Development of an Acute Biotic Ligand Model for Ni Toxicity to *Daphnia pulex* in Soft Water: Effect of Ca, Mg, Na, K, Cl, pH and Dissolved Organic Matter

Development of an Acute Biotic Ligand Model for Ni Toxicity to *Daphnia pulex* in Soft Water:

Effects of Ca, Mg, Na, K, Cl, pH and Dissolved Organic Matter

by

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Title: Development of an acute Biotic Ligand Model for Ni toxicity to *Daphnia pulex* in soft water: effect of Ca, Mg, Na, K, Cl, pH, alkalinity and dissolved organic matter.

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Abstract

In this study the influence of several water chemistry parameters on the toxicity of Ni to *Daphnia pulex* in soft water were tested. A reconstituted soft water (pH 7.8, hardness 31.5 mg/L $CaCO_3$) was used as the basis for culture and testing. *Daphnia pulex* was chosen as a typical cladoceran, one which can be acclimated to very soft water. An understanding of the influence of water chemistry on Ni toxicity in soft water is relevant because metals have higher bioavailability in soft water. The 48h EC50 in the reconstituted soft water (RSW) was 974 µg/L (16 µM) dissolved Ni (95% CI 830 – 1081 µg/L). The following factors were examined for their potential for modifying Ni toxicity: Ca, Mg, Na, K, Cl, pH (3 different approaches used) and natural organic matter (NOM, 2 sources tested). Both Ca and Mg protected against Ni toxicity and the relative effect was greater for Ca. Varying the concentrations of Na, Cl or K did not alter the toxicity of Ni. Tests at different pH showed that as pH increased, Ni toxicity decreased. When the test solution pH was adjusted with the organic buffer 3-morpholinepropanesulfonic acid, there was a clear correlation between increasing pH and increasing EC50. The pH tests using bicarbonate to adjust pH did not show this relationship as clearly. Both types of NOM showed a protective effect on Ni toxicity with Nordic Reservoir NOM having a 4 fold greater effect than that of Suwannee River NOM. This research illustrated that the effect of alterations in water chemistry were generally as predicted within the context of the biotic ligand model (BLM) approach. The data provide the information required to develop a BLM for the acute effects of Ni in soft water.

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Table of contents

List of Tables

List of Figures

List of Equations

Abbreviations

Alk – alkalinity

AWWA – American Water Works Association

BLM – Biotic Ligand Model

CANMET MMSL – Canada Centre for Mineral and Energy Technology Mining and Minerals Sciences Laboratories

CETIS - Comprehensive Environmental Toxicity Information System

CPMAS ^{13}C NMR - cross polarization/magic angle spinning ^{13}C Nuclear Magnetic Resonance spectroscopy

DOM – Dissolved Organic Matter

EC50 – Effective Concentration for 50% immobilization of test-organisms

EPA – Environment Protection Agency of the United States

ICP-AES – Inductively Coupled Plasma Atomic Emission Spectroscopy

IHSS – International Humic Substances Society

LCL – Lower Confidence Limit

MATC – Maximum Acceptable Toxicant Concentration

MOPS - 3-morpholinepropanesulfonic acid

NOM – Natural Organic Matter

NRNOM – Nordic Reservoir Natural Organic Matter

RSW – Reconstituted Soft Water

SNOM – Suwannee River Natural Organic Matter

UCL – Upper Confidence Limit

YCT – Yeast Cerophyll Trout feed suspension

CHAPTER ONE

GENERAL INTRODUCTION AND PROJECT OBJECTIVES

1.1 Ni in the environment: occurrence, chemistry and toxicity.

1.1.1 Sources and Chemistry of Ni

Nickel is a common metal in the terrestrial and aquatic environment. In nature, Ni originates from multiple sources including erosion and weathering of bedrock materials, volcanic eruptions, forest fires, and deposition of cosmic dust (Rasmussen 1996). Also, plants and animals contain small amounts of nickel and following death, decay processes release this into the environment (Eskew et al., 1984; Watt and Ludden, 1999; Whitehead, 2000). The most common oxidation state of nickel is +2 and although 0, +1, +3 and +4 states can also exist, the divalent ion (Ni^{2+}) is the dominant form. In aquatic systems Ni occurs as the hydrated free ion $(Ni(H_2O)_6)^{2+}$ but it also, through complexation with negatively charged anions, forms neutral complexes. For example, Ni complexes with inorganic anions such as OH^-, SO_4^{2-}, and HCO_3^-, and also can bind to the carboxyl groups of natural organic matter (NOM; Schroeder et al., 1974; Nielsen, 1980). The degree of inorganic complexation and the formation of these complexes are dependent on the chemistry of the receiving water, including the concentration of Ni and NOM.

