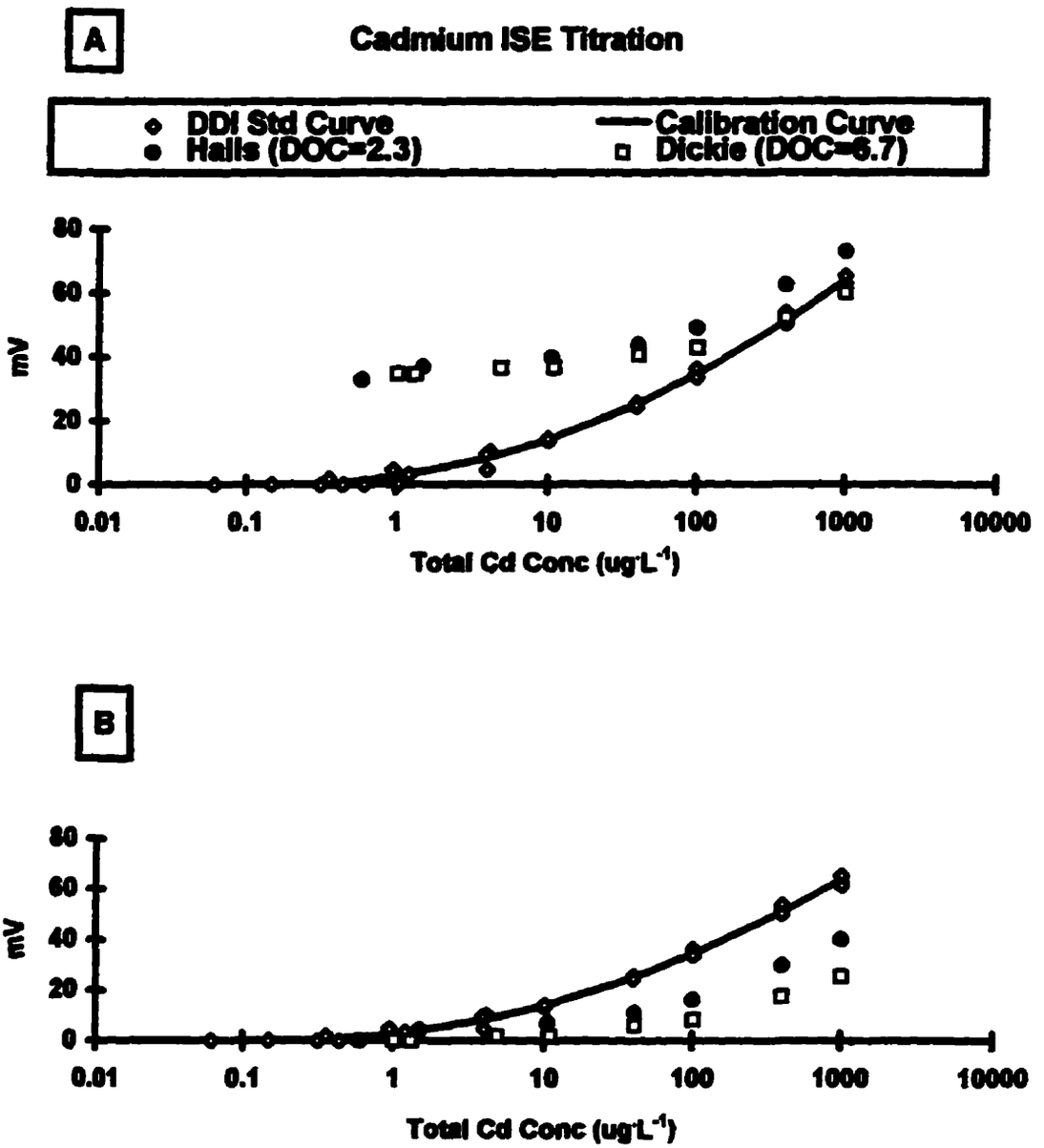


Figure 3.10 Cd ion titrations with Halls Lake and Dickie Lake water (0.01 M KCl). DDI std curve denotes standard curves with high purity water (n=3) determined the same days as Cd titrations in lake water. (A) mV reading in lake water not corrected; mV = 0.0 based on 0 ug·L^{-1} Cd in high purity distilled water. (B) mV reading in lake water tared at 0.0 mV.

Dickie and Halls Lake with no Cd added were tared to 0 mV. This approach of blank subtraction has also been utilized by Buckley et al. (1985).

Free Cd ion concentration was calculated in a similar fashion as free Cu ion concentration, using the regression equation:

$$\text{Log free Cd ion} = -0.299\ [\pm 0.071] + (0.092\ [\pm 0.007] \bullet \text{tared mV}) - (0.0007\ [\pm 0.0001] \bullet \text{tared mV}^2) \qquad (r^2=0.99;\ p<0.001) \qquad (13)$$

This quadratic regression was calculated from the high purity water standard curves prepared on the same days as the Cd titrations in Dickie and Halls Lake water. A linear regression was not used since the linear portion of the Cd standard curve only occurred at Cd concentrations > 30 $\mu g \cdot L^{-1}$.

3.4.4 Metal-DOC binding Characteristics

The average log K' calculated using FITEQL based on free and total metal concentrations for lake water DOC were 7.47 and 7.99 for Cu and 5.31 and 6.04 for Cd in Halls and Dickie lakes, respectively (Table 3.5). The binding capacity for Cu ranged from 0.07 to 0.16 μmoles Cu bound·mg DOC in Dickie and Halls Lake, respectively, and for Cd from 4.7 to 12.7 μmoles Cd bound·mg DOC in Dickie and Halls Lake, respectively (Table 3.5). These average log K' and CC values provide a general approximation of the metal binding

Table 3.5 **Average metal binding characteristics of Dickie and Halls Lake calculated using FITEQL (see text for details).**

Lake	Metal	Conditional Stability Constant (Log K^1)	Binding Capacity (μmole Me mg DOC^{-1})	Total Capacity (μM Me)
Halls Lake	Cd	5.31	12.7	29.2
Dickie Lake	Cd	6.04	4.7	31.5
Halls Lake	Cu	7.47	0.16	0.37
Dickie Lake	Cu	7.99	0.07	0.47

characteristics of the lake water DOC since they combine the effects of all dominant ligands. Due to the matrix effect observed with the Cd ion electrode, the Cd ion estimates may be biased and the resulting metal binding characteristics of the lake water DOC less precise.

It was not possible to fit a 2 ligand model using FITEQL, probably because of the low number of data points required to estimate 4 unknown variables. Scatchard plots were generated using interpolated data from the metal titrations of lake water from both Dickie and Halls Lake. An example of a Scatchard plot identifying the individual linear portions of the curve is provided for Dickie Lake - Cu metal titration (Figure 3.11). The Scatchard plots for lake water titrated with either Cu, and to some extent, Cd were non-linear, indicating at least 2 dominant ligand types in the sample (Figure 3.12 and 3.13). The Cu plots are typical of a 2 ligand model; one ligand with high affinity for the metal ion and low number of binding sites and the other ligand with low affinity and high number of binding sites (MacRae et al. 1995). The Cd plots are not typical of Scatchard plots; a large number of the datapoints have the fraction of bound over free Cd increasing as the bound Cd concentration increased (Figure 3.13). This pattern is not typical and is probably due to the error associated with estimating the free Cd ion at these low total Cd ion concentrations. For both the Halls Lake and Dickie Lake - Cd titration data, only one ligand was estimated using the non-linear regression model. However, 2 ligands were estimated from the Halls Lake - Cd Scatchard plot.

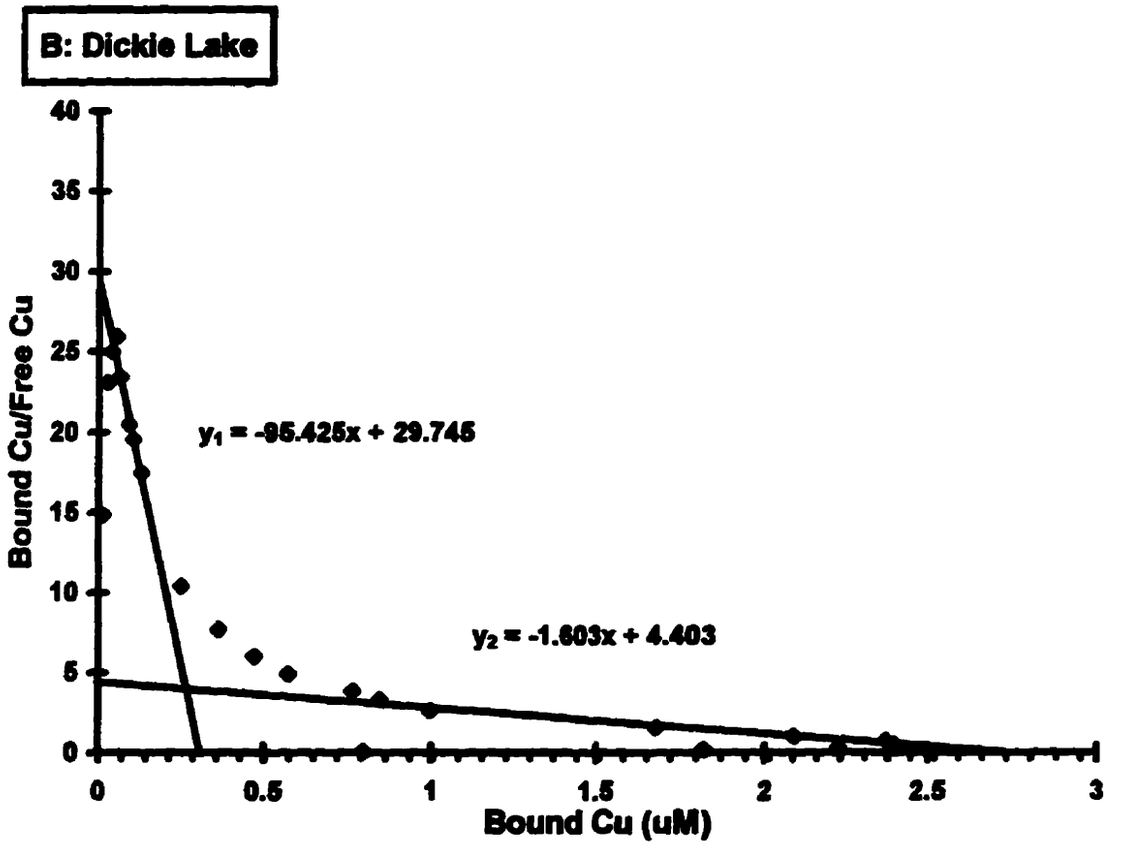


Figure 3.11 Example Scatchard Plot for Cu binding to DOC in Dickie Lake water. Conditional stability constants (K) are equal to the negative slope of the individual linear components multiplied by 10^6 to convert units to M. Complexation capacity (CC) is determined by the x-intercepts of the individual linear components.

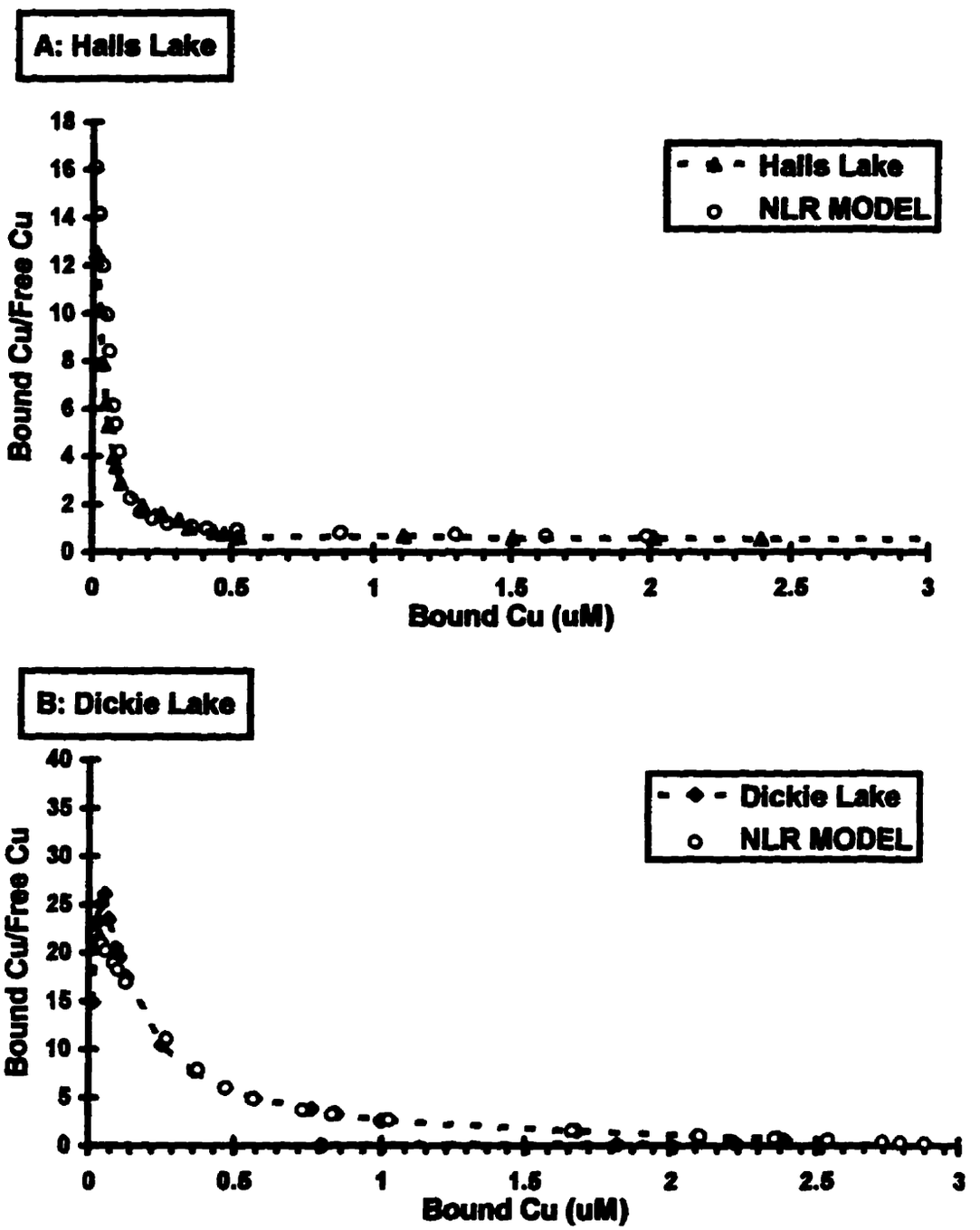


Figure 3.12 Scatchard Plots of Cu binding to DOC in Halls (A) and Dickie (B) lake water. Initial conditional stability constants (K) and complexation capacity (CC) estimated from the linear segments of these plots and used as starting values in nonlinear regression models (NLR). The relationship predicted by the 2-ligand NLR is denoted by open circles (o).

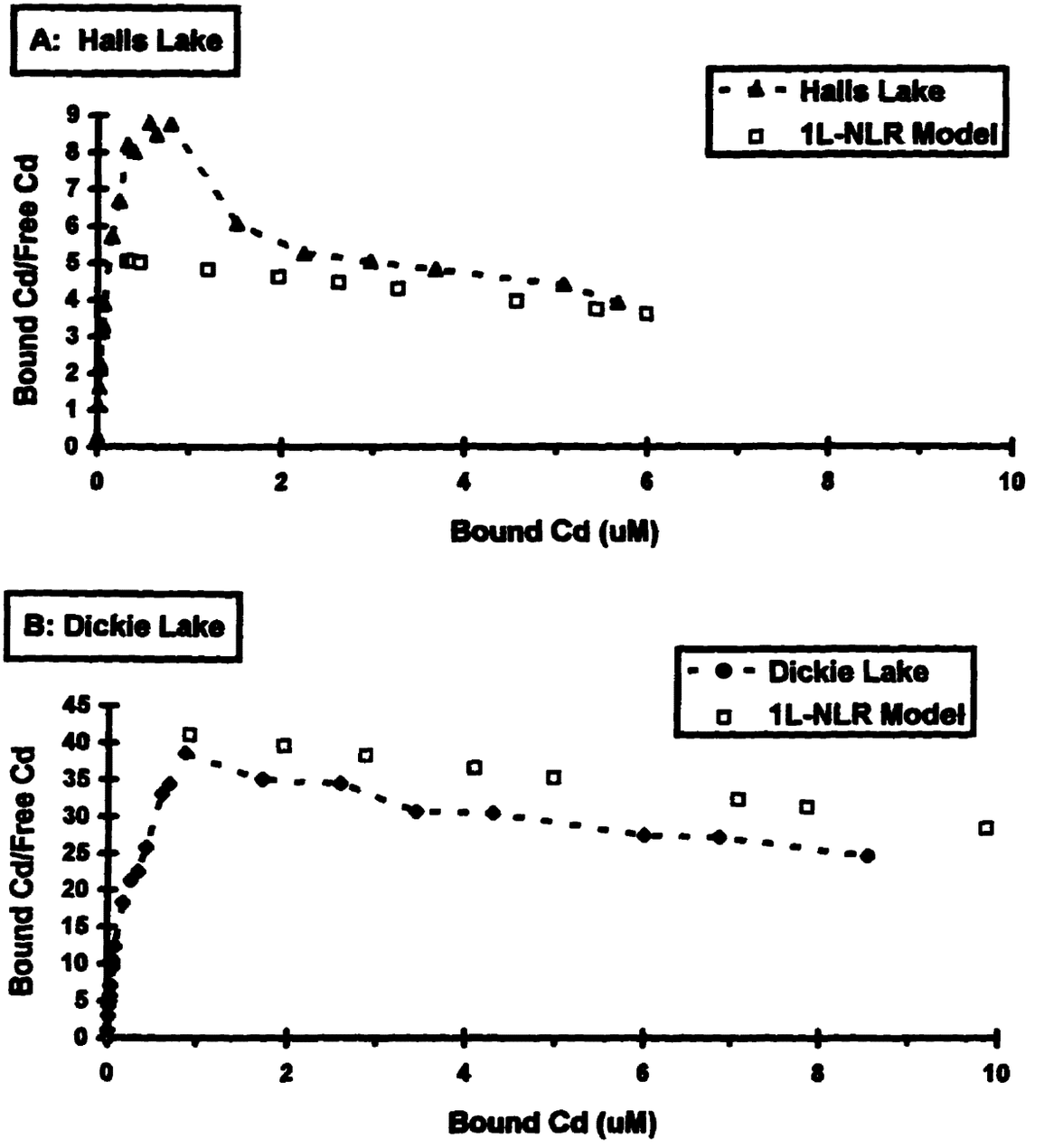


Figure 3.13 Scatchard Plots of Cd binding to DOC in Halls (A) and Dickie (B) lake water. Initial conditional stability constants (K) and complexation capacity (CC) estimated from the linear segment of these plots were used as starting values in nonlinear regression models (NLR). The relationship predicted by the1-ligand NLR model is denoted by open squares (□). The 2-ligand NLR would not converge for Halls Lake or Dickie Lake Cd titration data.

The estimates from the Scatchard plots for log K' and CC for each dominant ligand were used in a 1 or 2 ligand nonlinear regression model. The 2-ligand nonlinear regression model (5) confirmed the estimates of log K_1' , log K_2', and CC_1 and CC_2 for Cu binding characteristics generated from the Scatchard plot (Figure 3.12; Table 3.6). However, only the 1-ligand nonlinear regression model (5) and (6) could predict the binding characteristics for Cd in Halls and Dickie Lake water (Figure 3.13; Table 3.6).

3.5 Discussion

Metal speciation at environmentally realistic total Cu and Cd concentrations was determined in natural lake water samples with minimal metal contamination. The close agreement in metal concentrations between measured and expected standard water samples (Table 3.1) demonstrates the lack of contamination, and high precision and accuracy of the analytical methods used to measure total Cu and Cd.

Lake water was filtered through a 5-μm filter and not through a 0.45 μm filter. As a result, reported metal concentrations do not represent the typical total "dissolved" fraction. Filtering a sample through a 0.45 μm filter can disrupt the equilibrium between the metal ion and ligands (Buffle 1988a) and is arbitrary, since it can include some colloidal materials as well as dissolved (Florence 1982). In addition, the 0.45 μm "dissolved" total metal concentration is specific for the type of filter and technique used (Horowitz et al. 1996). Therefore our total metal estimates include some particulate material (including bacteria), as well as

Table 3.6 Predicted Conditional Stability Constants (log K_i'), Complexation Capacities (CC_i) and Total Binding Capacity in Lake Water containing natural DOC. Log K_i' and CC_i were estimated from comparisons of free and bound metal ions using Scatchard plots, FITEQL and a 1-ligand or 2-ligand least squares nonlinear regression model.

Lake	Metal	Method	Log K_1	CC_1 (μmoles Me·mg DOC^{-1})	Total Capacity (μM Me)	Total Capacity (μg Me·L^{-1})	Log K_2	CC_2 (μmoles Me·mg DOC^{-1})	Total Capacity (μM Me)	Total Capacity (μg Me·L^{-1})
Halls	Cd	S-Plot	5.49	29.6	68.1	7,650	4.63	53.9	124	13,900
		1L-NLR	5.59	8.3	19.1	2,150	----	----	----	----
		FITEQL	5.31	12.7	29.2	3,280	----	----	----	----
Dickie	Cd	S-Plot	6.23	3.3	22.1	2,490	----	----	----	----
		1L-NLR	6.33	4.5	30.2	3,390	----	----	----	----
		FITEQL	6.04	4.7	31.5	3,540	----	----	----	----
Halls	Cu	S-Plot	8.08	0.04	0.09	5.85	5.05	2.87	6.6	419
		2L-NLR	8.23	0.04	0.09	5.85	4.89	4.35	10.0	636
		FITEQL	7.47	0.16	0.37	23.4	----	----	----	----
Dickie	Cu	S-Plot	7.98	0.05	0.34	21.3	6.20	0.36	2.41	153
		2L-NLR	7.93	0.06	0.40	25.6	6.11	0.41	2.75	175
		FITEQL	7.99	0.07	0.47	29.8	----	----	----	----

colloidal and dissolved metal species (Buffle 1988a). Dialysis provided an estimate of the "dissolved fraction" based on mobility through a 1000 MWCO membrane, approximately equal to 0.001 to 0.01 μm (Buffle 1988a).

3.5.1 Dialysis

The dialysis fraction estimates the combined concentration of free metal ion, inorganic metal species and low molecular weight organic metal species. KCl was added as an ionic strength adjustor to maximize the pore size of the dialysis membrane, and minimize any Donnan effects (Backes and Tipping 1987). Donnan effects occur when the ion of interest fails to reach equilibrium because of an electrostatic effect of unequal ion distribution. Dialysis membrane pore size was an important factor in time to equilibrium. The 500 MWCO membranes appeared to have an effective pore size that is too small to allow equilibrium to be reached even after 96 h. The addition of 0.01 M KCl had a minor effect on Cu speciation but at that low concentration, the changes in Cu speciation were minimized (Backes and Tipping 1987). From the MINEQL+ modelling results, the addition of 0.01 M KCl resulted in CdCl complexes but not significant CuCl complexes (Table 3.4). These CdCl complexes will not influence the Cd estimate from the dialysis experiments since CdCl complexes would be membrane permeable. In addition, the CdCl complexes did not appear to bias the free Cd ion estimates (see below).

3.5.2 Metal ISEs

Metal ISEs are specific for free metal ion activity but also measure possibly interfering ions. Buffle (1988a) recommends a mV meter sensitive to 0.1 mV to reduce errors in free metal ion estimates and recording the mV potential based on a potential-time curve where the mV drift doesn't change appreciably (< 0.02 $mV \cdot min^{-1}$). These conditions were met in this study; the mV meter was sensitive to 0.1 mV and a mV reading was recorded after an asymptote was reached.

3.5.2.1 Cu ISE

By controlling the temperature, pH, ionic strength, electrical interferences and light intensity, problems with poor reproducibility and unstable potentials with the Cu ISE were reduced. The long equilibration time at low total Cu concentrations is troublesome. Shifts in the baseline over time may occur while waiting for a stable reading in the response-time curve. Daly et al. (1990) observed that 85% of the complexation of Cu occurred within the first 20 minutes at low $\mu g \cdot L^{-1}$ concentrations but was not complete even after an additional 8 hours. If the electrode response time is the same for this study as they observed, then the measured free Cu ion concentration will be slightly lower than the actual free ion concentration. Since the same criteria were used to determine when a stable mV reading was recorded, this bias should be consistent in all the samples.

The Cu ISE is sensitive to interfering substances at low total Cu levels. The decrease in mV readings when the electrode was placed in natural lake water (Figure 3.9) may be due to a complexation reaction between the lake water DOC and interfering substances affecting the electrode response (e.g., electrode-bound Ag). Interfering substances may bind to the Cu ISE and increase the electrode mV response (i.e., resulting in a higher baseline mV reading). Alternatively, the DOC in solution could influence the baseline mV reading by binding directly to the electrode (Daly et al. 1990).

3.5.2.2 Cd ISE

Unlike the Cu ion electrode, equilibrium times between the Cd ion electrode and the sample were rapid and appeared stable. The matrix effect when the electrode was placed in natural lake water resulted in increased baseline mV readings. The usefulness of the Cd ion estimates are suspect, since the mV output was "tared" to 0 mV after the electrode was calibrated in high purity distilled water. In addition, the metal titration curves were not typical of titration plots. The mV response in the lake water (an estimate of the free metal ion) should approach the mV response in the standard curve (as seen with the Cu titration plots) as the concentration of free metal ion predominates in solution. The lack of a typical binding curve in the lake water titrations may be due to the low total Cd concentrations in relation to the sensitivity of the electrode or the bias as a result of the lake water matrix effect. Laegreid et al. (1983) also had problems with the Cd ion electrode, where it gave good results for one lake but not for another.

In addition to the lake water matrix effect, the Cd ion speciation was predicted to be changed by addition of 0.01 M KCl (MINEQL+ predicted 50% CdCl complexes in distilled water at pH 6.3). No apparent effect of this binding was detected with the Cd titration plots. The standard curve with both KCl or $NaNO_3$ had similar mV outputs at the same total Cd ion concentration. Apparently, the Cd ion electrode can measure CdCl complexes in addition to Cd ion.

3.5.3 DOC and DOC-metal interactions

Fulvic acids typical of aquatic environments have a molecular weight (MW) between 500 and 2,000 (Suwanne river source: Cabaniss and Shuman 1988a) but may contain a significant portion (approximately 10%) of 5,000 to 10,000 MW molecules derived from soil fulvic acids (Beckett et al. 1987). These relatively few large MW fulvic acids may be very important with respect to metal binding (Bartschat et al 1992), especially if they represent ligands with high affinity for metal ions. Evans et al. (1989) determined the average MW of DOC in freshwater Ontario lakes (between 1,200 and 2,200) to be primarily a function of DOC concentration and secondarily a function of the ratio of catchment area to lake area. Using their model, the average MW was calculated to be 1,407 and 1,565 in Halls and Dickie lakes, respectively (Table 3.7). In addition, the percent of DOC that is made up of fulvic acids, as determined by the absorbance at 250 nm, is less in Halls Lake (42%) than in Dickie Lake (73%) (Table 3.3). Therefore, the DOC in Dickie Lake appears to be made up of larger MW organic components dominated more by fulvic acids than the

Table 3.7 **Morphometric characteristics of study lakes. Watershed Area (Ad) and Lake Area (Ao) for Halls Lake and Dickie Lake from Reid et al. (1987) and Dillon and Rigler (1974), respectively. Mean molecular weight of lake DOC determined by the formula: mean DOC MW = 1245 + (50.7 * DOC) + (5.88 * Ad/Ao) (Evans et al. 1989).**

Lake	Watershed Area (Ad) (km^2)	Lake Area (Ao) (km^2)	Ratio (Ad/Ao)	Mean DOC ($mg \cdot L^{-1}$)	Mean DOC (MW)
Halls	270	5.32	50.8	2.3	1407
Dickie	5	0.94	5.3	6.7	1565

constituents of the Halls Lake DOC. Hence, the nature of the DOC pool in the two lakes is different based on size and nature of the constituent organic molecules. The higher MW DOC in Dickie lake may be a result of increased input of allochthonous organic matter (Evans et al. 1989).

Metal ions bind preferentially to high affinity binding sites before binding to lower affinity binding sites. At low metal concentrations, there are many metal binding sites available on organic molecules and as these sites become saturated, less and less of the free metal will bind because of steric hindrance, charge repulsion or lower negative charge of the organic molecule (Cabaniss and Shumann 1988b). Since toxicity occurs at low total metal concentrations, the total available metal binding sites are probably not saturated. However, the majority of bound metal ions will be bound to high affinity ligands and depending on the total metal concentration, these ligands can become saturated. For instance, the total binding capacity of the high affinity ligands for Cu based on the 2 ligand non-linear regression was 0.09 μM Cu (5.85 $\mu g \cdot L^{-1}$ Cu) in Halls Lake water and 0.40 μM Cu (25.6 $\mu g \cdot L^{-1}$ Cu) in Dickie Lake water (Table 3.6).

3.5.3.1 Geochemical Models

The use of geochemical models of humic and fulvic acids to predict the effect of natural organic substances on trace metal speciation and toxicity is currently limited (Bartschat et al. 1992; Cabaniss and Shuman 1988b). "The structure and chemistry of these

macromolecules (humic substances) are complex such that it is almost impossible to characterize a complete molecule. As a result, thermodynamic data on these molecules are unavailable for modelling" (Bassett and Melchior 1990). However, because of the importance of organic substances on trace metal binding and speciation, these substances must be incorporated into geochemical speciation models for accurate and meaningful predictions of trace metal speciation in natural waters (Bassett and Melchior 1990). Allison and Perdue (1994) in the geochemical speciation program MINTEQA2 have successfully modeled the binding of metal ions to carboxylic acid sites on natural humic substances but have been unable to model the phenolic sites. As a result, site specific information on the binding to the DOC with the metal of interest, as well as other dominant, possibly competing ions (H, Ca, Mg, etc.), need to be determined before the metal binding characteristics of natural DOC can be included in the geochemical speciation models. In this study, only the metal binding characteristics of Cu and Cd to the lake water DOC have been determined.

Bresnahan et al (1978) recommend that metal-humic interactions in natural waters be measured by modelling the natural mixture of humic material with several individual organic acids. The humic material must be characterized based on functional (acid base solubilities) or physical characteristics (number of carboxylic and phenol binding sites), and suitable organic acid analogs selected. This surrogate organic acid mixture containing individual organic molecules (each having a known number of binding sites and defined stability constants) can be used in geochemical speciation programs in place of the

chemically complex humic acid mixture. Sposito (1981) used a 9 organic-acid model (arginine, lysine, ornithine, valine, citric, maleic, phthalic, benzenesulfonic and salicylic acids) to mimic sewage sludge fulvic acid. Campbell and Stokes (1985) tested natural soft water shield lakes at pH levels between 4 and 7 with this organic ligand model (minus benzenesulfonic acid) and found that only Cu and Al (out of 10 metals tested including Cd) chemically interacted with the 8-ligand DOC surrogate. Recently, this approach has been used with some success to characterize DOC in a soft water creek. Three organic acids, (malonic, oxalic and dipicolinic acids) effectively mimicked the metal binding characteristics of DOC in Panther Creek in Idaho, USA (MacRae et al. 1995). These acids were then used in toxicity experiments to determine the effect of this site specific DOC analog on the speciation and toxicity of Cu to trout (Marr et al. 1995).

3.5.3.2 Cu Stability Constants

Due to the shape of the Cu Scatchard plots (Figure 3.12), the Cu binding characteristics of the DOC was modelled with a 2 ligand model. These estimates (Table 3.6) are in agreement with published literature values for lake water DOC (McKnight et al. 1983) . Daly et al. (1990) examined natural waters at DOC concentrations of approximately 8 and 13 $mg \cdot L^{-1}$ using ISE and ASV (anodic stripping voltametry) and calculated mean log K'_i in soft water

(hardness = 16 $mg \cdot L^{-1}$ as $CaCO_3$, pH = 7, I = 0.01 M) of 7.7 and 5.6 . MacRae et al. (1995) had similar values (log K_1 = 7.26, CC_1 = 0.21 $\mu mole \cdot mg$ DOC; log K_2 = 5.13, CC_2 = 2.67 $\mu mole \cdot mg$ DOC) in moderately soft water (hardness 25 $mg \cdot L^{-1}$ as $CaCO_3$, pH 7.5, I = 0.01 M).

Vanden Berg and Kramer (1979) determined an average log K' (I = 0.01 M) and CC for Dickie Lake using an ion exchange technique with MnO_2 and differential pulse ASV (log K' = 7.8, CC= 2.19 μM Cu total DOC). This compares favourably to our estimate of the average log K' for Cu in Dickie Lake of 7.99 but is higher than our average CC estimate of 0.47 μM total DOC (0.07 $\mu M \cdot mg$ DOC) (Table 3.5).

3.5.3.3 Cd Stability Constants

Based on the shape of the Scatchard plots (Figure 3.11), the Cd binding characteristics of the DOC was modelled with a 1-ligand model for Dickie and Halls Lake. The increasing bound-Cd to free-Cd ratio at low bound-Cd concentrations was an artifact probably due to errors in predicting the free Cd ion concentration at low total Cd concentration. Even though the Cd titration plots were influenced by apparent matrix effects and the Scatchard plots are not typical, the estimated conditional stability constants for Cd ion complexation with DOC are still consistent with expected comparisons between Cu and Cd conditional stability constants. Saar and Weber (1982) noted that the conditional stability constants of Cd-fulvic acid complexes (3.6 to 4.1) are about 100 times lower than the conditional stability

constants of Cu-fulvic acid complexes. At pH 6, conditional stability constant for Cd-fulvic acid complexes were 3.6 to 4.1 compared with conditional stability constants for Cu-fulvic acid complexes of 6.0 (Saar and Weber 1982). Our estimates for both Cd and Cu are 2 orders of magnitude higher than the estimates of Saar and Weber (1982). The estimates for Cd binding capacity are very large and much higher than the estimates for Cu binding capacity (Table 3.6). Since our Cu binding capacity estimates are in agreement with other studies, this excessively large Cd binding capacity is probably erroneous and due to errors in measuring the free Cd ion.

3.5.4 Conclusions

In this study, Cu and Cd speciation was determined in natural lake water (spiked with metal) by both dialysis and metal ion specific electrodes. This metal speciation scheme determines the metal ion fraction bound to membrane permeable inorganic and low MW organic ligands (via size exclusion equilibrium dialysis) and the free metal ion activity directly. From these measurements, the metal fraction associated with large MW organic ligands (total metal concentration minus the dialysis fraction) can be calculated.

The relative contribution of each of these metal fractions to the total metal concentration are shown in the two lake waters for comparison (Table 3.8). The dialysis fraction represents the combined concentrations of free metal ion, as well as membrane permeable (1000 MWCO) inorganic and low molecular weight organic metal species; 72% and 39% of Cu

Table 3.8 Cu and Cd speciation in Halls and Dickie water: (a) concentration of metal associated with large MW organic ligands determined from total metal concentration minus metal concentration in the dialysis fraction; (b) concentration of metal in the dialysis fraction based on the regression equations (8) to (11) (Figure 3.5-3.6); (c) free metal ion concentration (corrected for ionic strength) determined by visually reading the mV value at the total metal concentration from the metal titration plots with lake water (Figure 3.9 and 3.10) and calculating the free metal ion from equations (12) and (13).

Metal	Water	Metal Concentration ($\mu g \cdot L^{-1}$)			
		Total Metal	Metal bound to Large MW Organic Ligands[a]	Dialysis Fraction[b]	Free Metal Ion
Cu	Halls	1	0.3	0.7	----
		10	2.9	7.1	3.1
		100	28.6	71.4	75
	Dickie	1	0.6	0.4	----
		10	6.1	3.9	0.7
		100	60.7	39.3	34
Cd	Halls	1	0.1	0.9	0.8
		10	0.9	9.1	3.1
		100	9.0	91.0	20
	Dickie	1	0.4	0.6	0.7
		10	4.0	6.0	1.0
		100	39.8	60.2	3.7

and 91% and 60% of Cd was membrane permeable in Halls Lake (DOC = 2.3 mg L^{-1}) and Dickie Lake (DOC = 6.7 mg L^{-1}) water, respectively. Hence, more Cu and Cd was bound to large MW organic compounds (>1000 MWCO) in Dickie Lake than in Halls Lake water. In addition, as predicted from metal-organic binding characteristics, more Cu was bound to large MW organic compounds (>1000 MWCO) than Cd in both lake waters.

The metal ion specific electrodes measure the free metal ion activity. More free Cu and Cd ion were present in Halls Lake than in Dickie Lake water. Comparisons between the amount of free Cu verses free Cd concentrations at a given DOC concentration within a lake are inappropriate since the free Cd ion estimates were less precise than free Cu ion estimates. In addition, the free Cd ion estimates were biased due to confounding effects of interfering substances. However, comparisons of free Cd ion between lake waters are appropriate as the relative bias in the Cd ion estimates are similar between the two lake waters tested.

Cu and Cd binding characteristics of the lake water DOC was determined by metal ion titrations. The average conditional stability constants calculated using the metal speciation model FITEQL, for lake water DOC were 7.47 and 7.99 for Cu and 5.31 and 6.04 for Cd in Halls Lake and Dickie Lake water, respectively. Total metal binding capacity was higher in Dickie Lake than in Halls Lake water for both Cu and Cd. Based on Scatchard analysis, two dominant ligands were present in the lake water for Cu and one or two dominant ligands

were present for Cd. Very large complexation capacity estimates for Cd (3.3 to 29.6 μmole Cd·mg DOC^{-1}), but not for Cu (0.04 to 0.16 μmole Cu·mg DOC^{-1}), are indicative of biased free Cd ion estimates.

Armed with this site-specific metal speciation data, the following chapter examines the chronic toxicity of Cu and Cd to larval fathead minnow and correlates the metal speciation with the toxicity observed.

4.0 Influence of dissolved organic carbon on the chronic toxicity of Cu and Cd to larval fathead minnow (*Pimephales promelas*) in soft water.

4.1 Abstract

Larval fathead minnow (*Pimephales promelas*) were exposed for 21 days to low concentrations of Cu and Cd (<32 $\mu g \cdot L^{-1}$) in water collected from two soft water lakes, Halls Lake and Dickie Lake, at pH 6.3. These lakes are typical soft water lakes (low calcium, alkalinity and conductivity) but have different DOC concentrations: mean lake water DOC concentration was 2.4 $mg \cdot L^{-1}$ in Halls Lake and 7.2 $mg \cdot L^{-1}$ in Dickie Lake. Both Cu and Cd are extremely toxic to larval fathead minnow. Based on total metal concentrations, Cu toxicity was higher in water collected from Halls Lake than water collected from Dickie Lake. Incipient lethal levels for Cu exposure ranged from 6.2 $\mu g \cdot L^{-1}$ in Halls Lake to 17.2 $\mu g \cdot L^{-1}$ in Dickie Lake. The lowest observed effect concentrations (LOEC) for total length in fish exposed to Cu in Halls Lake was 5.3 $\mu g \cdot L^{-1}$ and the LOEC for total length and dry weight in fish exposed to Cu in Dickie Lake was 16.1 $\mu g \cdot L^{-1}$. The LOEC for increased whole body Cu concentration in Cu exposed fish ranged from 2.5 $\mu g \cdot L^{-1}$ in Halls Lake to 3.6 $\mu g \cdot L^{-1}$ in Dickie Lake. Hence, fish had reduced growth in Halls Lake water at total Cu concentrations 3 times lower than in Dickie Lake water, but had increased Cu tissue concentrations in Halls Lake water at total Cu concentrations only 1.5 times lower than in Dickie Lake water.

Based on lethality, Cd was more toxic in Halls Lake than in Dickie Lake. Incipient lethal levels after Cd exposure ranged from 1.2 $\mu g \cdot L^{-1}$ in Halls Lake water to 2.0 $\mu g \cdot L^{-1}$ in Dickie Lake water. However, sub-lethal indicators of Cd toxicity were similar between the two exposure waters. LOECs for total length and dry weight could not be statistically determined for fish exposed to Cd in water from Halls Lake. However, the LOEC for total fish length from Halls Lake water was estimated to be approximately 2.9 $\mu g \cdot L^{-1}$. LOECs for total length and dry weight for fish exposed to Cd in Dickie Lake water were 2.9 $\mu g \cdot L^{-1}$. In addition, LOECs for increased whole body Cd concentration in fish were 0.5 $\mu g \cdot L^{-1}$ in Halls Lake water and in Dickie Lake water; both at the lowest Cd exposure concentrations used in the experiments.

Cu and Cd speciation was measured in the exposure water from both Halls and Dickie Lake (see chapter 3). Differences in the observed toxic effects between the 2 lakes when expressed as total Cu were reduced when expressed as the dialysis fraction. When effects from the Cu exposure were expressed as a function of the free Cu ion, the toxicity was roughly similar between the two lakes. The opposite result was observed for Cd. Differences in the observed toxic effects between the 2 lakes were reduced when expressed as total Cd. When expressed as the dialysis fraction, the effects of Cd exposure were more pronounced in Dickie Lake than in Halls Lake. Free Cd ion estimates also indicated that the effects of Cd exposure were more pronounced in Dickie Lake water.

These results suggest that the free ion activity model (FIAM) of organism-metal interaction may not be applicable to all divalent metals. One central tenet of the FIAM is that the free metal ion activity is proportional to the observed toxicity. With natural DOC, the model was validated for Cu (a metal that forms strong covalent bonds with organic ligands) but results were ambiguous for Cd (a metal that forms weak electrostatic bonds with organic ligands); free Cd ion estimates indicate that Cd ion may be more toxic in high DOC water (Dickie Lake water) than in low DOC water (Halls Lake water). Because of uncertainty in the free Cd ion estimates, it was not possible to rigorously test the FIAM with respect to Cd toxicity (i.e., is the observed toxicity a function of the free Cd ion concentration or not). However, it is clear that Cd toxicity is not due to the free Cd ion concentration alone, since based on dialysis results, some Cd is bound to large MW complexes (>1000 MWCO) in Dickie Lake water and yet observed sub-lethal toxic effects are similar between the two lakes based on total Cd exposure.

Metal binding characteristics of DOC ligands and fish gill ligands, as well as competition between them for metal ions, appear to explain this discrepancy. The high affinity binding sites on the fish gill outcompete the low affinity DOC ligands for metal ions. A steady state or equilibrium condition, between the metal ions bound to the low affinity DOC ligands and the high affinity fish gill ligands occurs, apparently only in the vicinity of the fish gill, creating a "depletion" zone. Measuring or predicting the metal speciation based solely on the chemical characteristics of the water, without the organism present, may be flawed

since, at the site of metal uptake, the organism may change the quantity and quality of metal ligands and affect metal speciation.

4.2 Introduction

Numerous studies, mostly on Cu, Cd and Zn, have demonstrated that organic compounds and soil derived humic acids reduce the toxicity of metals to aquatic biota (Borgmann and Ralph 1984; Poldoski 1979; Stackhouse and Benson 1988; Winner 1986) and that the toxicity observed appears to be proportional to the free metal ion concentration (Allen et al. 1980). Examining the impact of isolated organic compounds on metal toxicity removes the variability associated with natural DOC. It is difficult to extrapolate the results of these studies to the aquatic environment since both isolated organic compounds and soil derived humic acids are poor surrogates for natural DOC. Natural aquatic DOC is composed of a population of organic compounds, each with different metal binding capacities and metal ligand binding site densities. In Precambrain Shield lakes, DOC is dominated by low molecular weight (1,200 - 2,200 MW) fulvic acids (Evans 1989), compounds that are much smaller that the high molecular weight (up to 300,000 MW) soil derived humic acids (Marley et al. 1992).

The free ion activity model (FIAM) of metal-organism interaction predicts that the effect of metal exposure on organisms is a function of the free ion activity, a measure of the reactive

free metal ion concentration (Morel 1983). The model is based largely on experiments with synthetic organic ligands and has not been adequately tested with naturally occurring DOC (Campbell 1995).

The bioavailability of metal bound to organic ligands is unclear. Although some Cu-organic ligand complexes (Borgmann and Charlton 1984; Winner 1985) and Cd-organic ligand complexes (Giesy et al. 1977; Poldoski 1979; Winner 1986) appear to contribute to toxicity in some organisms, these metal complexes are usually hydrophobic (Campbell 1995) and rapidly diffuse through biological membranes (Florence 1986).

From the acute experiments described in Chapter 2, it is apparent that the acute toxicity of Cu, but not of Cd, to larval fathead minnow and *Hyalella azteca* in soft water is modified by organic complexation. Chemical characterization of the metal speciation in two soft water lakes (Halls and Dickie Lake), similar to the water used in the acute experiments, indicate that the free Cu and Cd ion, and membrane permeable (1000 MWCO) inorganic and low molecular weight organic Cu and Cd species are reduced in the presence of DOC (Chapter 3). Organic complexation was greater for Cu than for Cd, as indicated from less Cu being associated with the free ion or membrane permeable metal fraction than Cd (Table 3.8).

According to the FIAM and from the predicted metal speciation, a reduction in the acute Cd toxicity would have been expected in the acute Cd exposure experiments. Since the acute

toxicity experiments were static, the free metal ion concentration in the exposure beakers may have changed over the course of the experiment and influenced the toxicity observed.

This study was designed, in part, to test the FIAM of metal-organism interaction in soft water with naturally occurring DOC. The chronic toxicity of Cu and Cd to *P. promelas* in water from two soft water lakes, Halls Lake (DOC = 2.3 $mg \cdot L^{-1}$) and Dickie Lake (DOC = 6.7 $mg \cdot L^{-1}$), was examined over 21-d under continuous flow conditions. Toxicity was interpreted in relation to the measured concentrations of the metal species in the lake water. Evaluation of the FIAM under realistic conditions provides direction on the use of total or free metal ion concentration for both environmental effects monitoring and the development of more realistic water quality objectives for soft water lakes.

4.3 Materials and Methods

Larval fathead minnows (*P. promelas*), were exposed to low concentrations (< 32 $\mu g \cdot L^{-1}$) of Cu or Cd over 21 days. Fish were exposed to both metals separately in water collected from Dickie Lake (DOC = 6.7 $mg \cdot L^{-1}$) and Halls Lake (DOC = 2.3 $mg \cdot L^{-1}$). Fish were exposed in duplicate to 6 Cu or Cd concentrations plus a control. Toxicity was characterized in terms of total and bioavailable metal species and was based on lethal and sublethal biological responses. Whole body tissue metal concentrations were also determined for individual fish to compare tissue residues with lethal and sub-lethal effects and as a biological measure of metal bioavailability.

Metal toxicity experiments with larval fathead minnow were conducted at low total Cu and Cd concentrations (0.05 to 30 $\mu g \cdot L^{-1}$) in water collected from two soft water lakes. In order to minimize metal contamination, the experiments were carried out in a class-100 clean room facility located at the Dorset Research Centre, Ontario Ministry of Environment and Energy. All exposure tanks and plumbing were made out of plastic (polypropylene or polyethylene) and the use of metal parts was kept to a minimum. Access to the clean laboratory was restricted and proper clean room attire was worn by personnel at all times. Air was filtered through 6 high efficiency particle filters (HEPA), which removed the bulk of the particulate matter from the air. Independent tests confirmed that the air had less than 10 particles > 5 μm per cfm and less than 500 particles > 0.5 μm per cfm (H.E.P.A. Filter Services Inc, Concord, Ontario).