Background levels of Ni in uncontaminated natural waters are usually about 100 times lower than the concentrations that are toxic to most aquatic organisms (Eisler, 1998; Keithly et al., 2004). Typical concentrations for Ni in uncontaminated fresh waters range from 1 to 10 μg/L while for marine systems this range would be 0.2 to 0.7 μg/L (Jenkins, 1980; Eisler, 1998).). Threshold concentrations for *Daphnia magna* are 10-20 μg/L Ni or 0.17-0.34 μM (as $NiSO_4$) in waters containing 0.5 mM Ca. Maximum acceptable

toxicant concentration (MATC) for Ni varies among countries and in many jurisdictions; this varies depending on hardness of water. Within Canada, the CCME (Canadian Council of Ministers of the Environment) water quality guideline value for Ni in freshwater ranges from 25 μg/L or 0.42 μM when hardness is less than 60 mg/L $CaCO_3$ to 150 μg/L or 2.5 μM at hardness values above 180 μg/L (CCME 2006). This value represents a conservative assessment of chronic toxicity endpoints. The USEPA acute water quality criteria (CMC) is calculated based on a hardness equation and ranges from 120 μg/L or 2.0 μM at hardness value of 20 to 470 or 8.0 μM at a hardness of 100 mg/L $CaCO_3$ (US EPA 2007).

Increases in Ni concentrations in the environment can occur naturally, for example due to volcanic processes and forest fires (Eisler, 1998), and can also occur due to anthropogenic emissions. Elevated Ni concentrations in the environment result from mining, smelting, refining, alloy processing, fossil fuel combustion, and waste incineration activities (NAS, 1975; WHO, 1991; Eisler, 1998). In highly contaminated fresh waters, dissolved Ni concentrations may reach as high as a 1 mg/L and in extreme cases, for example lakes near Sudbury ON, up to 200 mg/L (Eisler, 1998).

1.1.2 Nickel as an essential micronutrient.

Ni has been recognised as an essential micronutrient for living organisms. The role of Ni in metabolism varies among different taxa. For example, in animals Ni plays roles in lipid metabolism, hematopoeisis, and the delivery of oxygen to cells. Ni has important roles in the enzyme systems of terrestrial and aquatic animals (Nielsen, 1980, 1991; Calamari et al., 1982; Phillips et al., 2002). For example, NiFe-hydrogenases, urease, and coenzyme F430 contain nickel (Szilagyi, 2004). The role of Ni in metabolic processes of aquatic organisms is not well known, and needs further investigation (Anke

et al., 1995; Muyssen et al., 2004). The vital importance of Ni as an essential micronutrient has been shown for algae (USEPA, 1986; Eisler, 1998; Szilagyi, 2004). Ni is consistent with the actions of other micronutrient transition metals such as Fe, Cu and Zn (Szilagyi, 2004; Simkiss and Taylor, 1989).

1.1.3 Ni toxicity.

1.1.3a. Aquatic chemistry of Ni in relation to toxicity

As with all elements, Ni is toxic above a threshold concentration. This threshold is different for different organisms (Eisler, 1998; Mussen et al., 2004). As was mentioned above, Ni occurs in aquatic systems as a mixture of free and complexed species. The bioavailability of Ni species varies and therefore the development of toxicity will be dependent on the underlying water chemistry and the bioavailability of the Ni species that are present. The most toxic form of Ni in the aquatic environment is the free Ni^{2+} ion (Anderson and Morel, 1978). Ni complexes are usually less toxic than free ions.

1.1.3b. Sensitivity of species to Ni

While the toxicity of Ni in aquatic media is influenced by the chemistry of the solution, there are also dramatic differences among species in their response to Ni. Several aquatic species such as *Ceriodaphnia dubia* (Schubauer-Berigan et al., 1993; Keithly et al., 2004) and *Hydra sp.* (Mandal et al., 2002; Santiago-Fandino, 1983) appear to be notably more sensitive to short term Ni toxicity (48h LC50s about 200 and 150 μg/L respectively). On the resistant side of the spectrum, caddisfly (*Clistoronia magnifica*) larvae and the oligochaete, *Tubifex tubifex*, are highly tolerant of Ni toxicity with reported 48h LC50 values above 10000 μg/L (Keithly et al, 2004). A detailed review of the sensitivities of species to Ni is given by Eisler (1998). Smaller and younger organisms are usually more

sensitive to Ni compared to older and larger organisms of the same species (Grosell et al., 2002; Bossuyt and Janssen, 2005).

1.1.4 Mechanism of nickel toxicity

In comparison to some other divalent metals (e.g. Cu and Zn), the mechanisms of Ni toxicity for aquatic organisms are not well understood. Ni uptake and intracellular fate are different from those of other metals. According to Gunther et al. (1986), Ni enters cells through a Mg^{2+}-HCO_3^- co-transport pathway. This was further supported by investigations conducted on *Salmonella typhimurium* (Snavely et al., 1991) and *Daphnia magna* (Pane et al., 2003a, 2004a) showing linkages between Mg balance and Ni. As well, it has also been demonstrated in *in vitro* studies using rabbit hepatocytes and high metal concentrations that Ni^{2+} can substitute for Ca^{2+} in annexin proteins and stimulate the Ca^{2+}–dependent process of protein binding to phospholipid membranes (Mel'gunov et al., 2000). Ni^{2+} also can form triple complexes with liposomes and annexins (Genge et al., 1989) instead of Ca^{2+}. The significance of this substitution and binding is not clear but it illustrates that Ni^{2+} and Ca^{2+} can be linked physiologically.