All labware (tanks, buckets, pipettes, etc.) were cleaned prior to use by soaking in 1% contrad-70 soap solution (an anionic detergent) for a minimum of 24 h, rinsing with high purity water and soaking in either a 1% sulphuric acid bath (routinely used for all labware) or in a 5 or 10% nitric acid bath (used for vials associated with tissue digestion). After the acid soak, labware was rinsed at least 7 times with high purity water and stored dry. Large holding and transport tanks, and exposure tanks were cleaned in a similar fashion except that they were rinsed with sand-filtered St. Mary's Lake water or with 5 μm filtered Halls or Dickie lake test water. Soap and acid solutions were also pumped through all PVC and tygon tubing that came in contact with the test water. Viton tubing, used to deliver metal stock solution to exposure tanks, was cleaned by pumping 5% nitric acid through the tubing

for a minimum of 4 h and rinsed by pumping test water through the tubing for a minimum of 24 h. Nytex mesh cages, used to contain fish during the experiment, were also cleaned with soap and acid as noted above. Due to problems removing the acid from the mesh with high purity water, the acid was removed by rinsing and soaking the cages in batches of 50-L sand-filtered St. Marys Lake water. This rinse and soak procedure was repeated 3 times and the cages were stored dry until needed.

4.3.1 Site Identification and Water Collection

A database of 777 soft water lakes on the Precambrian Shield in the Muskoka/Haliburton region was examined to identify 2 lakes that were within 1 h of travel time from the laboratory and had approximately 3 $mg \cdot L^{-1}$ Ca, pH 6.3, and DOC concentrations of either 2 or 6 $mg \cdot L^{-1}$, the 25^{th} the 75^{th} quartiles for the range of DOC in Ontario soft water lakes (Neary et al. 1990). Halls Lake and Dickie Lake, which met the criteria, were selected to supply the experimental water. Water was collected from the littoral zone of Halls Lake and Dickie Lake (15 m offshore and 0.5 m deep) during the ice-free season of 1994 and the spring of 1995. The water was pumped into 2 polyethylene tanks (total capacity 2200 L) with a metal free pump at 22 $L \cdot min^{-1}$. Lake water was sampled immediately prior to entering these tanks on approximately every third sampling trip for chemical characterization. Minimal manipulations were done to this water. The water was transported back to the laboratory within 1 hour of collection and transferred into the first of two 4500 L polyethylene tanks located adjacent to the clean laboratory. The water was filtered through a

20-μm particle filter before being pumped into the first 4500 L holding tank. The water was further filtered through a 5 μm particle filter before being pumped into the second 4500 L holding tank. The water in these storage tanks was continuously filtered through the 20 or 5 μm filters and aerated by the return water splashing back into the tank. Water was progressively transferred from holding tank 1 to holding tank 2 as the water was drawn from holding tank 2 for the toxicity experiment. Some mixing of water collected between sampling trips occurred in these 4500 L storage tanks. The pH was adjusted to 6.3 ■ 0.1 in these tanks by adding 1 M sodium hydroxide or 5% nitric acid.

4.3.2 Experimental System

Filtered water collected from the lakes and stored in the holding tanks was pumped into a head tank in the clean laboratory from holding tank 2 and heated to 24 °C with a teflon coated immersion heater. The heated water was vigorously aerated to avoid supersaturation of dissolved gases and slowly drained into a second head tank. Water was then pumped using peristaltic pumps at a rate of 30 $mL \cdot min^{-1}$ through tygon tubing from the second head tank into the exposure tanks. A total of 14 exposure tanks were used in the toxicity experiments. Prior to entering the exposure tank, the test water was mixed with metal stock solution (either Cu or Cd) in mixing chambers (see below). Water in the first head tank was sampled for chemical characterization every 3-4 days during an experiment.

Concentrated metal stock solutions (10 and 100 $mg \cdot L^{-1}$ Cu or Cd) were made from either $CuSO_4 \cdot 5H_20$ or $3CdSO_4 \cdot 8H_20$. Metal exposure stock solutions (30 times the nominal exposure concentrations) were made by diluting concentrated metal stock solutions in 18-L of high purity water in 20-L polyethylene buckets. The metal exposure stock solutions were pumped by peristaltic pumps (1 $mL \cdot min^{-1}$) into the metal mixing chamber of each exposure tank. The mixing chambers consisted of two 5 mL pipette tips placed inside one another that drained into a standpipe with an opening at the bottom. Both the water and the metal solution were pumped into the mixing chamber and mixing occurred as the solutions travelled from the first to the second chamber and finally through the standpipe into the exposure tank. Mixing within each 10 L exposure tank (and into the mesh cages) was ensured by twice daily lifting the mesh cages halfway out of the water and then replacing them. Natural mixing also occurred, as the drain was located at the end of the tank opposite to the mixing chamber. The metal concentration of the water was well mixed as indicated by similar metal concentrations at various locations in the exposure tank and inside the mesh containers prior to the mesh cages being disturbed (data not reported).

4.3.3 Fish Culturing and Initiation of Experiments

Fathead minnow larvae were cultured as described in Chapter 2 with the exception that eggs were hatched in the test water that was used in the experiment (either Halls or Dickie Lake) at pH 6.3. In addition, tests were carried out with offspring from first and second generation wild fish stock. Once eggs hatched, the larval fish were kept in unaerated 2-L Nalgene

beakers until used in toxicity tests. The fish were fed newly hatched brine shrimp after the first day. Debris and any dead fish were siphoned off and approximately 50% of the water was changed daily. In initial range finding tests, we observed unusual and erratic mortality in larval fish when we started toxicity tests with <24 h old larval fish. We attributed this increased mortality to either handling stress (Arthur and Dixon 1994), difficulty in feeding (Hutchinson and Williams 1994), or failure to thrive. To reduce this mortality, toxicity tests were initiated with fish between 24 and 72 hours old (occasionally 96 h) that had already been fed newly hatched brine shrimp. Fish were randomly grouped into sets of 4 in small weigh boats and then randomly assigned to one mesh container. Care was taken to transfer as little water as possible with the larval fish and to ensure that the fish were not trapped against the walls of the mesh container. Between 16 and 20 fish were added to each 10-L exposure tank at the start of an experiment. Nytex mesh cages (500 μm mesh size) were used to separate fish into groups of 4 to reduce density dependent growth differences (Arthur and Dixon 1994). The mesh containers were similar to the ones used by Arthur and Dixon (1994) except that they were 7 cm in diameter instead of 10 cm. The cages were rinsed with test water prior to being added to the exposure tanks. Water and metal stock solutions were pumped into the exposure tanks 24 to 48 hours prior to the start of an experiment.

Fish were exposed to 2 replicates of a geometric series of 6 contiguous metal concentrations ranging between 0.56, 1, 1.8, 3.2, 5.6, 10, 18, and 32 $\mu g \cdot L^{-1}$ and a control for 21 days. Replicates were started 2 to 3 days apart due to limited numbers of larval fathead minnows

and in an attempt to have a true replicate, rather than a duplicate, of the test conditions. In all but one experiment, replicate A was started first.

Larval fish were fed newly hatched brine shrimp (Argentemia® certified brine shrimp eggs, San Francisco, CA; protein 61.8%, carbohydrates 21.5%, ash 11.5%) three times daily on weekdays and 2 times daily on weekends. Artemia cysts (2 g) were added to 1.5 L of test water in a 2 L separatory funnel with 10 g marine salt (0.11 M NaCl) and kept suspended by gentle aeration. Nauplii hatched within 24 hours and were fed to fish within 48 hours. Nauplii were collected by removing the air source and letting them settle to the bottom of the separatory funnel. They were successively re-suspended three times in 1 L of test water to rinse off any remaining salt before feeding. Fish were fed brine shrimp dropwise from a dilute slurry of brine shrimp and lake water. Feeding started at 25 brine shrimp per fish and increased by 25 brine shrimp each week: the number of brine shrimp was estimated by counting the number in 3 to 4 drops prior to feeding. On weekends, since fish were fed only twice, the number of brine shrimp was increased to either 40, 75 or 110.

Mortality was checked twice daily before morning and afternoon feeding. Any dead fish were immediately removed. When required, to ensure similar fish densities between cages within an exposure tank (3-4 fish per mesh cage), surviving fish were gently transferred using a wide bore pipette to other mesh cages. pH and temperature was measured once daily in the exposure tanks. The flow rate of both the toxicant and water were checked daily in

each tank and adjusted if rates deviated from 30 ± 2 $mL \cdot min^{-1}$ for water or 1 ± 0.1 $mL \cdot min^{-1}$ for metal stock solution.

Small larval fish occasionally became trapped in the seam of the nytex mesh cages. In cases where this occurred, the mesh cage was replaced and the remaining fish were gently transferred using a wide bore pipette to a new mesh cage. Any fish trapped in a mesh cage were treated as not being present in the experiment (since death was not due to metal exposure but to physical trauma). Out of a total of 999 fish, 45 became trapped in the seam of the mesh container, usually within the first 3 days of the experiment. Fish that died during the first 18 hours of the experiment (35 overall) were replaced; death was attributed to handling stress (Arthur and Dixon 1994). The majority of these fish (21) died within the first 3 hours of the experiment. The remaining fish (14) died overnight (within 18 hours of the experiment) and were replaced when observed after the mortality check in the morning. No pattern with metal exposure concentration was apparent with either fish entrapped in mesh cages or fish replaced in the initial 18 hours.

4.3.4 Fish sampling at 120 h and after 21 days

In order to determine sublethal effects after acute metal exposure, up to 4 fish from the same mesh enclosure for each exposure concentration were sampled after 120 h. In addition, all surviving fish were sampled at the end of the experiment (21 d). Fish collected at the end of the experiment had not been fed in the previous 18-24 hours. Fish were gently

transferred to a plastic weigh boat with a wide bore pipette tip and sacrificed by immersion in ice cold high purity water for a maximum of 30 seconds. Fish total length was measured with plastic vernier calipers under a magnifying glass to the nearest 0.05 mm. Fish dry weight was measured by drying the fish at 60 °C for 48 hours and weighing to the nearest 0.001 mg on a Cahn electrobalance. Total metal body concentration was also determined in fish that survived after 21 d metal exposure.

4.3.5 Fish Tissue Digestion Method

Dried whole fish (1 to 2 mg dry weight) were digested in sealed conical 5 mL teflon vials at 80°C for 3 hours in 250 uL concentrated nitric acid and 100 uL 50% hydrogen peroxide. The digestate was diluted with 3.15 mL of high purity water and stored in 7 mL polyethylene scintillation vials until analysis by GFAAS. The final solution matrix was 5% nitric acid and 1.4% hydrogen peroxide. The digestions were not performed in the clean laboratory because a fume hood was not available. Instead, digestions were performed in a laminar flow fume hood located in a nearby analytical chemistry laboratory. Because of the increased risk of contamination, 20% of all digestion samples were operational blanks. Vials were handled in sets of 5 with the 5th vial being the operational blank. This blank was treated exactly as any other sample except no fish or tissue standard was added. Each digestion batch processed a maximum of 32 fish samples, 4 tissue standard samples and 9 acid blanks. Initial digestion samples had unacceptably high contamination and were discarded. Improved cleaning procedures (where teflon vials were soaked in 10% nitric acid

instead of 5% nitric acid) minimized the contamination. Average metal concentrations were determined in the tissue standards and acid blanks for each digestion block. The amount of metal in the fish tissue was determined by subtracting the amount of metal in the mean acid blank after corrections for sample dilution was taken into account. Tissue concentrations were determined by dividing the amount of metal in the fish by the dry weight.

Dogfish muscle (DORM-1, National Research Council, Ottawa, Canada) was used as a tissue standard for digestion and subsequent analysis to verify the digestion method. The dry weight of DORM-1 used was similar to the weight of the fish (2 - 5 mg), well below the minimum weight of 250 mg recommended. Because of problems with static using the DORM-1 powder, a slurry was made by adding high purity water to an aliquot of DORM-1 standard in a 1 mL disposable centrifuge tubes and dried. Dried chunks of DORM-1 were then chipped into smaller pieces with a teflon coated probe and weighed.

4.3.6 Chemical analysis of water samples from lake and exposure system.

Lake water and test water were analyzed for major anions and cations, trace metals, and DOC following the same methodology as described in Chapter 2. Al speciation was also performed on these water samples. Total Al was analyzed as before and included inorganic and organic monomeric Al, Al colloids, and polymeric Al. Ion exchange was used to separate total Al into inorganic monomeric Al, and combined inorganic and organic monomeric Al. Al in these fractions were determined using the same method as for total Al.

Total Cu or Cd concentrations was determined in each exposure tank up to 7 times over the course of an experiment. Samples were taken from each exposure tank every 3-4 days throughout the experiment as long as there were surviving fish. Ten mL of test water was sampled by collecting two 5 mL aliquots, one from each of two mesh cages in the exposure tank.

4.3.7 Metal Speciation

Sublethal effects of metal exposure determined as a function of total metal concentrations were compared to predicted metal dialysis fraction (free metal ion plus membrane permeable low molecular weight complexes) and the free metal ion concentration. Using the regression equations generated in Chapter 3 (equations 8 through 11), the metal concentration in the dialysis fraction at the total metal concentration where lethal and sublethal effects were observed was predicted. The free metal ion concentration was estimated at the total metal concentration where lethal and sublethal effects were observed by visually determining the mV at the total metal concentration from the lake water metal titration plots (Figure 3.8 and 3.9) and predicting the free metal ion, using the regression equations generated from Chapter 3 (equations 12 and 13). The free metal ion concentration was then corrected for ionic strength differences between the exposure water and the metal titrations used to generate the regression equations.

4.3.8 Statistics

Cu or Cd concentrations in each exposure tank were tested for differences between tests and within replicates by ANOVA. Means were compared by Bonferroni adjusted means test or by Student's t-test only if the effect appeared significant based on the ANOVA analysis ($p < 0.05$). For each chronic experiment, biological effects data (mortality, length, dry weight, whole body concentration) were pooled from the two replicates. Differences between replicates were first tested by ANOVA and, when present, differences were tested with Student's t-test or Bonferroni adjusted means test. When appropriate, data were also analyzed separately for both replicates. All statistical analysis except for LC50 determination were completed using SYSTAT (Wilkinson 1989).

Median lethal metal concentrations (LC50s) were determined based on mortality data at a given time, and measured total Cu or Cd water concentrations by the trimmed Spearman-Karber method (Hamilton et al. 1977). For LC50 determination, the number of fish present at the start of an experiment is required. This was adjusted after fish were sampled at 120 hours. The total number of fish after sampling at 120 h that would generate equivalent percent mortality as before sampling was calculated in each exposure tank and rounded off to the nearest 1. These estimates were then used to calculate LC50s in the exposure tanks after 120 h. In the Dickie Lake - Cd experiment and the Halls Lake - Cd experiment, in

some of the replicates, all of the surviving fish in the 5.6 $\mu g \cdot L^{-1}$ and 10 $\mu g \cdot L^{-1}$ Cd exposure tanks were removed during sampling at 120 hours if the number of surviving fish was ≤ 2. As a result the LC50 estimate after this time may be slightly inflated.

4.4 Results

4.4.1 Water quality of lake water and exposure water

Collecting, transporting and filtering the lake water did not appreciably change the chemical composition of the water (Table 4.1). Because the water was adjusted to pH 6.3 in the holding tanks, the test water pH, alkalinity, and dissolved inorganic carbon concentration were all significantly different from the lake water: Dickie Lake water pH was adjusted from 5.85 to 6.35 while Halls Lake water pH was adjusted from 6.75 to 6.30. Small reductions in the colour, DOC and Al concentrations in the test water occurred and were probably due to filtering the lake water through the 20 and 5 μm particle filters. No increases were observed in Cu, Cd, Pb or Zn indicating that no metal contamination occurred during collection, transport or storage of the water. With the notable exception of DOC concentration, water quality characteristics were similar between the two study lakes, although due to the high precision in the mean estimates, most water variables were measurably different between the test exposure waters (Table 4.1). Other parameters known to influence trace metal toxicity, namely Ca and Mg concentration and pH (Welsh et al.

Table 4.1 Water quality characteristics (Mean ± SD) of lake water and test water used in metal exposure experiments. p-value indicates significant differences between lake and test water (*) or between test waters (†) as determined by Student's t-test. Lake water was collected after pumping from the littoral zone of the lake, test water was collected from the head tank from each experiment. In determining mean water quality characteristics, values reported as below detection limit were included as one half the detection limit instead of being treated as missing values.

	Halls Lake			Dickie Lake			Test Water
Water Quality Characteristic	Lake Water	Test Water	p-value*	Lake Water	Test Water	p-value*	p-value †
Conductivity ($\mu S \cdot cm^{-1}$)	33.2 ± 1.6	35.3 ± 1.3	0.003	32.9 ± 0.8	33.2 ± 0.8	0.481	<0.001
Colour (Hazen units)	6.18 ± 1.07	5.38 ± 0.84	0.057	61.8 ± 21.9	43.3 ± 11.1	0.097	<0.001
DIC ($mg \cdot L^{-1}$)	0.83 ± 0.07	0.33 ± 0.07	<0.001	0.80 ± 0.13	0.40 ± 0.07	<0.001	0.040
DOC ($mg \cdot L^{-1}$)	2.43 ± 0.17	2.32 ± 0.11	0.082	7.15 ± 1.77	6.74 ± 1.06	0.616	<0.001
pH (pH units)	6.75 ± 0.11	6.29 ± 0.12	<0.001	5.84 ± 0.21	6.35 ± 0.10	0.001	0.254
Hardness ($mg \cdot L^{-1}$ as $CaCO_3$)	10.8 ± 1.00	10.7 ± 0.95	0.805	9.50 ± 0.83	9.28 ± 0.61	0.587	<0.001
Al ($\mu g \cdot L^{-1}$)	4.67 ± 4.83	2.25 ± 4.33	0.210	71.5 ± 18.9	45.3 ± 19.7	0.021	<0.001
Al (inorganic, $\mu g \cdot L^{-1}$)	1.0 ± 0.0	1.0 ± 0.0	----	32.5 ± 11.6	22.7 ± 7.3	0.101	----
Al (inorg. + organic, $\mu g \cdot L^{-1}$)	1.0 ± 0.0	1.0 ± 0.0	----	52.7 ± 16.6	32.3 ± 10.8	0.029	----
Cd ($\mu g \cdot L^{-1}$)	0.01 ± 0.01	0.01 ± 0.02	0.723	0.04 ± 0.04	0.03 ± 0.02	0.500	0.153
Cu ($\mu g \cdot L^{-1}$)	0.41 ± 0.28	0.41 ± 0.28	0.991	0.48 ± 0.15	0.42 ± 0.11	0.433	0.896
Pb ($\mu g \cdot L^{-1}$)	0.07 ± 0.09	0.07 ± 0.08	0.990	0.06 ± 0.05	0.01 ± 0.01	0.076	0.034
Zn ($\mu g \cdot L^{-1}$)	3.57 ± 1.21	3.60 ± 1.17	0.962	4.76 ± 2.83	5.96 ± 2.42	0.402	0.006
Ca ($mg \cdot L^{-1}$)	3.11 ± 0.35	3.10 ± 0.32	0.923	2.74 ± 0.26	2.65 ± 0.19	0.504	<0.001
K ($mg \cdot L^{-1}$)	0.53 ± 0.04	0.53 ± 0.04	0.968	0.42 ± 0.03	0.41 ± 0.04	0.443	<0.001
Mg ($mg \cdot L^{-1}$)	0.74 ± 0.06	0.72 ± 0.04	0.459	0.65 ± 0.05	0.65 ± 0.03	0.942	<0.001
Na ($mg \cdot L^{-1}$)	1.23 ± 0.08	1.28 ± 0.07	0.110	1.98 ± 0.10	2.26 ± 0.24	0.005	<0.001
Cl ($mg \cdot L^{-1}$)	1.45 ± 0.19	1.51 ± 0.13	0.393	3.44 ± 0.20	3.32 ± 0.19	0.232	<0.001
SO4 ($mg \cdot L^{-1}$)	6.88 ± 0.37	6.90 ± 0.18	0.890	4.77 ± 0.32	5.00 ± 0.61	0.317	<0.001

1996), were also similar between the two lakes, although the Ca and Mg levels were lower in Dickie Lake. Of the trace metals measured, only Zn and Al were higher in Dickie Lake than in Halls Lake.

DOC concentration in the test water was relatively consistent between sampling trips (over a 3 week period) for each experiment except for the Dickie Lake - Cd experiment in the fall of 1994 (Figure 4.1). Here, DOC concentrations were elevated from approximately 6.3 $mg \cdot L^{-1}$ to between 8.5 and 9.0 $mg \cdot L^{-1}$ during the first week of collecting water. This increase in DOC was probably due to poor mixing in the lake and/or high inflows from a nearby wetland.

4.4.2 Metal concentration in exposure tanks

Extensive determination of total metal concentrations in exposure tanks were performed to ensure accurate metal exposure levels. Overall, the metal dosing system was good; measured metal concentrations were between 80 and 115% of nominal levels except for the 1.0 and 1.8 $\mu g \cdot L^{-1}$ Cu exposures where the metal concentrations were 218 and 137% nominal, respectively (Table 4.2). Metal concentrations in the exposure water tended to increase as the nominal metal concentration increased, except at low exposures (< 5 $\mu g \cdot L^{-1}$ Cu and < 1 $\mu g \cdot L^{-1}$ Cd) where metal exposure tanks (especially for Cu) were not significantly different from each other (Table 4.2). Even though the work was performed in a class 100 clean laboratory, some contamination occurred in the Cu experiments (but not in the Cd experiments) where Cu concentrations were higher than expected at low nominal concentrations. Sources of Cu contamination may be a

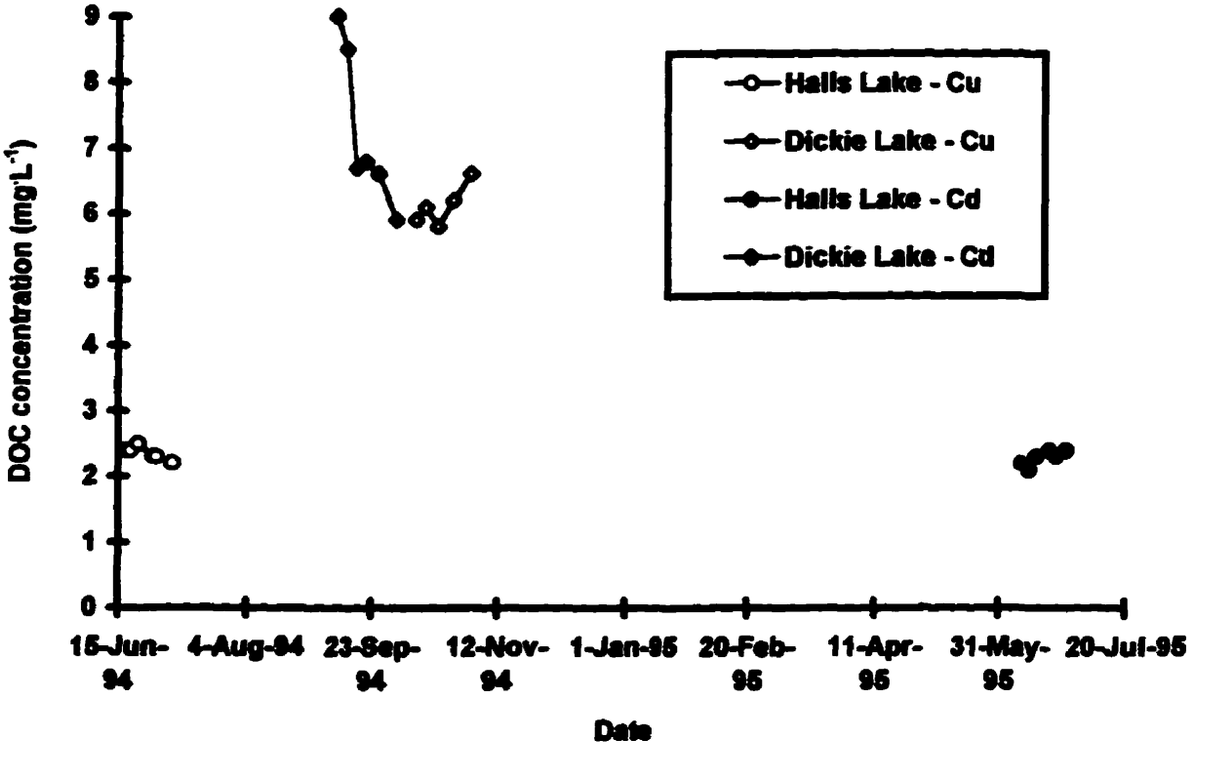


Figure 4.1 Variation in DOC concentration ($mg \cdot L^{-1}$) in test water used in metal exposure experiments. Note the high DOC levels that were present at the beginning of the Dickie Lake - Cd experiment.

Table 4.2 **Measured metal concentrations in Halls and Dickie lake water Cu and Cd exposure tests. Grand means with a common letter are not significantly different as determined by Bonferroni adjusted means test. An asterisk (*) denotes significant differences between mean Cu exposure between treatment tanks from each lake water based on Students t-test ($p<0.05$). The percent nominal (nom. %) denotes the ratio between the grand mean metal concentration and the nominal metal concentration (x 100).**

	Mean ± SD (n) Metal Concentration ($\mu g \cdot L^{-1}$)					
Metal	Nominal Conc.	Halls Lake Water	Dickie Lake Water	Grand Mean	Nom (%)	Bon. Mean
Cu	0.0	1.17±0.97 (12)	1.04±0.82 (10)	1.11±0.89 (22)	----	a
	1.0	2.18±0.93 (12)	----	2.18±0.93 (12)	218	ab
	1.8	2.50±0.92 (12)	2.44±1.05 (10)	2.47±0.97 (22)	137	ab
	3.2	3.62±0.89 (12)	3.54±0.91 (10)	3.58±0.87 (22)	112	bc
	5.6	5.44±0.86 (12)	5.18±1.52 (12)	5.31±1.22 (24)	95	c
	10	9.43±0.82 (9)	12.62±6.54 (11)	11.18±5.05 (20)	112	d
	18	15.43±0.91 (5)	16.43±1.86 (11)	16.12±1.66 (16)	90	e
	32	----	26.99±3.49 (11)	26.99±3.49 (11)	84	f
Cd	0.0	0.02±0.01 (15)	0.10±0.29 (15)	0.06±0.20 (30)	----	a
	0.56	0.44±0.09 (15)*	0.57±0.15 (14)*	0.50±0.14 (29)	89	ab
	1.0	0.74±0.10 (15)*	1.03±0.27 (15)*	0.89±0.25 (30)	89	b
	1.8	1.67±0.27 (17)	1.84±0.89 (15)	1.75±0.64 (32)	97	c
	3.2	2.67±0.42 (16)	3.15±1.56 (15)	2.90±1.13 (31)	91	d
	5.6	4.78±0.42 (7)	5.55±2.48 (11)	5.25±1.95 (18)	94	e
	10	8.65±1.14 (6)	7.48±0.84 (8)	7.98±1.11 (14)	80	f

result of feeding, contamination during sampling and/or technician error while measuring pH and temperature in the exposure tanks. Variation in Cu and Cd concentrations within exposure tanks over the 21 day test was minimal as indicated by the low standard deviations (Table 4.2). With a few exceptions (e.g., Dickie Lake - Cu experiment), the standard deviation for Cu levels in exposure tanks were consistent at approximately 0.9 $\mu g \cdot L^{-1}$. A similar pattern was apparent for Cd, where standard deviations for Cd levels in exposure tanks were usually < 0.4 $\mu g \cdot L^{-1}$. Some exceptions to this general pattern were noted. In these cases, the main cause of the variation in the total metal concentration in metal exposure tanks was due to water or metal pump delivery problems. This was especially notable for the Dickie Lake - Cd experiment where Cd concentrations were about 3 times nominal for 12-24 hours due to a pump failure. The relative error in total metal concentration was higher at lower metal concentrations since the standard deviation was relatively constant at increasing total metal concentrations for both Cu and Cd (Table 4.2). All metal concentration data were used to calculate mean exposure levels, including spiked exposure levels due to occasional pump failures, since fish were exposed to these elevated concentrations. Removing these high data points resulted in slightly lower mean estimates with reduced variation.

Cu concentrations in the exposure tanks did not vary between tests or within replicates (Table 4.3). However, total Cd concentrations in the exposure tanks varied within replicates (Table 4.3) and between experiments (Table 4.4). The variation in Cd levels between replicates was due to differences in the Dickie lake - Cd experiment where significant differences were measured between replicates (Table 4.4): differences were detected between the replicates for 1.8 and 3.2

Table 4.3 **Statistical analysis of differences in metal concentrations in exposure tanks. Data for 1.0 $\mu g \cdot L^{-1}$ (Halls) and 32 $\mu g \cdot L^{-1}$ (Dickie) nominal Cu exposure were removed from the ANOVA analysis to balance the design.**

ANOVA TABLE					
Metal	**Source**	**df**	**MS**	**F-ratio**	**p-value**
Copper	Conc (C)	5	550.57	106.73	< 0.001
	Rep (R)	1	11.37	2.20	0.141
	Test (T)	1	1.26	0.24	0.623
	C*R	5	9.82	1.90	0.100
	C*T	5	2.97	0.57	0.719
	R*T	1	0.06	0.01	0.912
	C*R*T	5	2.28	0.44	0.818
	Error	102	5.16		
Cadmium	Conc (C)	6	129.93	188.84	< 0.001
	Rep (R)	1	3.37	4.90	0.028
	Test (T)	1	0.02	0.02	0.877
	C*R	6	0.68	0.99	0.433
	C*T	6	1.18	1.72	0.119
	R*T	1	5.87	8.53	0.004
	C*R*T	6	1.01	1.46	0.194
	Error	156	0.69		

Table 4.4 **Statistical analysis of differences in Cd concentrations in exposure tanks in water collected from Halls and Dickie Lake.**

ANOVA TABLE					
Test	**Source**	**df**	**MS**	**F-ratio**	**p-value**
Both	**Conc (C)**	**6**	**152.43**	**206.40**	**< 0.001**
	Test (T)	**1**	**0.49**	**0.66**	**0.416**
	C*T	**6**	**1.43**	**1.94**	**0.078**
	Error	**170**	**0.74**		
Dickie Lake	**Conc (C)**	**6**	**56.86**	**46.68**	**< 0.001**
	Rep (R)	**1**	**8.84**	**7.26**	**0.009**
	C*R	**6**	**1.57**	**1.29**	**0.272**
	Error	**79**	**1.22**		
Halls Lake	**Conc (C)**	**6**	**74.22**	**514.21**	**< 0.001**
	Test (T)	**1**	**0.18**	**1.22**	**0.272**
	C*T	**6**	**0.07**	**0.45**	**0.840**
	Error	**77**	**0.14**		

nominal Cd exposure (data not shown). Replicate Cd levels in exposure tanks were not different for the Halls Lake - Cd experiment (Table 4.4). Differences in total Cd exposure concentrations between experiments was due to higher Cd concentrations in the Dickie Lake experiment, especially in the 0.56 and 1.0 nominal Cd exposures (Table 4.2).

In determining the significance of replicate or experiment on Cu concentrations in the exposure tanks (Table 4.3), the data for 1.0 $\mu g \cdot L^{-1}$ (Halls Lake) and 32 $\mu g \cdot L^{-1}$ (Dickie Lake) nominal Cu exposure were removed from the ANOVA analysis to balance the design: no corresponding Cu exposure treatment was present in the respective lake water experiment (Table 4.2).

4.4.3 Mortality in Control and Metal Exposure Tanks

For all four experiments, fish mortality in replicate A control tanks did not exceed the USEPA guideline of 20% for 7-d fathead minnow toxicity tests (DeGraeve et al. 1991) (Figure 4.2). However, for some unexplained reason, fish mortality in the replicate B control tanks surpassed 20% after 5 to 9 days and increased progressively to the end of the 21-d tests (Figure 4.2). This occurred in all tests; after 21-d total control mortality in replicate B ranged from 30% to 63%. This pattern of elevated fish mortality in replicate B was also apparent in the metal exposure tanks in all experiments (Figure 4.3). For metal exposure tanks where replicate A data indicated that metal concentrations were not lethal, fish mortality in replicate B seemed to increase between days 4 and 8 and then stabilize.

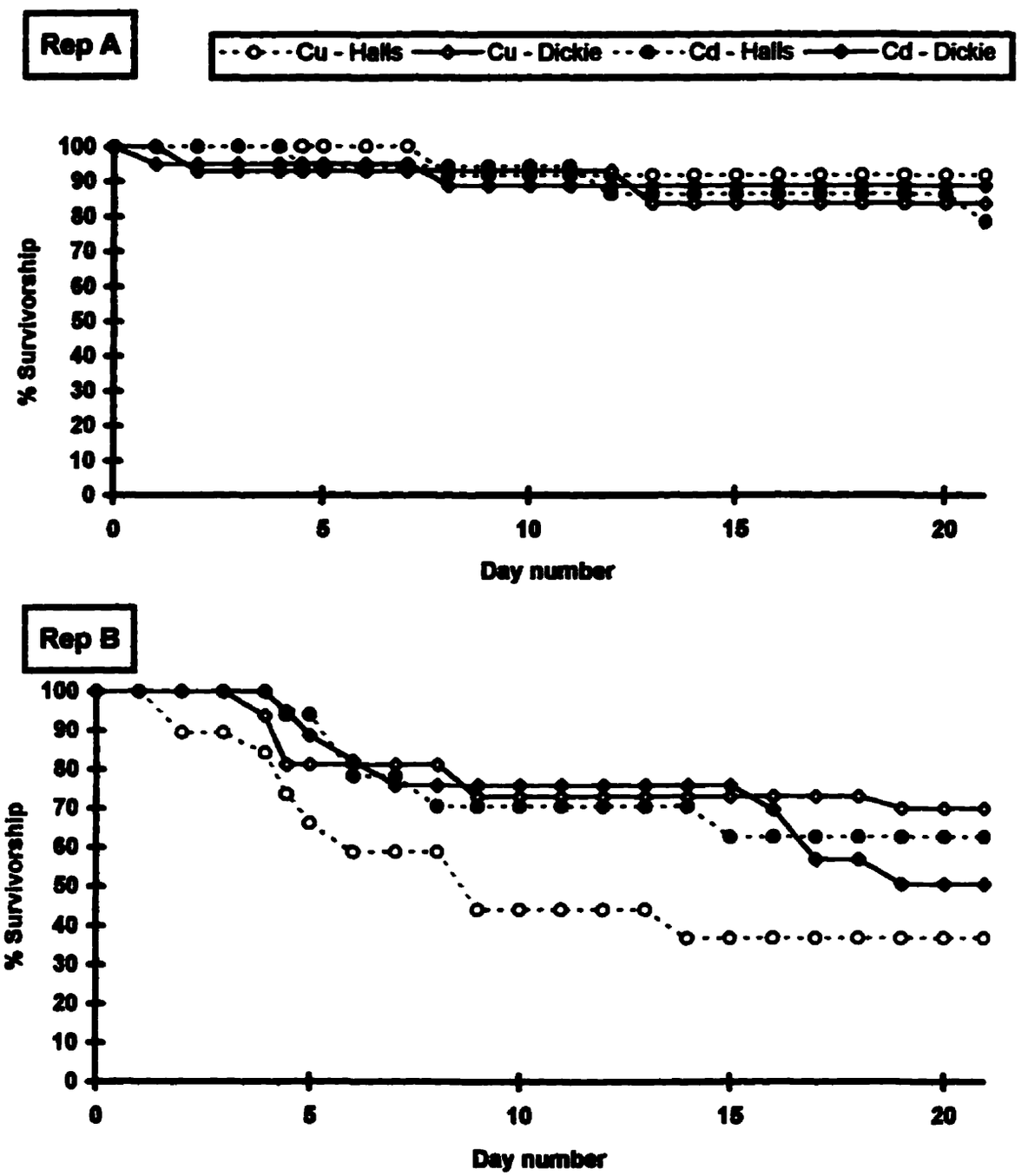


Figure 4.2 Fish survivorship in control tanks for replicate A and replicate B during the four experiments. Total number of fish at the start of the experiments was between 16 and 20.

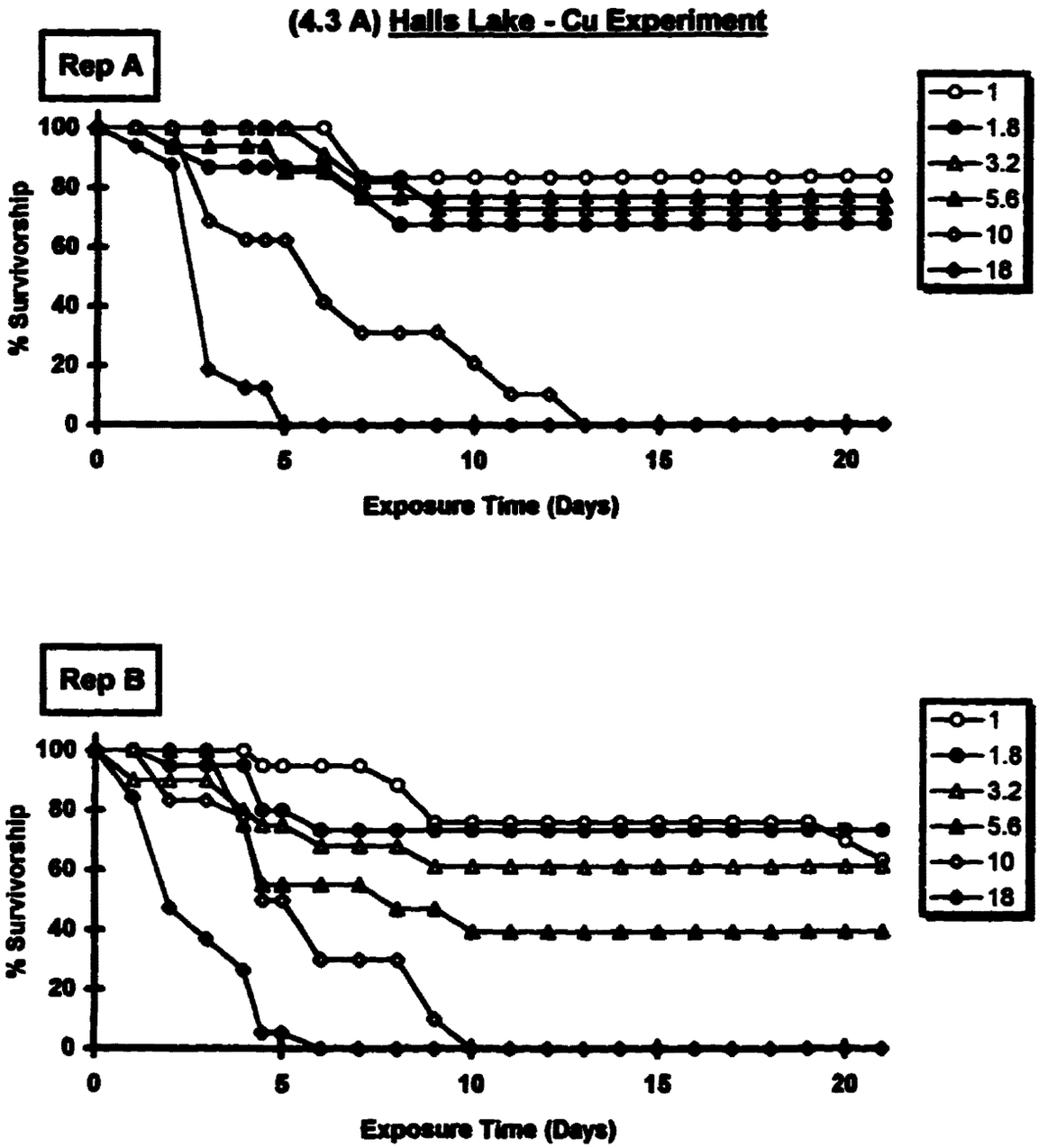
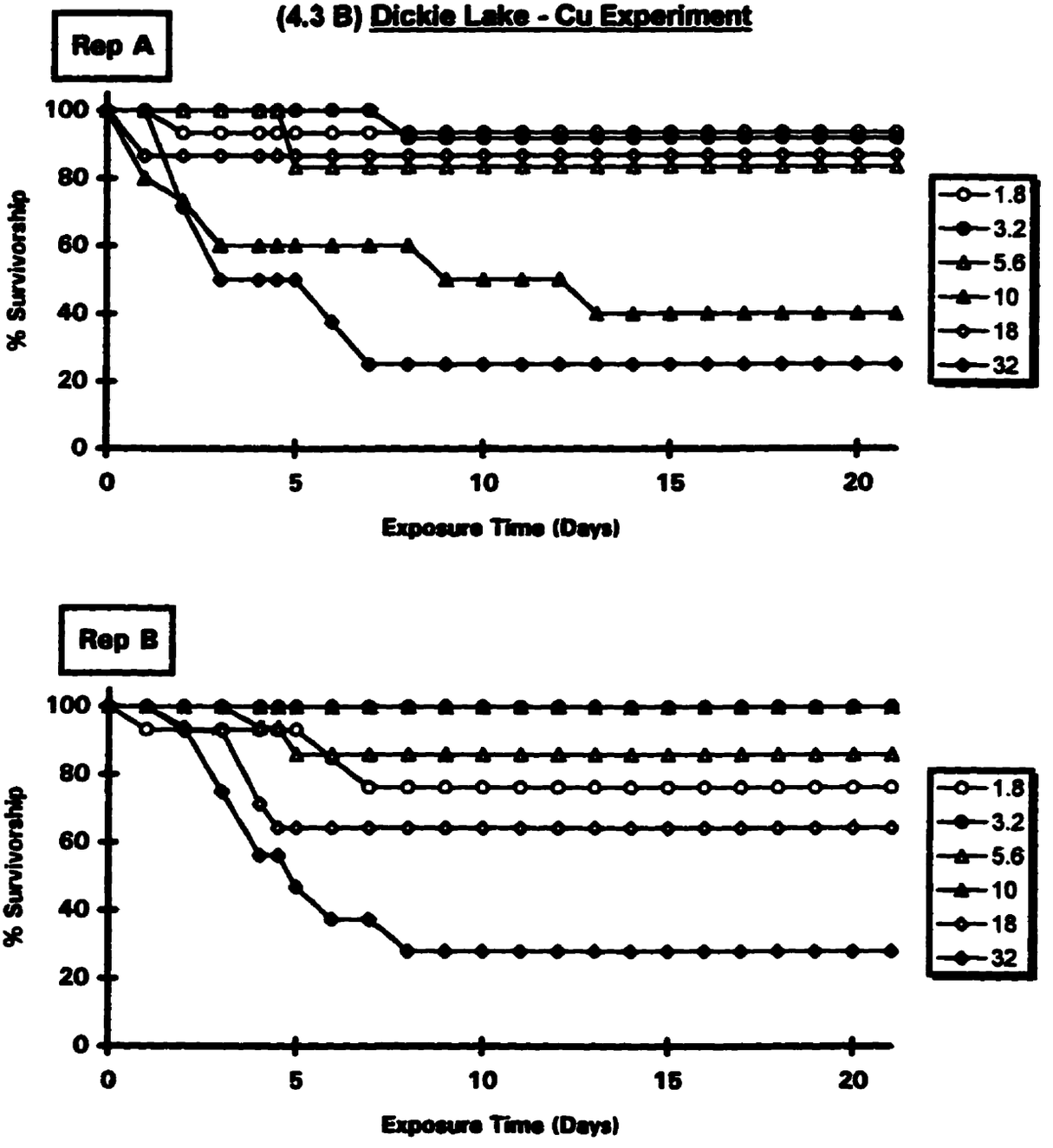
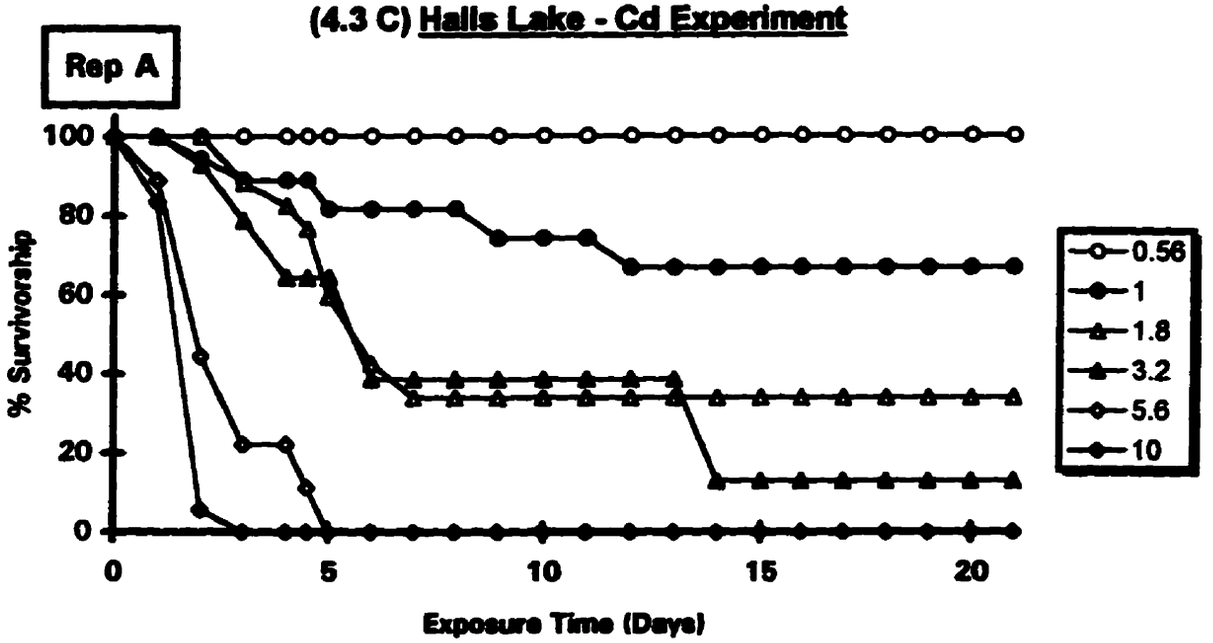
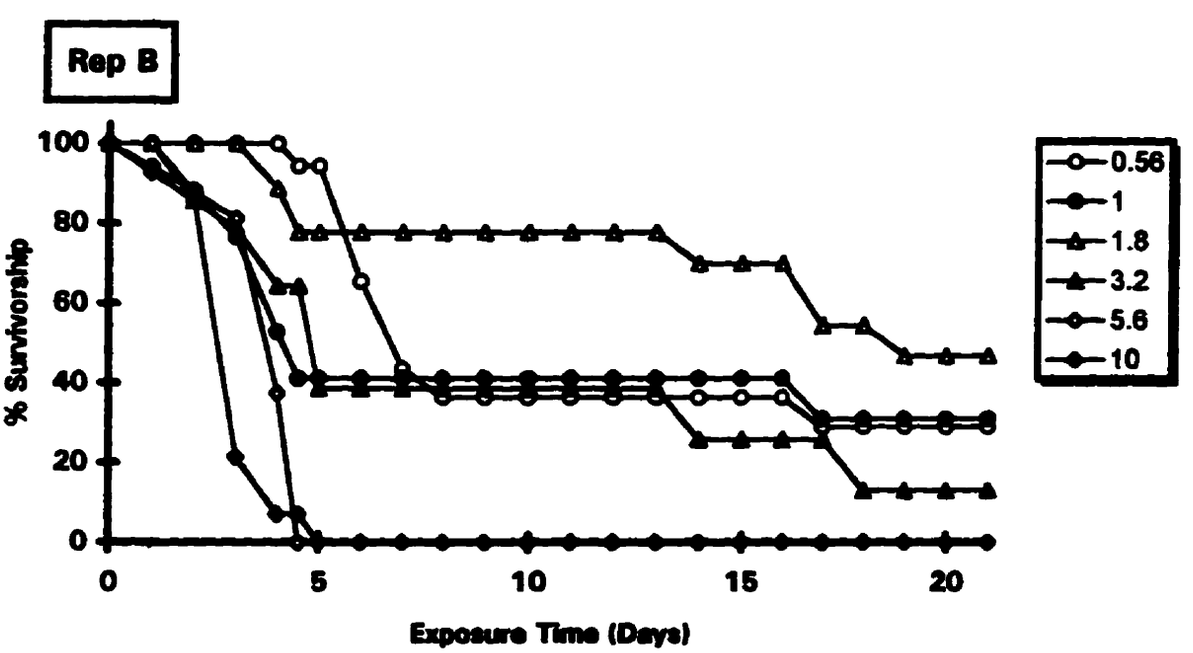


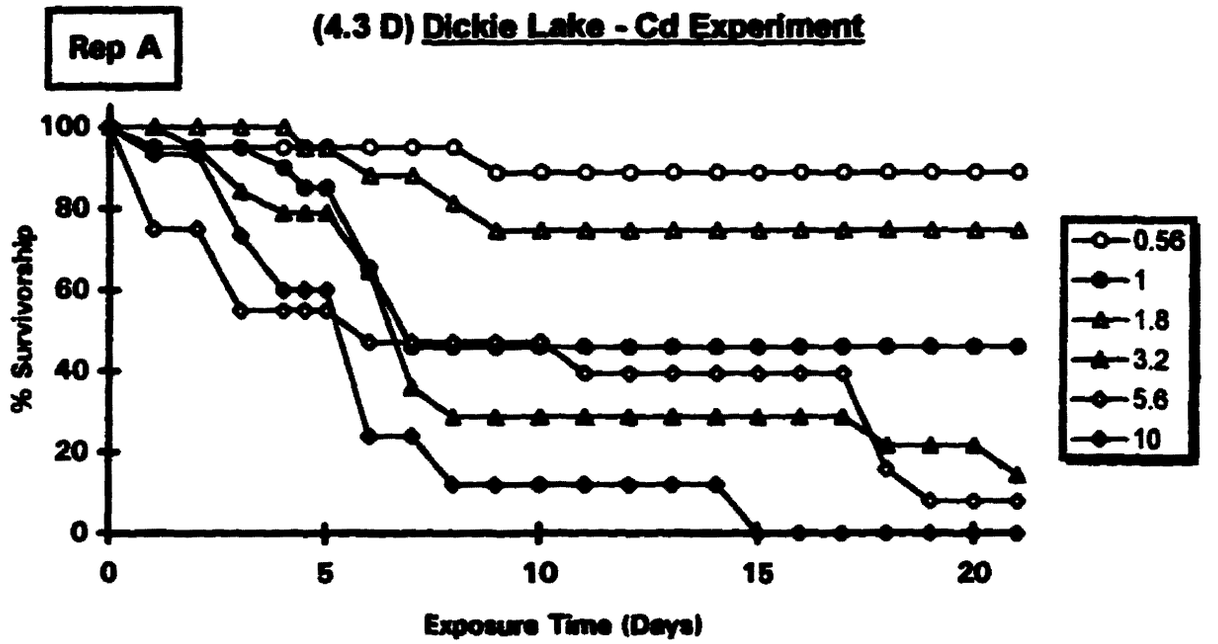
Figure 4.3 Survivorship of larval fathead minnows exposed to Cu ($ug \cdot L^{-1}$) and Cd ($ug \cdot L^{-1}$) in Halls Lake and Dickie Lake exposure water: (A) Halls Lake - Cu experiment; (B) Dickie Lake - Cu experiment; (C) Halls Lake - Cd experiment; (D) Dickie Lake - Cd experiment.

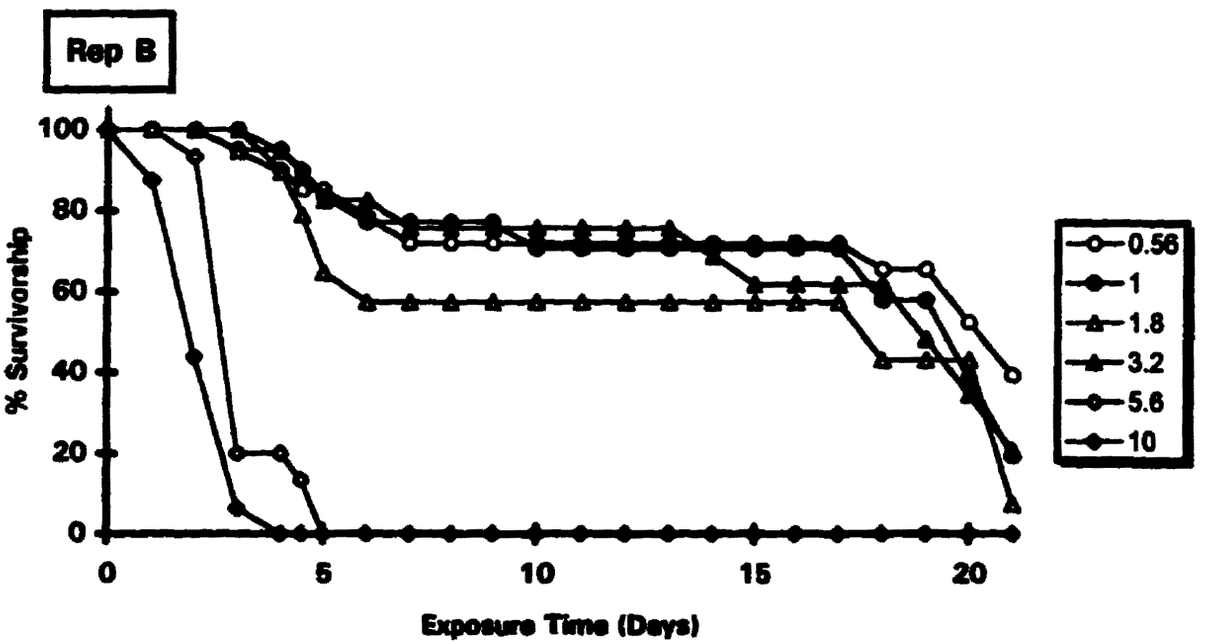

(4.3 B) Dickie Lake - Cu Experiment
Rep A
100
80
60
40
20
0
% Survivorship
0
5
10
15
20
Exposure Time (Days)
1.8
3.2
5.6
10
18
32
Rep B
100
80
60
40
20
0
% Survivorship
0
5
10
15
20
Exposure Time (Days)
1.8
3.2
5.6
10
18
32

(4.3 C) Halls Lake - Cd Experiment






Rep A
(4.3 D) Dickie Lake - Cd Experiment
% Survivorship
100
80
60
40
20
0
0
5
10
15
20
Exposure Time (Days)
0.56
1
1.8
3.2
5.6
10


Rep B
% Survivorship
100
80
60
40
20
0
0
5
10
15
20
Exposure Time (Days)
0.56
1
1.8
3.2
5.6
10

Increased fish mortality, predominantly in replicate B and to a lesser extent in replicate A, also occurred in the last week, but only in the Cd experiments (Figure 4.3). This mortality did not appear to be related to Cd exposure as it was observed in the replicate B control fish of the Dickie Lake - Cd experiment (but not in the control fish of the Halls Lake - Cd experiment) (Figure 4.2). The fish that died appeared healthy and were observed with stomachs (in some cases ruptured) full of brine shrimp. It appears that the fish probably died as a result of overfeeding or ingestion of brine shrimp cysts. Because of the unexplained mortality from replicate B, the biological effects data from replicate B were compared to the data from replicate A before the data were pooled to calculate effects based on the sum of the two replicates. Where appropriate, data is presented for replicate A and B separately to illustrate any possible differences in effects between the two replicates.

4.4.4 Median Lethal Concentrations

Median lethal metal concentrations (LC50s) were calculated daily based on combined mortality data, as well as on replicate A data only. Because of the erratic fish mortality in replicate B, no adjustments were made for control mortality. Differences in calculated LC50s based on combined data versus replicate A data were apparent in the Cu experiments (Figure 4.4) but minimal in the Cd experiments (Figure 4.5). Because of these differences, and because of the unexplained mortality in the replicate B exposure tanks, LC50s are reported based on replicate A data only. The vast majority of the mortality occurred over the first 14 days of metal exposure (Figure 4.4 and 4.5). The incipient lethal level (ILL), the point where mortality does

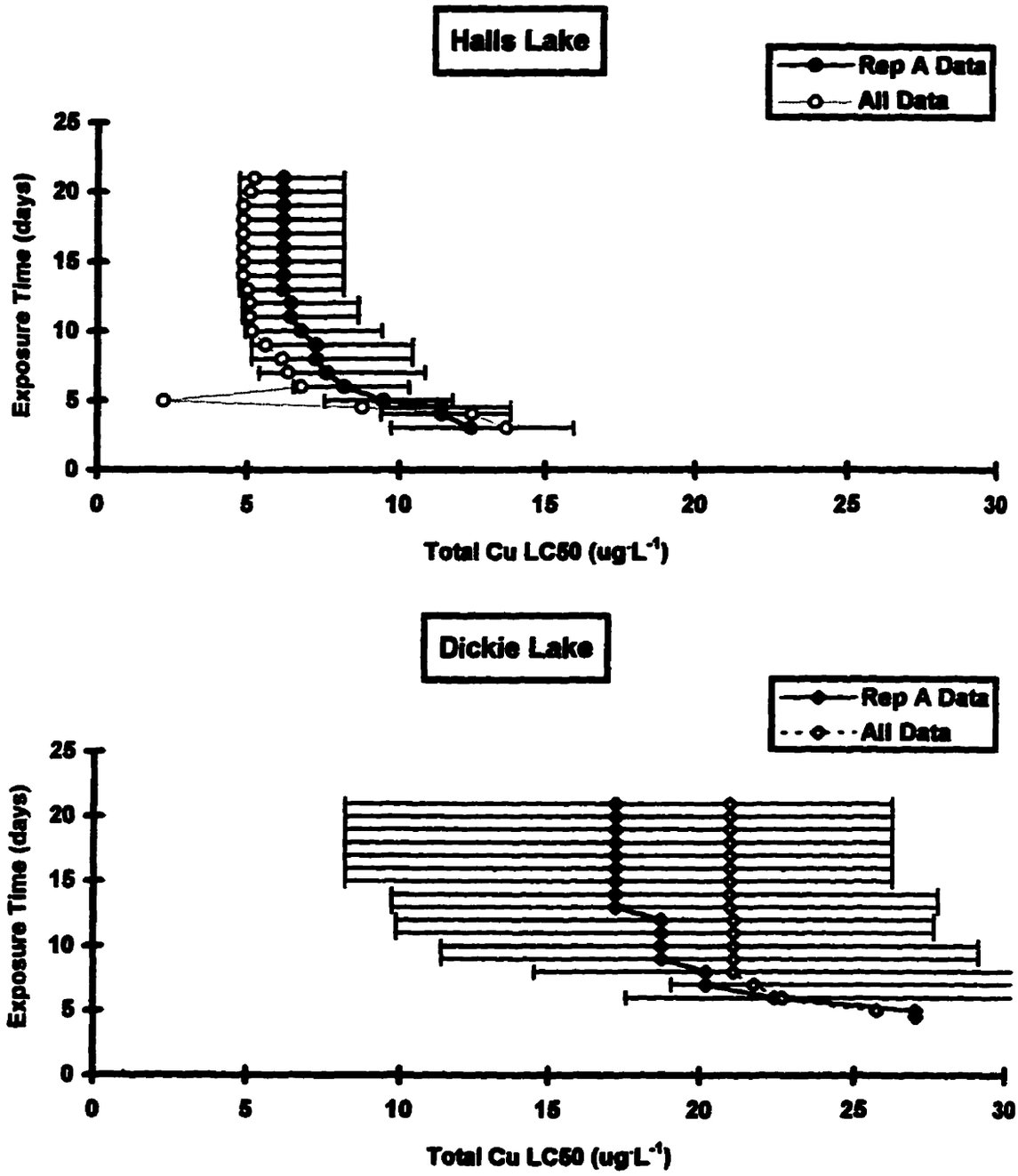


Figure 4.4 Calculated Spearman-Karber Cu LC50s for larval fathead minnow in Halls and Dickie lake water. LC50s were calculated based on all of the data (replicate A plus replicate B) and on replicate A only (see text for details). For clarity, 95% fiducial limits are shown for replicate A data only.

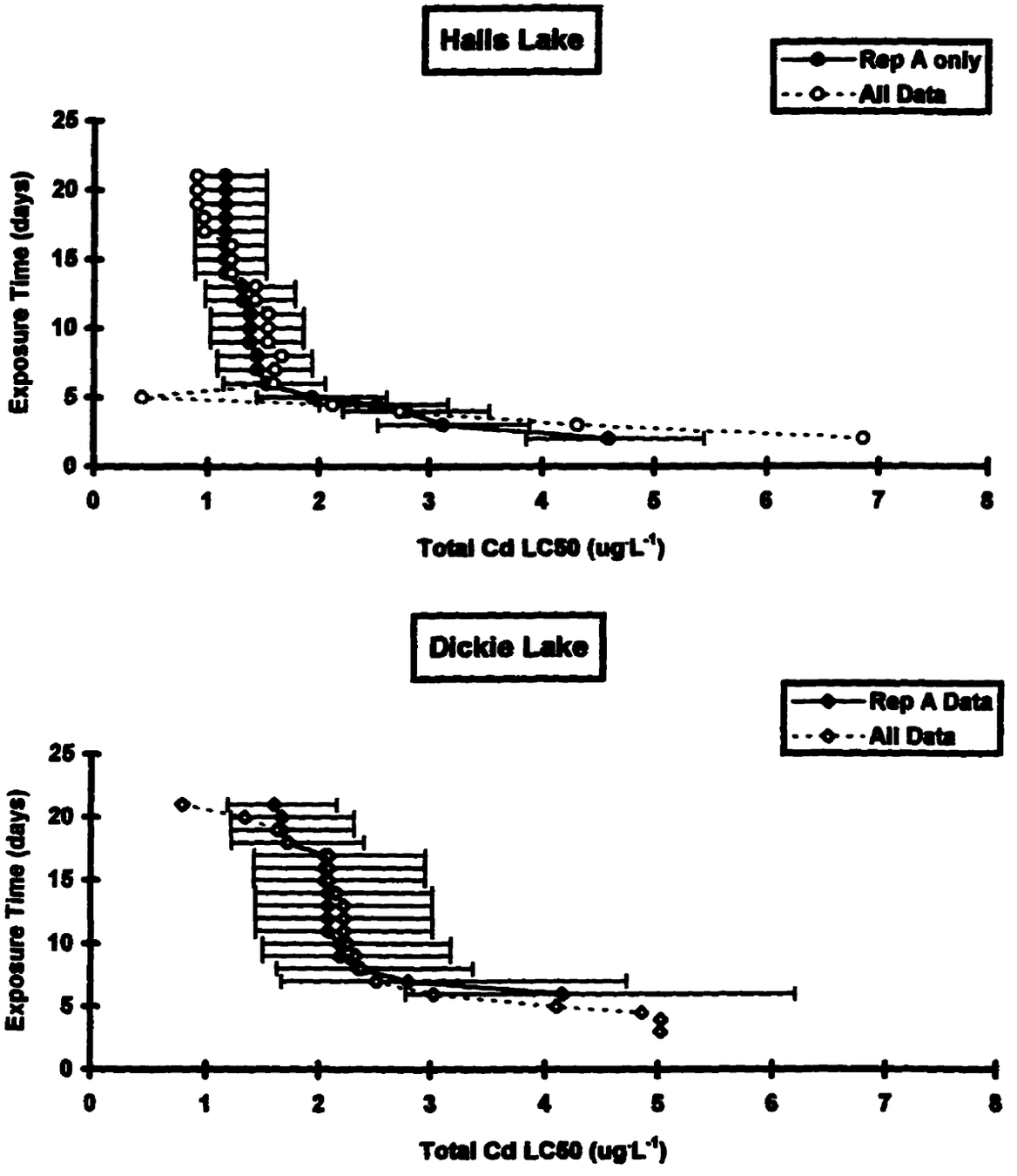


Figure 4.5 Calculated Spearman-Karber Cd LC50s for larval fathead minnow in Halls and Dickie lake water. LC50s were calculated based on all of the data (replicate A plus replicate B) and on replicate A only (see text for details). For clarity, 95% fiducial limits are shown for replicate A data only.

not further increase with exposure time (Sprague 1969), was calculated based on the LC50 after 21 days exposure using replicate A data only. For the Dickie Lake - Cd experiment only, the ILL was based on the LC50 after 17 days exposure since the additional mortality after day 17 was apparently not due to Cd exposure. The ILL was 6.2 $\mu g \cdot L^{-1}$ and 17.2 $\mu g \cdot L^{-1}$ for Cu in water collected from Halls and Dickie lake, respectively, and 1.2 $\mu g \cdot L^{-1}$ and 2.0 $\mu g \cdot L^{-1}$ for Cd in water collected from Halls and Dickie lake, respectively.

4.4.5 Sublethal Effects of Cu and Cd Exposure

Total length and dry weight were used as sublethal indicators of metal exposure and combined to give an indication of the effect of metal exposure on growth. Sublethal effects of metal exposure on growth were determined after 5 and 21 days for both Cu and Cd exposures.

Total length and dry weight of fish exposed to Cu for 21 days in Halls and Dickie Lake water were significantly different between replicates for each experiment (Table 4.5). While replicate B fish tended to be smaller and lighter than replicate A fish, differences between replicates (by Students t-test) were only significant at a few metal exposure concentrations (Table 4.6). As a result, the total length and dry weight data for the Cu exposures were pooled.

No differences in fish total length or dry weight were observed in fish exposed to Cu in Dickie Lake water after 5 days. However, both total length and dry weight were reduced in fish exposed to > 16.1 $\mu g \cdot L^{-1}$ Cu in Dickie Lake water after 21 d (Figure 4.6 and 4.7). When

Table 4.5 Statistical analysis of difference in total length (mm) and dry weight (mg) in Cu exposed fish.

			ANOVA TABLE			
Lake	Variable	Source	df	MS	F-ratio	p value
Halls	Total Length	Conc (C)	4	7.74	4.53	0.002
		Rep (R)	1	14.99	8.78	0.004
		C*R	4	2.46	1.44	0.229
		Error	75	1.71		
	Dry Weight	Conc (C)	4	0.61	3.23	0.017
		Rep (R)	1	1.93	10.22	0.002
		C*R	4	0.11	0.56	0.692
		Error	75	0.19		
Dickie	Total Length	Conc (C)	6	10.61	20.10	<0.001
		Rep (R)	1	4.74	8.97	0.003
		C*R	6	1.80	3.42	0.004
		Error	102	0.53		
	Dry Weight	Conc (C)	6	1.23	9.56	<0.001
		Rep (R)	1	0.02	0.19	0.663
		C*R	6	0.34	2.65	0.020
		Error	102	0.13		

Table 4.6 Total length (mm) and dry weight (mg) of fish exposed to Cu for 21 days (mean±SD (n)). Values marked with an asterisk are significantly different from each other as determined by Student's t-test ($\alpha = 0.05$). Values marked with a † are significantly different from the control as determined by Dunnetts test (performed on grand mean estimates only). For Halls Lake only, values marked with a ‡ are significantly different from the 1.0 $\mu g \cdot L^{-1}$ Cu exposed fish as determined by Dunnetts test. Total length and dry weight of fish exposed to Cu for 5 days are shown in Figure 4.6 and 4.7.

	Measured Cu Conc.	Total Length (mm)			Dry Weight (mg)		
Lake	($\mu g \cdot L^{-1}$)	Replicate A	Replicate B	Grand Mean	Replicate A	Replicate B	Grand Mean
Halls	1.11	9.78±1.32 (11)	9.15±1.00 (4)	9.61±1.24 (15)	0.84±0.42 (11)	0.58±0.26 (4)	0.77±0.39 (15)
	2.18	10.94±1.62 (10)	9.78±1.42 (11)	10.33±1.59 (21)	1.33±0.63 (10)	0.88±0.49 (11)	1.10±0.59 (21)
	2.47	9.82±1.50 (7)	10.00±0.95 (11)	9.93±1.16 (18)	0.86±0.42 (6)	0.79±0.31 (11)	0.81±0.34 (17)
	3.58	10.48±1.72 (8)	9.69±1.00 (9)	10.06±1.40 (17)	1.20±0.67 (8)	0.77±0.30 (8)	0.98±0.49 (16)
	5.31	9.53±0.96 (9) *	7.48±1.16 (5) *	8.80±1.42 (14) ‡	0.83±0.31 (9) *	0.40±0.40 (4) *	0.70±0.35 (13)
Dickie	1.11	12.24±0.49 (9) *	10.28±0.57 (4) *	11.64±1.07 (13)	1.73±0.25 (9) *	1.04±0.27 (4) *	1.52±0.41 (13)
	2.47	11.26±0.54 (11)	11.47±0.83 (9)	11.36±0.68 (20)	1.35±0.25 (11)	1.60±0.44 (9)	1.46±0.36 (20)
	3.58	11.14±0.61 (11)	10.99±1.07 (13)	11.06±0.87 (24)	1.27±0.31 (11)	1.41±0.48 (13)	1.34±0.41 (24)
	5.31	11.29±0.82 (10)	10.61±0.93 (11)	10.94±0.92 (21)	1.28±0.32 (10)	1.34±0.49 (10)	1.31±0.40 (20)
	11.18	11.15±0.90 (4)	11.06±0.66 (13)	11.08±0.69 (17)	1.32±0.61 (4)	1.44±0.38 (13)	1.41±0.43 (17)
	16.12	9.96±0.42 (10) *	9.11±0.45 (6) *	9.64±0.59 (16) †	0.94±0.18 (10) *	0.69±0.15 (6) *	0.85±0.21 (16) †
	26.99	8.30±0.07 (2)	8.50±0.15 (3)	8.42±0.16 (5) †	0.42±0.06 (2)	0.54±0.02 (3)	0.49±0.07 (5) †

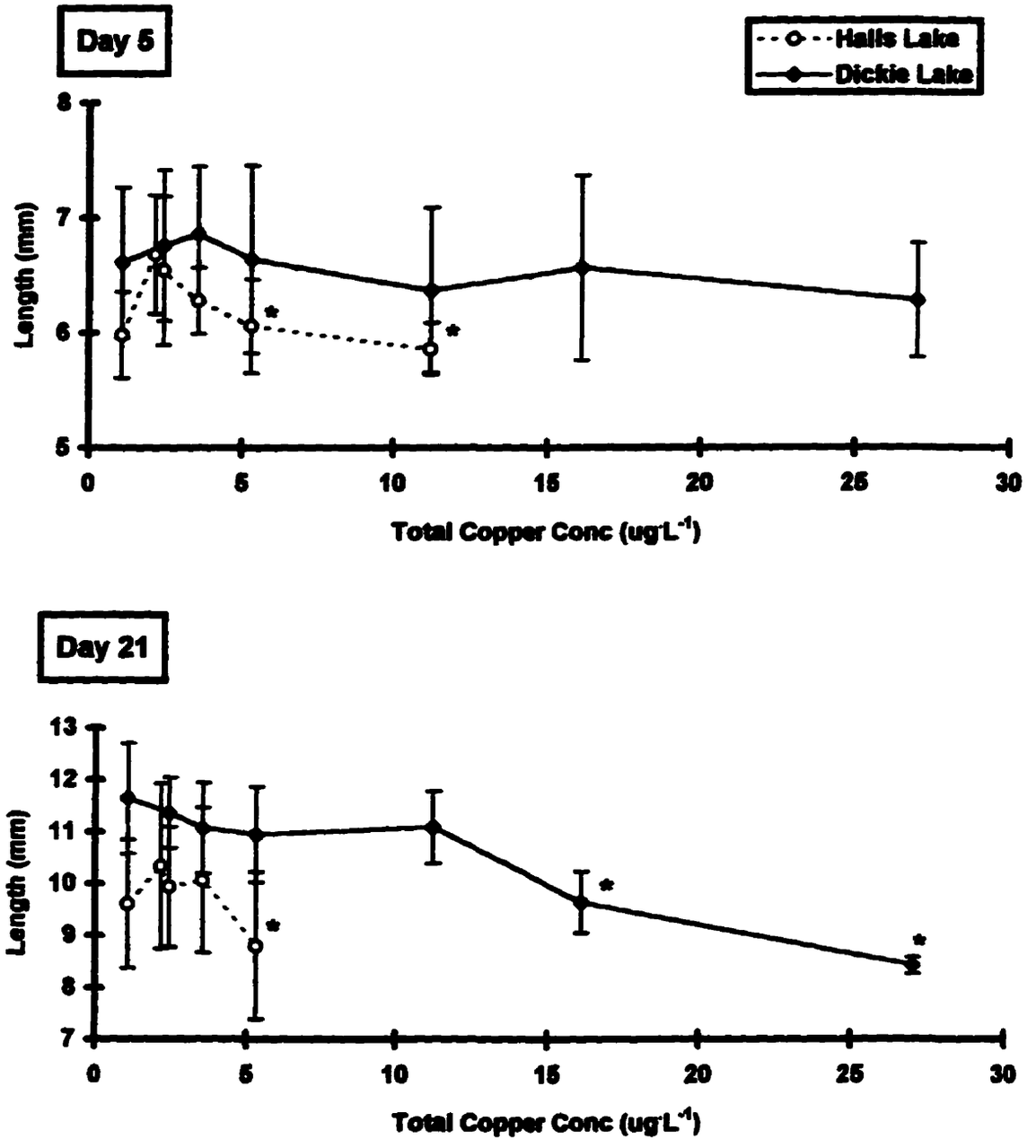


Figure 4.6 Effect of sublethal Cu exposure on fish length. Mean total fish length (+/- SD) after 5 days and 21 days Cu exposure. Values denoted by an * are different from control values as determined by Dunnetts test. For Halls lake only, values denoted by an * are different from fish in the 1.0 nominal Cu exposure as determined by Dunnetts test and not different from control values (see text for details).

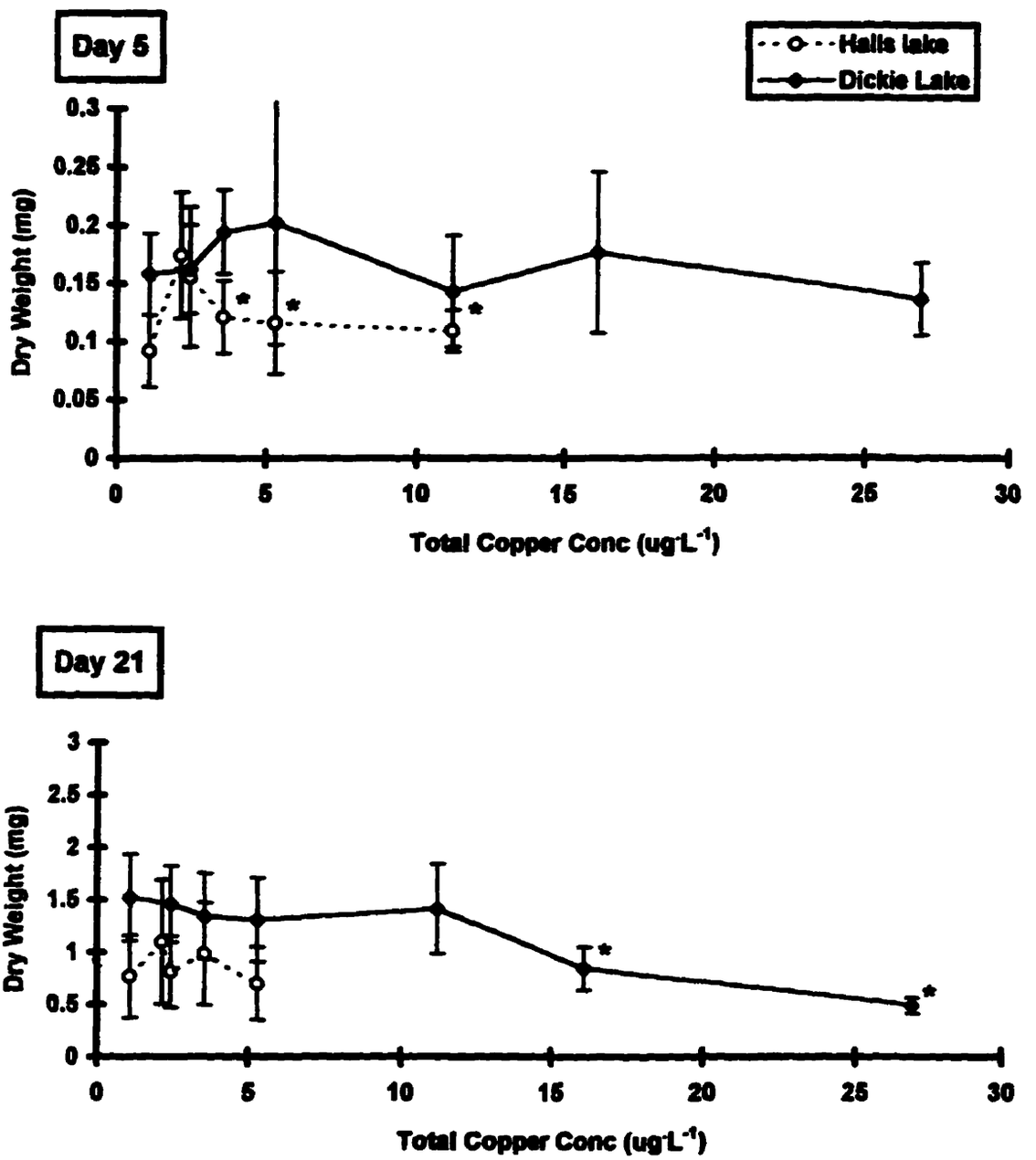


Figure 4.7 Effect of sublethal Cu exposure on fish weight. Mean dry fish weight (+/- SD) after 5 days and 21 days exposure to Cu. Values denoted by an * are different from control values as determined by Dunnetts test. For Halls lake only, Dunnetts test was performed against the mean dry weight for the fish in the 1.0 nominal Cu exposure and not control.

compared to control fish, no differences were detected in total length and dry weight between Cu exposed fish in Halls Lake water after 5 or 21 days Cu exposure. Since total length and dry weight were significantly lower in the control fish of Halls Lake Cu exposure after 5 and 21 days, differences were also tested by comparing total length and dry weight to the lowest Cu exposure concentration (1.0 $\mu g \cdot L^{-1}$ Cu nominal) and not to the control. In this instance, fish exposed to > 5.3 $\mu g \cdot L^{-1}$ Cu in Halls Lake water had reduced total length after 5 days and 21 days (Figure 4.6). Dry weight data were more variable; fish dry weight was reduced after exposure to > 3.6 $\mu g \cdot L^{-1}$ Cu for 5 days in Halls Lake water but no difference was detected after 21 days (Figure 4.7).

As with the Cu exposures, differences were detected between replicates in total length of fish exposed to Cd for 21 days in Halls and Dickie Lake water and dry weight of fish exposed to Cd in Dickie Lake water only (Table 4.7 and 4.8), although differences between replicates appear to be a function of unequal sample sizes. The number of fish in replicate B of the Dickie Lake Cd experiment was very low due to unexplained mortality in the last week as previously mentioned (Figure 4.3 d). As before, the total length and dry weight data for the Cd exposures were pooled.

Total length was reduced in fish exposed to 5.3 $\mu g \cdot L^{-1}$ Cd (but not 8.0 $\mu g \cdot L^{-1}$ Cd) in Halls Lake water and in fish exposed to 8.0 $\mu g \cdot L^{-1}$ Cd in Dickie Lake water after 5 days (Figure 4.8). Dry weight was significantly reduced in fish exposed to 8.0 $\mu g \cdot L^{-1}$ Cd in Dickie Lake water only after 5 days (Figure 4.9). No difference was detected in total fish length and dry weight in fish

Table 4.7 Statistical analysis of differences in total length (mm) and dry weight (mg) in Cd exposed fish after 21 days.

		ANOVA TABLE				
Lake	Variable	Source	df	MS	F-ratio	p value
Halls	Total Length	Conc (C)	4	8.16	4.87	0.002
		Rep (R)	1	3.89	2.32	0.134
		C*R	4	4.46	2.66	0.043
		Error	51	1.68		
	Dry Weight	Conc (C)	4	0.87	3.88	0.008
		Rep (R)	1	0.28	1.22	0.274
		C*R	4	0.39	1.71	0.161
		Error	51	0.22		
Dickie	Total Length	Conc (C)	4	2.30	2.87	0.031
		Rep (R)	1	5.04	6.31	0.015
		C*R	4	0.62	0.77	0.548
		Error	59	0.80		
	Dry Weight	Conc (C)	4	0.65	3.04	0.024
		Rep (R)	1	1.20	5.64	0.021
		C*R	4	0.08	0.36	0.836
		Error	59	0.21		

Table 4.8 Mean total length (mm) and dry weight (mg) to fish exposed to Cd for 21 days (mean±SD (n)). Total length and dry weight of fish exposed to Cd for 5 days shown in Figures 4.8 and 4.9. Values marked with an asterisk are significantly different from each other as determined by students t-test (α=0.05). Values marked with an † are significantly different from the control as determined by Dunnetts test (performed on grand mean estimates only). Values marked with an § denote no t-test was done since one of the replicates had a sample size of 0 or 1.

	Measured Cd conc.	Total Length (mm)			Dry Weight (mg)		
Lake	($\mu g L^{-1}$)	Rep A	Rep B	Grand Mean	Rep A	Rep B	Grand Mean
Halls	0.06	11.00±1.06 (10)	11.70±0.96 (8)	11.31±1.05 (18)	1.21±0.35 (10)	1.41±0.36 (8)	1.30±0.36 (18)
	0.50	11.04±1.54 (15)	8.65±1.73 (4)	10.53±1.83 (19)	1.26±0.48 (15) *	0.55±0.42 (4) *	1.11±0.54 (19)
	0.89	11.96±0.96 (9)	11.58±1.37 (3)	11.87±1.02 (12)	1.61±0.49 (9)	1.42±0.59 (3)	1.56±0.50 (12)
	1.75	12.09±1.28 (4)	11.51±1.44 (6)	11.74±1.34 (10)	1.67±0.53 (4)	1.52±0.68 (6)	1.58±0.59 (10)
	2.90	10.4 (1) §	9.4 (1) §	9.90±0.71 (2)	1.00 (1) §	0.87 (1) §	0.93±0.09 (2)
Dickie	0.06	11.88±0.80 (14)	12.09±0.74 (8)	11.96±0.77 (22)	1.62±0.44 (13)	1.91±0.50 (8)	1.73±0.47 (21)
	0.50	11.65±1.20 (14)	11.86±0.42 (6)	11.71±1.02 (20)	1.51±0.59 (14)	1.69±0.26 (6)	1.57±0.51 (20)
	0.89	11.12±0.47 (7)	11.88±0.55 (3)	11.35±0.59 (10)	1.33±0.25 (7)	1.63±0.51 (3)	1.42±0.35 (10)
	1.75	11.59±1.15 (11) §	13.35 (1) §	11.74±1.21 (12)	1.52±0.48 (11) §	2.33 (1) §	1.58±0.51 (12)
	2.90	10.25±0.07 (2)	11.08±0.45 (3)	10.75±0.56 (5) †	0.90±0.15 (2)	1.17±0.28 (3)	1.06±0.26 (5) †
	5.25	9.35 (1) §	----	9.35 (1)	0.57 (1) §	----	0.57 (1)

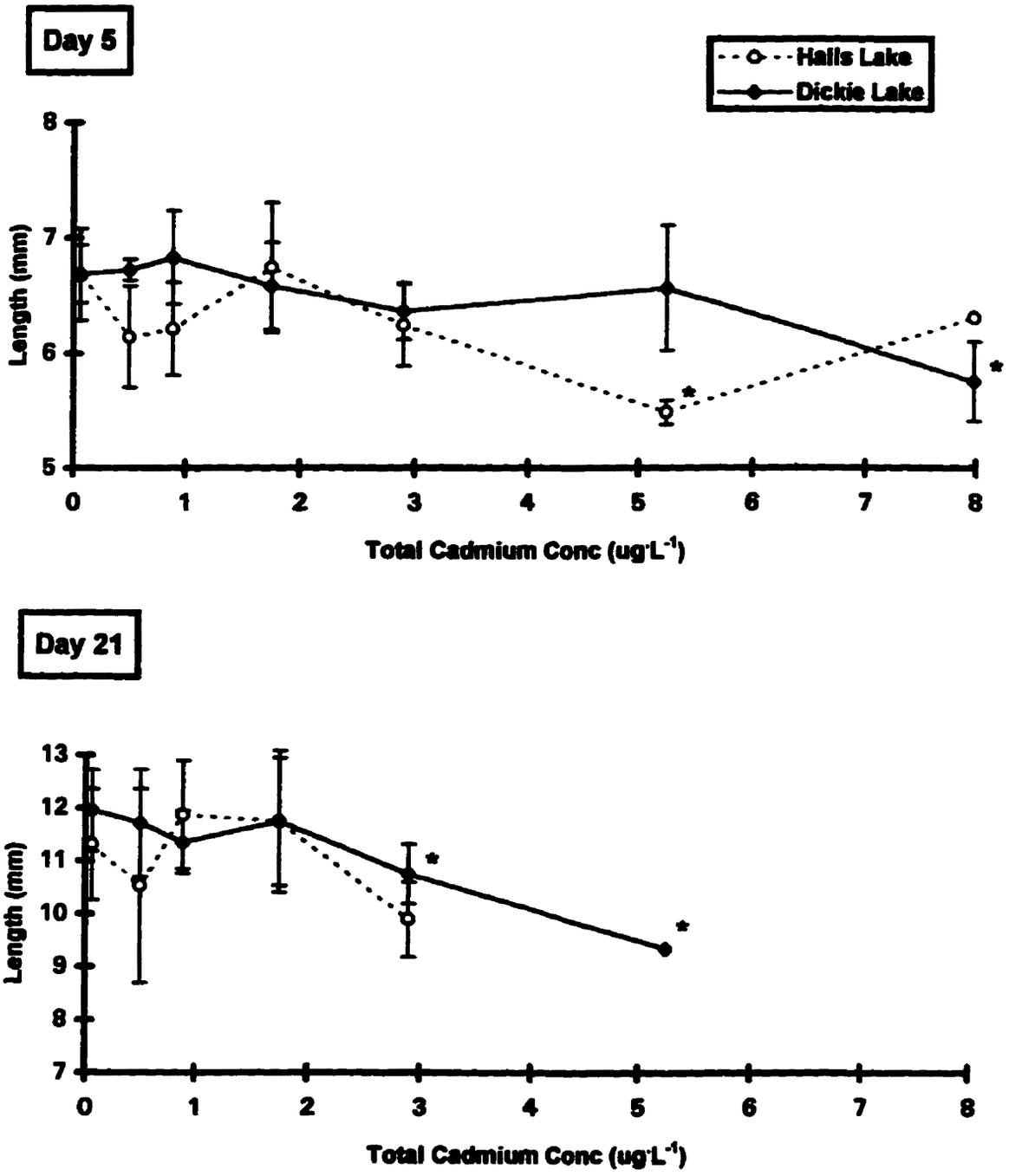


Figure 4.8 Effect of sublethal Cd exposure on fish length. Mean total fish length (+/- SD) after 5 days and 21 days exposure to Cd. Values denoted by an * are different from control values as determined by Dunnetts test.

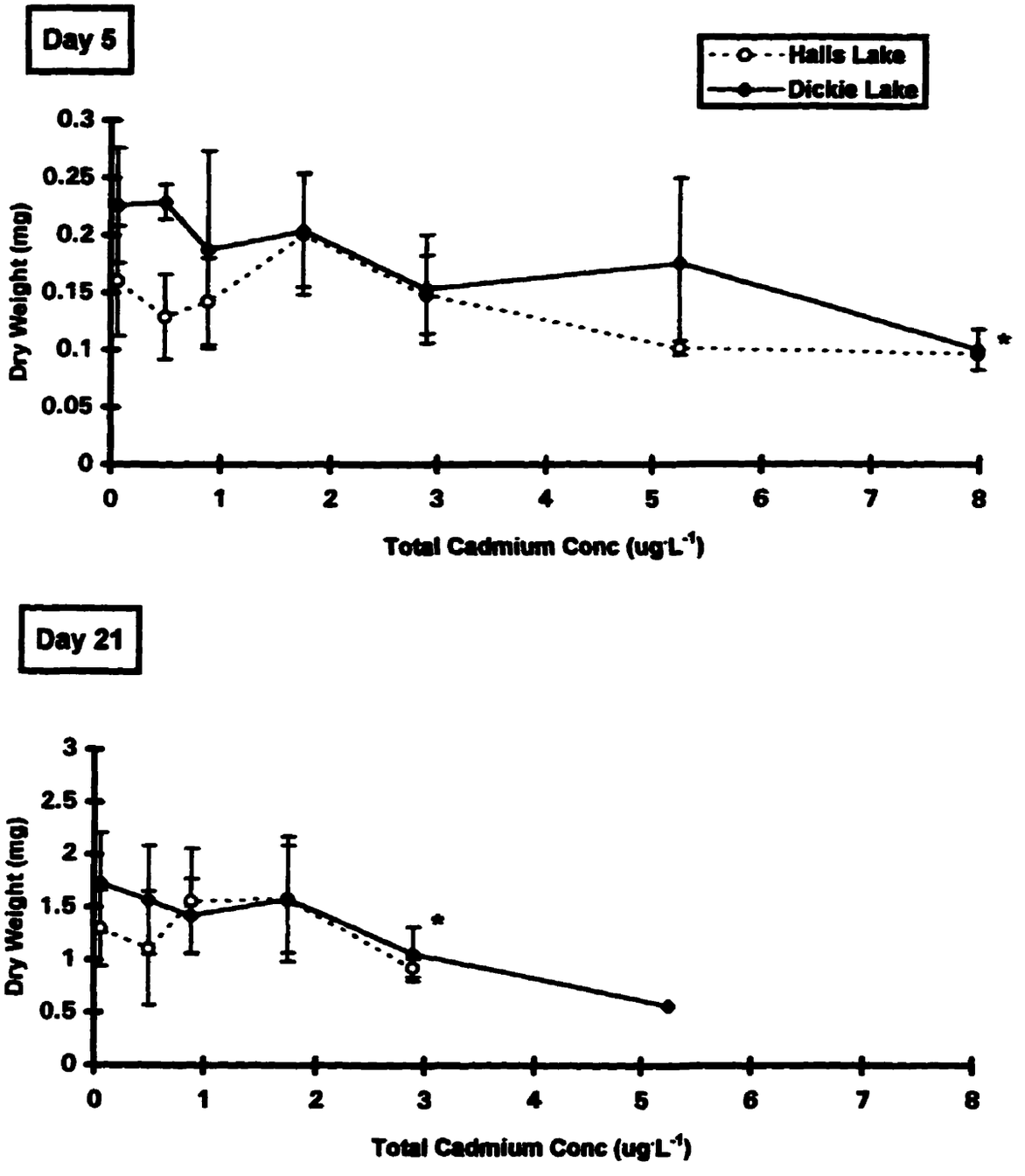


Figure 4.9 Effect of sublethal Cd exposure on fish weight. Mean dry fish weight (+/-SD) after 5 days and 21 days exposure to Cd. Values denoted by an * are different from control values as determined by Dunnetts test.

exposed to Cd in Halls Lake water after 21 days. However, both total length and dry weight were reduced in fish exposed to 2.9 $\mu g \cdot L^{-1}$ Cd for 21 days in Dickie Lake water.

4.4.6 Whole Body Metal Concentration in Fish

Whole body concentration (WBC) of Cu or Cd was determined in individual metal exposed fish. There was a significant block effect in the mean Cu concentration in the acid blanks for the digestions (ANOVA results not shown). The mean Cu concentration in the acid blanks for two digestion blocks were lower than the mean acid blanks in the remaining three digestion blocks. As a result, the concentration of Cu in the mean acid blank was calculated for each set and these values were used instead of the overall mean acid blank for blank correction of the fish tissue samples (Table 4.9). There was also a significant block effect in the mean Cd concentration in the acid blanks used in the Cd exposed fish digestions (ANOVA results not shown). The concentration of Cd in the mean acid blank was calculated for each block and used instead of the overall mean acid blank for blank correction of these fish tissue samples (Table 4.9).

The acid digestion of fish tissue was complete as indicated by the 92% recovery of the Cu concentration in the dogfish muscle tissue standard (DORM-1) (Table 4.9). The elevated percent recovery with the Cd concentration in the DORM-1 standard is a result of contamination of the standard itself, perhaps as a result of drying the DORM-1 slurry in the plastic centrifuge tubes: these tubes were only acid washed with 1% sulphuric acid. In addition,

Table 4.9 **Concentration of Cu and Cd in high purity water, acid blank samples and standard dogfish muscle (DORM-1) (Mean±SD, (n)). High purity water was used to dilute the digestate sample.**

Metal	Sample	Measured Value ($\mu g \cdot L^{-1}$) or ($\mu g \cdot g^{-1}$)	Expected Value ($\mu g \cdot g^{-1}$)	% Recovery
Cu	High Purity Water	0.06±0.04 (16)	----	----
	Overall Acid Blank	0.24±0.13 (30)	----	----
	- Acid Blank (set #1)	0.28±0.14 (19)	----	----
	- Acid Blank (set #2)	0.17±0.06 (11)	----	----
	DORM-1 Tissue standard	4.82±1.41 (20)	5.22±0.33	92%
Cd	High Purity Water	0.01±0.02 (24)	----	----
	Overall Acid Blank	0.14±0.13 (30)	----	----
	- Acid Blank (block #14)	0.09±0.05 (9)	----	----
	- Acid Blank (block #15)	0.25±0.20 (5)	----	----
	- Acid Blank (block #18)	0.15±0.14 (8)	----	----
	- Acid Blank (block #19)	0.11±0.13 (8)	----	----
	DORM-1 Tissue standard	1.14±0.53 (43)	0.086±0.012	1331 %

the Cd concentration in the DORM-1 standard is quite low (0.09±0.01 $\mu g \cdot g^{-1}$), so contamination of 1 $\mu g \cdot g^{-1}$ Cd (1 ppb) appears excessive when expressed as a percentage (Table 4.9).

In contrast to the results with the sublethal effects of Cu or Cd exposure on growth, WBC in exposure tanks were only significantly different from replicates in the Halls Lake - Cu experiment (Table 4.10). In the other experiments, the WBC of the fish in the exposure tanks did not differ between replicates. The difference in the Halls Lake - Cu experiment was in the replicates for the 1 $\mu g \cdot L^{-1}$ Cu exposure where the fish in replicate A had elevated Cu concentrations over the fish in replicate B (replicate A, 8.37 $\mu g \cdot g^{-1}$; replicate B, 4.77 $\mu g \cdot g^{-1}$) (Table 4.11). This difference was not due to elevated Cu exposure since the mean aqueous Cu concentration in these exposure tanks was not significantly different between replicates as determined by Student's t-test (replicate A, 2.21±0.82 $\mu g \cdot L^{-1}$; replicate B, 2.07±0.10 $\mu g \cdot L^{-1}$).

In both Cu and Cd exposures, fish WBC increased with increasing metal exposure (Table 4.11). As a function of total metal concentration, fish in Halls Lake had higher Cu WBCs than fish in Dickie Lake at any given Cu concentration (Figure 4.10). Conversely, fish in Halls Lake and Dickie Lake had similar Cd WBCs at similar total Cd concentrations (Figure 4.10). Fish exposed to Cu at > 2.5 $\mu g \cdot L^{-1}$ in Halls Lake and > 3.6 $\mu g \cdot L^{-1}$ in Dickie Lake had significantly higher WBCs over control fish (Table 4.11). Cd exposed fish had higher WBCs after exposure to the lowest Cd concentrations in water from both lakes (0.50 $\mu g \cdot L^{-1}$ in Halls and Dickie lake water) (Table 4.12).

Table 4.10 **Statistical analysis of difference in whole body Cu or Cd concentration in Cu and Cd exposed fish.**

				ANOVA TABLE		
Metal	Lake	Source	df	MS	F-ratio	p value
Cu	Halls Lake	Conc (C)	4	108.69	32.57	<0.001
		Rep (R)	1	56.57	16.95	<0.001
		C*R	4	5.43	1.63	0.183
		Error	46	3.34		
	Dickie Lake	Conc (C)	5	38.07	36.03	<0.001
		Rep (R)	1	1.78	1.68	0.203
		C*R	5	1.40	1.33	0.274
		Error	37	1.06		
Cd	Halls Lake	Conc (C)	3	123.99	214.95	<0.001
		Rep (R)	1	0.07	0.11	0.739
		C*R	3	0.58	1.01	0.399
		Error	41	0.58		
	Dickie Lake	Conc (C)	4	153.84	162.60	<0.001
		Rep (R)	1	0.02	0.02	0.885
		C*R	4	1.45	1.54	0.212
		Error	37	0.95		

Table 4.11 **Mean whole body concentration to fish exposed to Cu for 21 days (mean±SD (n)). Values marked with an asterisk are significantly different from each other as determined by students t-test (∝=0.05). Values marked with an † are significantly different from the control as determined by Dunnetts test (performed on grand mean estimates only). Values marked with an § denote no t-test was done since one of the replicates had a sample size of 0 or 1.**

Lake	Measured Cu conc. ($\mu g \cdot L^{-1}$)	Whole Body Cu Concentration ($\mu g \cdot g^{-1}$) Rep A	Rep B	Grand Mean
Halls	1.11	6.34±2.12 (7)	4.15±1.42 (4)	5.54±2.13 (11)
	2.18	8.37±1.70 (5) *	4.77±1.30 (11) *	5.90±2.21 (16)
	2.47	8.98±1.69 (3)	8.95±1.65 (11)	8.96±1.59 (14) †
	3.58	16.09 (1) §	12.97±2.04 (7) §	13.36±2.19 (8) †
	5.31	14.98±2.66 (3)	11.26±2.57 (4)	12.86 ±3.10 (7) †
Dickie	1.11	1.43±0.21 (4)	2.05±0.48 (4)	1.74±0.48 (8)
	2.47	2.15±0.74 (4)	2.78±0.98 (6)	2.53±0.91 (10)
	3.58	4.36±1.43 (4)	3.26±0.38 (4)	3.81±1.14 (8) †
	5.31	3.82±0.46 (4)	4.19±0.47 (4)	4.00±0.48 (8) †
	11.18	4.56±0.67 (4)	4.88±0.75 (3)	4.70±0.66 (7) †
	16.12	7.19±1.05 (4)	8.65±2.46 (4)	7.92±1.92 (8) †
	26.99	7.12±0.12 (2) §	----	7.12±0.12 (2) †

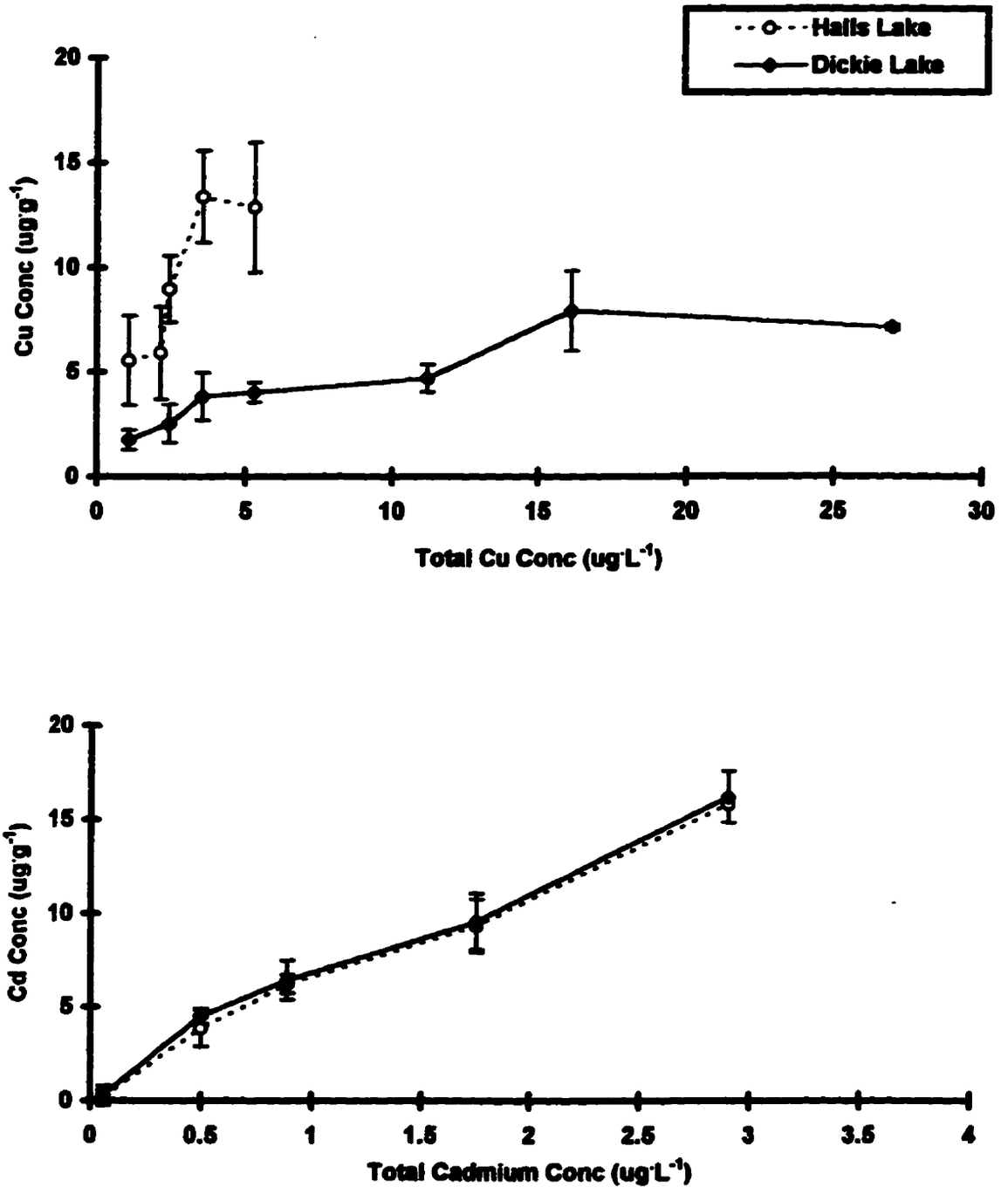


Figure 4.10 Whole Body metal concentration (mean +/- SD, $ug \cdot g^{-1}$) in fish exposed to either Cu or Cd for 21 days.

Table 4.12 **Mean whole body concentration to fish exposed to Cd for 21 days (mean±SD (n)). Values marked with an asterisk are significantly different from each other as determined by students t-test (α=0.05). Values marked with an † are significantly different from the control as determined by Dunnetts test (performed on grand mean estimates only). Values marked with an § denote no t-test was done since one of the replicates had a sample size of 0 or 1.**

	Measured Cd conc.	Whole Body Cd Concentration ($\mu g \cdot g^{-1}$)		
Lake	$\mu g \cdot L^{-1}$	Rep A	Rep B	Grand Mean
Halls	0.06	0.47±0.25 (10) *	-0.07±0.10 (8) *	0.23±0.34 (18)
	0.50	3.81±1.01 (9)	3.95±0.97 (4)	3.85±0.96 (13) †
	0.89	6.14±0.36 (9)	6.48±0.76 (3)	6.23±0.48 (12) †
	1.75	8.98 (1) §	9.42±1.56 (5) §	9.35±1.41 (6) †
	2.90	----	15.79 (1) §	15.79 (1) †
Dickie	0.06	0.77±0.50 (4)	0.04±0.35 (8)	0.28±0.52 (12)
	0.50	4.72±0.36 (4)	4.35±0.38 (6)	4.50±0.40 (10) †
	0.89	6.60±0.99 (7)	6.03±1.29 (3)	6.43±1.05 (10) †
	1.75	9.64±1.52 (11) §	8.66 (1) §	9.56±1.48 (12) †
	2.90	14.60 (1) §	----	16.18±1.38 (3) †

Data from 5 fish Cu WBC's from the Halls Lake - Cu experiment were outliers and not included in the data analysis. If these datapoints were included, the mean WBC for fish exposed to 3.6 $\mu g \cdot L^{-1}$ Cu would decrease from 13.4 to 10.4 $\mu g \cdot g^{-1}$ and for fish exposed to 5.3 $\mu g \cdot L^{-1}$ Cu would decrease from 12.9 to 10.8 $\mu g \cdot g^{-1}$ (Table 4.11). Data from 5 fish Cd WBC's from Halls Lake - Cd experiment and Dickie Lake - Cd experiment were also outliers and not included in the data analysis. These fish WBCs values were clearly contaminated, with data values 50 to 100% higher than other measured values.

4.4.7 Relationship between Metal Speciation and Biological Effects

Comparisons between the biological effects of metal exposure and metal speciation were made at the no observable effect concentration (NOEC) and the lowest observable effect concentration (LOEC) for total length, dry weight and WBC, and at the ILL (Table 4.13). Organic complexation reduced the toxicity of Cu to the fathead minnow. Observed toxic effects of Cu, based on total Cu concentration, were lower in Dickie Lake water (DOC = 6.7 $mg \cdot L^{-1}$) than in Halls Lake water (DOC = 2.3 $mg \cdot L^{-1}$). Differences between the lakes in LOECs for total length, dry weight and WBC in Cu exposed fish when expressed as total Cu, were less when expressed as the dialysis fraction and minimized when expressed as the free Cu ion concentration (Table 4.13). For example, in the Cu exposure experiments in Halls Lake and Dickie Lake water, respectively, the LOEC for growth decreased from 5.3 $\mu g \cdot L^{-1}$ and 16.1 $\mu g \cdot L^{-1}$ total Cu, to 3.5 $\mu g \cdot L^{-1}$ and 6.6 $\mu g \cdot L^{-1}$ dialysed Cu, to 1.1 $\mu g \cdot L^{-1}$ and 1.5 $\mu g \cdot L^{-1}$ free Cu ion (Table 4.13). Free Cu ion concentration estimates at the observed total Cu concentrations where

Table 4.13 No observable effect concentration (NOEC), lowest observable effect concentration (LOEC) and ILL for Cu and Cd exposed fish as a function of total, dialysis fraction (free metal ion plus membrane permeable low molecular complexes) and free metal ion. Values are measured mean total Cu or Cd concentrations ($\mu g \cdot L^{-1}$) in the exposure tanks and predicted dialysis fraction metal concentration or free metal ion concentration (corrected for ionic strength) at corresponding total metal concentrations (calculated using equations 8-13 in chapter 3).

			Halls Lake			Dickie Lake		
Metal	Effect	Parameter	Total	Dialysis	Free	Total	Dialysis	Free
Copper	NOEC	Total Length	3.6	2.3	0.6	11.2	4.7	0.8
		Dry Weight	----	----	----	11.2	4.7	0.8
		WBC	2.2	1.3	0.3	2.5	1.3	----
	LOEC	Total Length	5.3	3.5	1.1	16.1	6.6	1.5
		Dry Weight	----	----	----	16.1	6.6	1.5
		WBC	2.5	1.5	0.3	3.6	1.7	----
	ILL	Lethality	6.2	4.2	1.4	17.2	7.0	1.7
Cadmium	NOEC	Total Length	1.8	1.8	1.4	1.8	1.2	----
		Dry Weight	----	----	----	1.8	1.2	----
		WBC	----	----	----	----	----	----
	LOEC	Total Length	2.9	2.9	1.7	2.9	1.9	0.8
		Dry Weight	----	----	----	2.9	1.9	0.8
		WBC	0.5	0.7	----	0.5	0.5	----
	ILL	Lethality	1.2	1.3	1.0	2.0	1.4	0.7

toxicity is occurring are near the detection limit for the electrode. As a result, these estimates may have a higher degree of error associated with them.

Organic complexation had no or minimal impact on the toxicity of Cd to the fathead minnow. Observed toxic effects of Cd, based on total Cd concentration, were similar or slightly lower in Dickie Lake water (DOC = 6.7 $mg \cdot L^{-1}$) than in Halls Lake water (DOC = 2.3 $mg \cdot L^{-1}$). The ILL was lower in Halls Lake water (1.2 $\mu g \cdot L^{-1}$) than in Dickie Lake water (2.0 $\mu g \cdot L^{-1}$) indicating that Cd was more acutely toxic in Halls Lake water (Table 4.13). No differences were observed between the lake waters in calculated LOECs for total length, dry weight and WBC in Cd exposed fish when expressed as total Cd. However, differences in calculated LOECs for these sub-lethal toxic effects increased when expressed as the dialysis fraction (Table 4.13). Due to the low total Cd concentrations where toxic effects were observed and the uncertainty in measuring the free Cd ion, comparisons based on the free Cd ion concentration are inappropriate. Free Cd ion estimates are reported in Table 4.13 for interest only.

Comparisons between the fish WBC from the two lake waters and metal speciation were also made at all of the metal exposure concentrations. As observed with the NOEC/LOEC comparisons, the difference between the fish WBC when expressed as a function of total Cu, were reduced when expressed as a function of dialysed Cu and free Cu ion concentration (Figure 4.11). In contrast, difference between the fish WBC when expressed as a function of free Cd ion concentration, were reduced when expressed as a function of dialysed Cd and total Cd concentration (Figure 4.12).

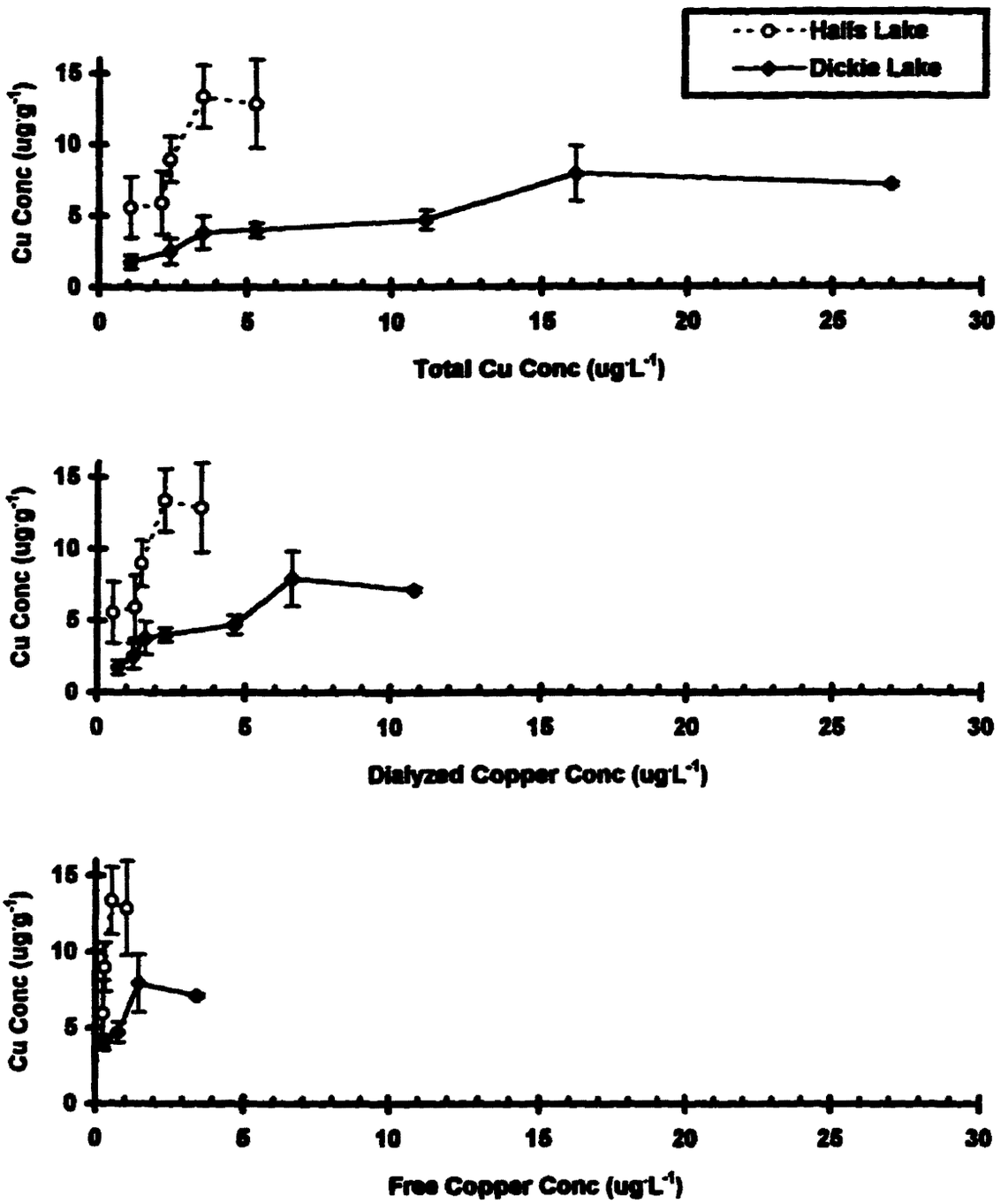


Figure 4.11 Mean (+/- S.D.) whole body Cu concentration ($ug \cdot g^{-1}$) in fish exposed to Cu for 21 days as a function of total Cu, dialyzed Cu or free Cu ion concentration ($ug \cdot L^{-1}$).

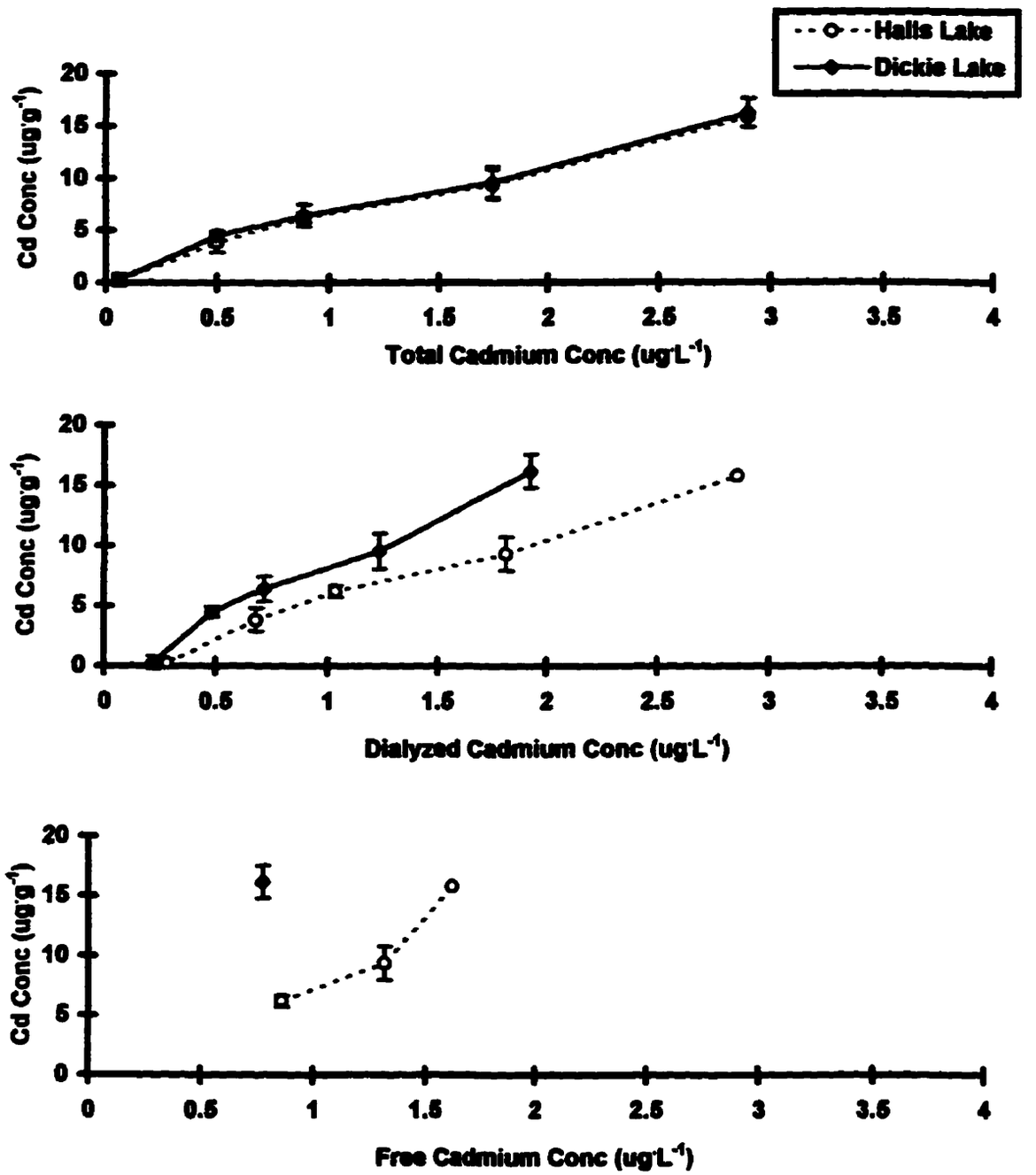


Figure 4.12 Mean (+/- S.D.) whole body Cd concentration ($ug \cdot g^{-1}$) in fish exposed to Cd for 21 days as a function of total Cd, dialyzed Cd and free Cd ion concentration ($ug \cdot L^{-1}$).

4.5 Discussion

4.5.1 Organic complexation and bioavailable metal species

One of the central tenets of the free ion activity model (FIAM) of organism-metal interaction is that the free metal ion activity is proportional to the observed toxicity (Campbell 1995). In this study, this model was tested by measuring metal speciation in two lake waters spiked with Cu or Cd and comparing the metal speciation with the chronic toxicity observed in *P. promelas*. Since the water chemistry is similar between the two lakes, except for DOC concentrations (Table 4.1), differences in the observed toxicity of either Cu or Cd should only be a function of organic complexation of the metal ions in solution. Hence, if the toxicity is a function of the free metal ion, similar effects from metal exposure should be observed between the two lake waters tested when expressed as a function of the free metal ion concentration.

The relationship between the free metal ion concentration and the observed metal toxicity to *P. promelas* between the two lake waters was not consistent for the two metals studied. While Cu toxicity appeared to be a function of the free Cu ion concentration as predicted from the FIAM, Cd toxicity did not appear to be a function of the free Cd ion concentration. Differences in toxic effects to *P. promelas* between the two lakes when expressed as total Cu, were reduced when expressed as the dialysate fraction (that fraction excluding the large molecular weight Cu bound complexes). When effects from the Cu exposure were expressed as a function of the free Cu ion, the toxicity was roughly similar between the two lakes (Table 4.13; Figure 4.11). The

opposite result was observed for Cd. Differences in toxic effects to *P. promelas* between the two lakes were reduced when expressed as total Cd. When expressed as the dialysis fraction, the effects of Cd exposure, except for lethality, were more pronounced in Dickie Lake water than in Halls Lake water (Table 4.13; Figure 4.12). Free Cd ion estimates also indicated that Cd toxicity was more pronounced in Dickie Lake water. These results confirm the acute toxicity observed in the static tests: based on total aqueous metal concentrations, Cu toxicity was a function of the DOC of the test water while Cd toxicity was independent of the DOC of the test water (chapter 2).

The apparent inconsistency of the FIAM can be explained if both the biological and geochemical systems are taken into account. Based on calculated conditional stability constants (log K') for the lake water DOC for Cu and Cd (chapter 3), and log K' values for fathead minnow gill tissue for Cu and Cd (Playle et al. 1993), the gill tissue has a much higher binding affinity for Cd ions (log K' = 8.6) than the DOC (average log K' = 5.3 - 6.0) (Table 4.14). In contrast, the binding affinity for Cu is similar between the gill tissue (log K' = 7.4) and the DOC (average log K' = 7.5 - 8.0).

Since toxicity was observed at low total metal concentrations, and based on the total binding capacity of the DOC in the lake waters (Table 3.6), it is likely that the high affinity ligands are the primary ligands interacting with, and binding to, the free metal ions in solution. Based on total Cu concentrations, toxicity is observed once the Cu concentration exceeds the total binding capacity of the high affinity ligand. For instance, in Halls Lake (based on the Scatchard plot

Table 4.14 Average log conditional stability constants (log K') of lake water DOC based on FITEQL estimates and adult *P. promelas* gill tissue in soft water (Playle et al. 1993).

Lake	Metal	Method	Log K_1'	Log K_2'	Average Log K'	Fish Log K'
Halls Lake	Cu	S-Plot	8.08	5.05	----	7.4
		2L-NLR	8.23	4.89	----	
		FITEQL	----	----	7.47	
Dickie Lake	Cu	S-Plot	7.98	6.20	----	
		2L-NLR	7.93	6.11	----	
		FITEQL	----	----	7.99	
Halls Lake	Cd	S-Plot	5.49	4.63	----	8.6
		1L-NLR	----	----	5.59	
		FITEQL	----	----	5.31	
Dickie Lake	Cd	S-Plot	----	----	6.23	
		1L-NLR	----	----	6.33	
		FITEQL	----	----	6.04	

and the 2-ligand non-linear regression), the total binding capacity of the high affinity ligand was 5.85 μg $Cu \cdot L^{-1}$ (Table 3.6), and reduced growth and the ILL were observed at total Cu concentrations of 5.3 and 6.2 $\mu g \cdot L^{-1}$, respectively (Table 4.13). Similarly, in Dickie Lake, the total binding capacity of the high affinity ligand was ~23.4 μg $Cu \cdot L^{-1}$ (Table 3.6), and reduced growth and the ILL were observed at total Cu concentrations of 16.1 and 17.2 $\mu g \cdot L^{-1}$, respectively (Table 4.13). Comparisons with the total binding capacity with Cd are inappropriate due to the bias in estimating the free Cd ion.

It appears that a kinetically controlled reaction creates a depletion layer near the surface of the gill where metal ions bound to lower affinity DOC ligands (with respect to higher affinity gill ligands) are transferred from the DOC ligands to the gill ligands. This competition reaction between organic ligands and the fish gill for metal ions, appears to occur only with the low affinity Cu ligands, but with all of the Cd ligands. The difference between the two metals is due to the log K' of the high affinity DOC-metal ligands. Since the log K' for the DOC-Cd ligand is 2-3 orders of magnitude below the fish gill ligands, DOC ligand-bound Cd can be outcompeted by the higher affinity fish gill ligand. The stability constant for the high affinity DOC-Cu ligand is similar or higher than the the fish gill (Table 4.14), so exchange reactions between the DOC ligands and fish gill ligands will not predominant. Binding site densities among the DOC ligands and the fish gill ligands, as well as the concentration of other competing ions, are also important in determining equilibrium conditions.

The bioavailable metal concentration appears to be not just a function of the aqueous free metal ion concentration (as predicted by the FIAM) but also of the kinetically controlled competition reaction between the free metal ion concentration, the metal binding ligands on the biological organism (in this case the fish gill) and the metal binding ligands in the external medium. Instead of a "pseudo-equilibrium" between the metal species in the bulk solution and the biological surface (Campbell 1995), there appears to be a "depletion layer" surrounding the organism, where bound metal may become bioavailable based on the kinetics of the dissociation of the metal complex within this layer (Guy 1991).

An alternate hypothesis is that some of the Cd-organic complexes are hydrophobic and uptake is increased due to the hydrophobic-metal complex diffusing through the plasma membrane (Block and Pärt 1986). Similar conclusions were drawn by Giesy et al. (1977) after they observed contradictory results when examining Cd toxicity to the cladoceran, *Simocephalus serrulatus*, and the mosquitofish, *Gambusia affinis*, in soft pond water (hardness 10 $mg \cdot L^{-1}$ as $CaCO_3$; DOC 15.2 $mg \cdot L^{-1}$). They found that organic ligands complexed Cd and reduced the acute Cd toxicity to the cladoceran but not to the mosquitofish and concluded that Cd can exert toxic effects even in the presence of excess organic complexation because either some Cd-organic ligand complexes are toxic (i.e., are bioavailable) or the equilibrium between the organic ligand and the fish favours the free Cd ion concentration (Giesy et al. 1977). Since in this study, toxicity tests were performed with both Cu and Cd using the same lake water and the increased toxicity in the high DOC lake water (Dickie Lake) was only found with Cd and not Cu, it is unlikely that the Cd-complexes are entering the fish as a hydrophobic complex and

the Cu-complexes are not. It is more likely, based on the metal binding characteristics of the DOC and the fish gill, that the uptake is based on kinetic effects. The difference observed by Giesy et al. (1977) between the toxicity of Cd-organic complexes in the cladoceran *S. serrulatus* and the fish *G. affinis* suggests that the stability constant of the fish gill is greater than that of the Cd-organic complex while the stability constant of the cladoceran is equal or less.

Other researchers have also concluded that a kinetic effect may be important in describing the bioavailability of metal complexes, in this case Cu, in the presence of organic compounds. Marr et al (1995) studied Cu toxicity on rainbow trout in reconstituted moderately soft water (hardness = 25 $mg \cdot L^{-1}$ as $CaCO_3$; pH 7.5) using a 3 organic acid mixture as an analog for natural DOC. The DOC mixture had similar conditional stability constants and number of metal binding sites as natural DOC in Panther Creek, ID, USA (MacRae et al. 1995). Marr et al. (1995) concluded that the inorganic Cu plus the Cu bound to low affinity organic ligands (log K' <7) were bioavailable to the fish. They concluded that the Cu bound to low affinity organic ligands were not bioavailable as an Cu-organic ligand complex but rather that the fish gill outcompeted the organic ligand for the Cu (Marr et al. 1996). Borgmann and Charlton (1984) also predicted toxicity of some Cu-organic ligand complexes with natural lake water DOC indirectly using the bioassay method (Borgmann 1981). This method compares the toxicity of the metal in the test water with and without the addition of a known ligand (in this case TRIS). Differences in toxicity provided an estimate of the concentration of metal bound to the added ligand and hence the free metal ion concentration could be calculated.

Data collected from field experiments, using natural DOC provides conflicting information on the importance of DOC in regulating Cd exposure and toxicity. Amyot et al. (1994) observed that Ca, pH and Cd concentration in the water (but not DOC) were key abiotic parameters in predicting Cd accumulation in the amphipod *Gammarus fasciatus*, while Stephenson and Mackie (1988) observed a similar relationship except DOC was significant (but moderately so) in predicting Cd accumulation in *H. azteca*. Hare and Tessier (1996) also observed that DOC as well as pH (but not Ca) were important in predicting Cd concentrations in *Chaoborus punctipennis*. In total, these studies indicate that DOC has a minor but significant effect on regulating the bioavailability of Cd. Of note, Amyot et al. (1994) observed that Cu accumulation in *G. fasciatus* was predicted primarily by pH and secondarily by DOC and sediment organic carbon. These studies were done with uncharacterized DOC; observed differences between Cd accumulation in these invertebrates may be a function of different concentrations and types of metal binding ligands in the DOC as well as possible differences in the affinity of the active binding site ligands on biological membranes as suggested here.

It does not appear from the results of this study, that any metal-organic complexes themselves were toxic. From a graphical illustration of whole body metal concentration (Figure 4.11 and 4.12), it appears that free Cu ion concentration and total Cd concentration (including free and membrane permeable Cd) are bioavailable. Based on the observed toxicity (NOEC and LOEC results), it also appears that free Cu and total Cd are bioavailable (Table 4.13). The results for Cu can not exclude the possibility of some low affinity Cu-ligands being bioavailable in addition to the free Cu ion.

4.5.2 Metal Toxicity Experiments

Overall, the toxicological diagnostics of the tests were satisfactory. The fish responded to the increasing metal concentrations in a concentration dependent fashion and aside from replicate B, control mortality was less than 20%. Unexplained and metal concentration independent mortality for fish in the B replicate was observed in all experiments (Figure 4.2 and 4.3). Mortality in replicate B exposure tanks was not due to any obvious metal contamination (as determined by measured metal concentration in samples taken every 3 days (Table 4.2) nor obvious technician error (in the Halls Lake - Cd experiment, replicate B was treated as replicate A such that feeding, pH, temperature and mortality checks were done on replicate B prior to replicate A with no difference in the mortality pattern). Comparisons of sublethal responses (length, dry weight, WBC) between replicates by ANOVA did show differences between the replicates (Tables 4.5, 4.7 and 4.10) but they were minor and not biologically relevant (Tables 4.6, 4.8 and 4.11). Overall, the reason for the increased mortality in replicate B is unknown, although it may have been due to some physical aspect of the laboratory that affected replicate B to a greater extent than replicate A exposure tanks.

The lake water DOC was constant in all experiments except the Dickie Lake - Cd experiment. In this experiment, the amount of DOC in the water over the first week of the test was higher than subsequent water collection DOC determinations. Since Cd toxicity in fathead minnow larvae was independent of DOC concentration, this increased DOC concentration in the first week appears to have had no consequences on Cd toxicity or bioavailability. However, the

increased DOC concentration would affect the Cd speciation, since the absolute number of Cd binding ligands (i.e., total binding capacity) would increase.

Both larval survival and growth (as determined by total length or dry weight) were sensitive parameters in determining the effect of metal exposure to fathead minnows. Sublethal LOECs for length and dry weight were similar or slightly less than the calculated ILLs for Cu but greater than the ILLs for Cd (Table 4.13). This result is typical for fathead minnow embryo-larval tests where often, the calculated LOEC for length and weight are at metal concentrations exceeding the LC50 and near the metal concentration that results in 100% lethality (Woltering 1984). LOECs for whole body metal concentration were consistently lower than LOECs for total length, dry weight or calculated LC50s. In other words, Cu or Cd residue could be measured in the whole fish tissue at concentrations significantly higher than control fish tissue, but at lower aqueous metal exposure concentrations than required to detect a reduction in growth or larval survival. In addition, whole body metal concentrations increased with increasing metal exposure.

Most of the mortality due to metal exposure occurred in the first 7-10 days of the metal exposures (Figure 4.4 and 4.5) indicating lethal effects were acute responses to Cu and Cd exposure. The impact on growth was an indicator of longer term metal exposures.

4.5.3 Toxic effects of metals to fish in soft water

One of the major results of this study is to reinforce the fact that Cu and Cd are extremely toxic to fish, especially larval fathead minnow, in soft moderately acidic waters. Lethal and sublethal effects reported in this study are at similar or lower concentrations than results published in the literature. Other researchers have observed sublethal effects on fish at similarly low total Cu levels. Marr et al. (1996) observed reduced growth in rainbow trout fry exposed to 4.6 $\mu g \cdot L^{-1}$ Cu in soft reconstituted well water (hardness, 25 $mg \cdot L^{-1}$ as $CaCO_3$) after 20 d exposure. Sauter et al. (1976 cited in Dave and Xiu 1991) measured reduced growth in Brook trout (*Salvelinus fontinalis*) larvae after 60 d exposure to 5 $\mu g \cdot L^{-1}$ Cu in soft well water (unknown hardness and DOC). Dave and Xiu (1991) reported reduced hatchability and delayed hatching times of the cyprinid *Brachydanio rerio* (Zebrafish) at extremely low total Cu levels (0.05 to 1 $\mu g \cdot L^{-1}$) in artificial soft water with no organic ligands present. They noted that other researchers have seen similar effects in soft water but with organic acids present at much higher Cu concentrations (reduced hatchability, 13-32.5 $\mu g \cdot L^{-1}$; delayed hatching, 31 $\mu g \cdot L^{-1}$; references cited in Dave and Xiu 1991).

Our Cd results are also in agreement with the literature with respect to the extreme toxicity of Cd to fish in soft water. The 30-d LC50 for fathead minnow in moderately soft water (hardness 50 $mg \cdot L^{-1}$ as $CaCO_3$) was 2.6 $\mu g \cdot L^{-1}$ (USEPA 1975 in Sprague 1987); a value comparable to the 1.2 $\mu g \cdot L^{-1}$ and 2.0 $\mu g \cdot L^{-1}$ ILLs determined in this study. Wicklund Gylnn (1992) observed

increased mortality in minnows (*Phoxinus phoxinus*) exposed for 140 days at Cd concentrations as low as 0.34 $\mu g \cdot L^{-1}$ in soft water (conductivity = 50 $\mu S \cdot cm^{-1}$; pH not given).

4.5.4 Whole Body Tissue Concentrations

Since the larval fathead minnow tested here are small (mean dry weight in control fish after 21 d was between 0.8 - 1.7 mg), whole fish were used as a surrogate to measure metal accumulation in the target organs (mostly gill, liver and kidney). The concentration of metal in the fish body is a biological measurement of metal bioavailability after 21-d exposure. Over the 21-d metal exposure period, the concentration in the fish tissue was assumed to have reached equilibrium based on previously published values. Dixon and Sprague (1981) noted that tissue residues in Cu exposed rainbow trout (0.5 - 0.9 g dry weight) appeared to reach equilibrium after 14 - 21 day exposure. Wicklund Glynn (1992) also observed that Cd kidney and Cd liver tissue concentrations stabilized after approximately 20 days in Cd exposed minnows (*Phoxinus phoxinus*; 0.2 - 0.6 g body weight). In contrast, however, Marr et al. (1995) noted Cu tissue levels in whole body rainbow trout (0.12 g wet wt.) fry stabilized after 40 days. In general, it is probable that a rapid increase in tissue Cu and Cd concentration occurred before 21-d exposure and that concentrations in the fish used in this study represent metal accumulation after 21-d metal exposure that are proportional to metal bioavailability. Borgmann et al. (1991) demonstrated with *H. azteca* that the body burden in the animal can be an accurate measure of the bioavailable fraction of the metal. They exposed *H.azteca* to Cd in the presence of different complexing agents in dechlorinated Lake Ontario tap water and observed that while 6 wk

EC50s varied widely (from 0.53 to 19 $\mu g \cdot L^{-1}$), bioaccumulated Cd was relatively stable at 38 to 44 $\mu g \cdot g^{-1}$ dry weight.

A threshold whole body tissue concentration (WBC), where presumably, fish with high metal tissue levels died as a result of metal toxicity can be estimated between 7 and 13 $\mu g \cdot g^{-1}$ dry weight for Cu and slightly greater than 16 $\mu g \cdot g^{-1}$ for Cd (Figures 4.11 and 4.12).

4.5.5 Water Quality Objectives

Based on the role of DOC in regulating Cu bioavailability and toxicity, the suitability of current water quality objectives (WQO) for Cu in soft water needs to be reevaluated. In soft water the current water quality objectives for Cu range from 2 $\mu g \cdot L^{-1}$ (CCREM 1986) to 5 $\mu g \cdot L^{-1}$ (MOEE 1994). In Ontario, an interim guideline of 1 $\mu g \cdot L^{-1}$ Cu has been set for soft water lakes. The EPA objective for Cu is based on a hardness regression equation for a 4 day average value not to exceed the value given by $e^{(0.8545[\ln(\text{hardness})]-1.465}$ more than once every 3 years (USEPA 1986). For water with a hardness of 10 $mg \cdot L^{-1}$ (similar to Dickie Lake and Halls Lake) the estimated 4 day average Cu concentration using the EPA regression is 1.65 $\mu g \cdot L^{-1}$. We observed toxic effects of Cu exposure at 5.3 $\mu g \cdot L^{-1}$ in Halls Lake water in the laboratory, 3 times the EPA WQO and equal to the WQO for Ontario.

Water quality objectives are set to protect aquatic biota during long term exposure, and as such an appropriate factor needs to be selected to achieve these goals. Often a safety factor of 10 is

applied to measured effects in the laboratory under the assumption that since we can not accurately mimic the field situation in the laboratory or test all species, a safety factor is a conservative measure to protect aquatic life (MOEE 1994). WQOs also tend to err on the side of caution since toxicants are regulated based on single exposures in the laboratory but in the environment, organisms are often exposed to multiple toxicants. For example, Enserink et al. (1991) has demonstrated that a mixture of 8 metals (Ar, Cd, Cr, Cu, Hg, Pb, Ni, and Zn) at maximum concentrations of the Dutch water quality criteria, were severely toxic to *Daphnia magna* and *Salmo gairdneri* resulting in 94% and 50% mortality, respectively. In fact, at concentrations 5 times lower than the Dutch water quality criteria the metal mixture was toxic resulting in a 10% decrease in density of *D. magna* populations and reduced growth in *S. gairdneri*. Spehar and Fiandt (1986) also observed a dramatic reduction in rainbow trout and *Ceriodaphnia dubia* survival at the maximum water quality criteria concentration for As, Cd, Cr, Cu, Hg, and Pb in Lake Superior water. If we apply a safety factor of 10 to the ILL for Halls Lake after 21-d Cu exposure (6.2 $\mu g \cdot L^{-1}$), then the recommended safe Cu concentration in this lake water would be 0.62 $\mu g \cdot L^{-1}$. Alternatively, applying a safety factor of 10 to the NOECs for reduced fish growth in Halls Lake would result in a WQO for Cu of 0.36 $\mu g \cdot L^{-1}$. These levels are extremely low and near the current Cu levels in many soft water lakes. An alternative to recommending one WQO for Cu to apply for all soft water lakes, is to take a site-specific approach that takes into consideration DOC as well as other modifying factors of Cu toxicity. In soft water lakes, these modifying factors of Cu toxicity are primarily DOC, pH and Ca concentrations (Welsh et. al 1993, 1996).

The lack of any relationship between DOC and Cd precludes the need to reexamine the WQO's for Cd with respect to the influence of DOC. Current WQO's for soft water are 0.2 $\mu g \cdot L^{-1}$ (MOEE 1994, CCREM 1986); levels an order of magnitude below the NOEC and LOEC for effects of Cd exposure to fathead minnows described in this study. The Canadian WQOs are similar to the USEPA objective for Cd which is also based on a hardness regression equation for a 4 day average value not to exceed the value given by $e^{(0.7852[\ln(\text{hardness})]-3.490}$ more than once every 3 years (USEPA 1986). For water with a hardness of 10 $mg \cdot L^{-1}$ as $CaCO_3$ (similar to Dickie and Halls lakes) the 4 day average Cd concentration is 0.186 $\mu g \cdot L^{-1}$.

4.5.6 The importance of dissolved organic carbon

DOC in soft water lakes is an important parameter in regulating Cu bioavailability, but not Cd bioavailability, to aquatic biota. In acute and chronic Cu exposure, DOC clearly modifies the Cu toxicity to larval fathead minnow. With very low calcium concentrations and moderately acid pH levels common in soft water lakes on the Precambrian Shield, organic complexation of metals in the water column is an important mechanism regulating the concentration of the free aquo metal ion.

DOC concentrations in soft water boreal lakes in North America have been declining as a result of acidification (Dillon et al. 1987), and increased water residence times due to warmer climates and reduced precipitation (Schindler et al. 1992). In moderately acidic Plastic Lake, Dillon et al. (1987) observed a reduction in DOC of 0.1 mg C/L/year (from approximately 2.7 to 2.1 $mg \cdot L^{-1}$

over 6 years) as the pH decreased from 5.8 to 5.6. These decreases in DOC as a result of acidification and climate warming have resulted in increased UV-B penetration in lakes (Schindler et al. 1996, Yan et al. 1996b). Photolytic byproducts of DOC from DOC-UV-B interactions may be more accessible to microbial degradation and the increased UV-B penetration and microbial breakdown may result in a positive feedback loop, further reducing the concentration of DOC in the water column (Schindler et al. 1996). The increase in UV-B penetration will only add to the stress of acidification on aquatic biota (Yan et al. 1996). In terms of metal-DOC concerns, reductions in DOC may have dramatic impacts on trace metal transport and retention through a watershed and metal speciation. Decreased concentrations of DOC will ultimately reduce the ability of the DOC to buffer the free Cu ion concentration (among other metals) and may result in shifts in chemical equilibrium favouring the free metal ion. The significance of these global pollution events (acidification, global climate change and increased UV radiation due to stratospheric ozone thinning) need to be considered in assessing the long term protective effect of DOC in soft water lakes on metal bioavailability and toxicity to aquatic biota.

4.5.7 Conclusions

The toxicity of Cu and Cd to larval *P. promelas* and the role that organic complexation has on modifying metal speciation and toxicity was examined in this study. Both Cu and Cd are extremely toxic to larval fathead minnow. Cu was toxic at or near current water quality

objectives set to protect aquatic life in soft water lakes. Organic complexation modified Cu toxicity but had little or minimal effect on Cd toxicity.

Based on total metal concentrations, Cu toxicity was higher in water collected from Halls Lake than water collected from Dickie Lake. Incipient lethal levels for Cu exposure ranged from 6.2 $\mu g \cdot L^{-1}$ in Halls Lake to 17.2 $\mu g \cdot L^{-1}$ in Dickie Lake. The lowest observed effect concentrations (LOEC) for total length in fish exposed to Cu in Halls Lake was 5.3 $\mu g \cdot L^{-1}$ and the LOEC for total length and dry weight in fish exposed to Cu in Dickie Lake was 16.1 $\mu g \cdot L^{-1}$. Based on lethality, Cd was more toxic in Halls Lake than in Dickie Lake. Incipient lethal levels after Cd exposure ranged from 1.2 $\mu g \cdot L^{-1}$ in Halls Lake water to 2.0 $\mu g \cdot L^{-1}$ in Dickie Lake water. However, sub-lethal indicators of Cd toxicity were similar between the two exposure waters. The LOEC for total fish length from Halls Lake water (estimated to be approximately 2.9 $\mu g \cdot L^{-1}$) was similar to the calculated LOECs for total length and dry weight for fish exposed to Cd in Dickie Lake water (2.9 $\mu g \cdot L^{-1}$).

Cu and Cd speciation was measured in the exposure water from both Halls and Dickie Lake (see chapter 3). Differences in the observed toxic effects between the 2 lakes when expressed as total Cu were reduced when expressed as the dialysis fraction. When effects from the total Cu exposure were expressed as a function of the free Cu ion, the toxicity was roughly similar between the two lakes. The opposite result was observed for Cd. Differences in the observed toxic effects between the 2 lakes were reduced when expressed as total Cd. When expressed as the dialysis fraction, the effects of Cd exposure were more pronounced in Dickie Lake than in

Halls Lake. Free Cd ion estimates also indicated that the effects of Cd exposure were more pronounced in Dickie Lake water.

These results suggest that the free ion activity model (FIAM) of organism-metal interaction may not be applicable to all divalent metals. With naturally occurring DOC, the model was validated for Cu (a metal that forms strong covalent bonds with organic ligands) but results were ambiguous for Cd (a metal that forms weak electrostatic bonds with organic ligands). Because of uncertainty in the free Cd ion estimates, it was not possible to rigorously test the FIAM with respect to Cd toxicity. However, based on dialysis results, it is clear that Cd toxicity is not due to the free Cd ion concentration only, since some Cd is bound to large MW complexes (>1000 MWCO) in Dickie Lake water and yet similar sub-lethal toxic effects were observed between the two lakes based on total Cd exposure.

Metal binding characteristics of DOC ligands and fish gill ligands, as well as competition between them for metal ions, appear to explain this discrepancy. Metal ions bound to low affinity DOC ligands appear to bind to the stronger high affinity binding sites on the fish gill. A steady state or equilibrium condition between the metal ions and the ligands on the DOC as well as the ligands on the fish gill occurs apparently only in the vicinity of the fish gill.

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6.0 Glossary

A250	Absorbance at 250 nm
Ad	Watershed Area
Ao	Lake Area
ASV	Anodic Stripping Voltammetry
CC	Complexation Capacity
DOC	Dissolved Organic Carbon
DORM-1	Standard tissue samples of Dogfish muscle
EDTA	Ethylene diamine tetraacetic acid
FIAM	Free Ion Activity Model
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
K	Conditional Stability Constant.
I	Ionic Strength
ILL	Incipient Lethal Concentration
ISA	Ionic Strength Adjuster
ISE	Ion Specific Electrode
MWCO	Molecular Weight Cut Off
LC50	Median Lethal Concentration
Log K	Log conditional Stability Constant
NTA	Nitrilo triacetic acid
ppm	Parts per million ($mg \cdot L^{-1}$)

ppb	Parts per billion ($\mu g \cdot L^{-1}$)
Tris	Tris-hydroxymethyl-aminomethane
WBC	Whole Body (metal) Concentration
WQO	Water Quality Objective