Intracellular mechanisms of Ni toxicity have been described for terrestrial organisms, in particular Ni-mediated damage to DNA and proteins as well as an inhibition of cellular antioxidant defenses (Suderman et al., 1984; Rodriguez et al., 1996). In aquatic animals however, the exact mechanisms of Ni toxicity in aquatic animals are poorly understood. However there appear to be significant differences between vertebrates and invertebrates. In fish Ni is a respiratory toxicant (Pane et al., 2003b; 2004b) and does not principally affect ion regulation. However, for *Daphnia magna*, Ni is an ionoregulatory toxicant and not a respiratory one (Pane et al., 2003a).

1.2 Water chemistry and nickel toxicity

1.2.1 Geochemical speciation

Water chemistry is an important determinant in the speciation of and therefore, the bioavailability of Ni. As a result, the chemical composition of the medium directly influences the acute and chronic toxicity of Ni. Water chemistry, particularly changes in chemistry, can also have a marked effect on aquatic organisms, for example in ion regulation dynamics and membrane permeability (Hunn, 1985; Pollard and Rojas, 1988; Taylor et al 2000). Therefore, understanding the bioavailability of metals under different water chemistry conditions and relating that to the potential for impacts are important areas of study. Previous studies in this field were done on Cu, Cd, Zn, and Ag (e.g. Sunda, 1976, 1978; Allen et al., 1980; Bury et al., 1999) but research on Ni is not as well advanced.

Characterization of the geochemical speciation of metal in aquatic systems has been an important approach for understanding metal toxicity. Central components in this research were chemical equilibrium models such as MINEQL+ (Westall et al., 1976, 1991) and also the free ion activity model FIAM (Morel, 1983; Campbell, 1995). Other models for predicting metal speciation include the Windermere Humic Aqueous Model (WHAM; Tipping and Hurley, 1992; Tipping, 1998) and the Non-Ideal Competitive Adsorption model (Benedetti et al., 1995). These models incorporate many geochemical and physical parameters that can be (potentially) important in modifying the bioavailability and hence the toxicity of metals. These include cationic competition, inorganic complexation and binding to natural organic matter (NOM).

1.2.2 Complexation

Factors than can influence (modify) the toxicity of metals in aquatic organisms are grouped into two categories, those that complex metals and those that compete for metal uptake. Complexation of the free ion form (i.e. Ni^{2+}) of the metal by a ligand generally reduces the bioavailability of that metal thereby making uptake, and subsequently toxicity, less likely. The strength of the ion-ligand interaction, as measured through conditional equilibrium constants (log K values), relative to the strength of the ion-organism (or ion-biotic ligand; BL) interaction provides a measure of how effective the change in bioavailability is. For example, strong metal-ligand interactions which have high log K values relative to the ion-BL are very effective at reducing metal toxicity. In the case of a weak ion-ligand interaction (i.e. comparatively lower log K for the metal-ligand interaction) the metal will still bind and be taken up by the organism and the ligand will not be effective in reducing toxicity. An example of this is NOM, which strongly binds and therefore has a strong protective effect against Cu, Zn and Cd toxicity (Smith et al., 2002; Richards et al., 2001), but provides weak protection against Ni toxicity (Delebeeck et al, 2007b;Wu et al., 2003). In general, anions that can bind metal and NOM are considered as complexing factors that have the potential to influence toxicity.

1.2.3 Ion competition.

The expression of metal toxicity also depends on the amount of free metal ions bound to biotic ligands or sites of toxic action, which are often considered to be ion channel or transport proteins (Pagenkopf, 1983; Meyer, 1999). Competition interactions that can reduce metal toxicity are focussed on the metal ion to biotic ligand interaction (Me^{2+}-BL). Cations that can interact at the site of Ni uptake have the potential to

compete with Ni^{2+} ions, displacing them, reducing Ni uptake and as a result, decreasing toxicity. The higher the concentration of competitive ion, the less bioavailable the metal becomes (Zitko, 1976; Di Toro et al., 2001; Wu et al., 2003). The protective effect of hardness is an example of cationic competition where Ca^{2+} and Mg^{2+} compete with Ni^{2+} for binding on the biotic ligand (Pane et al., 2003a; Deleebeeck et al., 2007a,b). Indeed hardness has been shown to provide a high level of protection against Ni toxicity (Chapman et al., 1980; Long et al., 2004; Keithly et al., 2004; Hoang et al., 2004).

Not just any ions can compete with each other for passage through the channels. They have to have some common properties related to relative affinity for binding to the biotic ligand. Examples of atomic features related to binding of uptake channels include radius and charge. However, ion radius can be slightly different depending on the coordination number of this ion in a complex (Shannon and Prewitt, 1969). According to Werner's theory of complex substances, coordination number can also vary dependently on type and concentration of ligands, as well as temperature of solution (De, 2003). Ionic charge does not always play a major role in the process of competition.

1.2.4 Effects of pH

The effect of altering pH combines both competition and complexation. As pH increases, the complexation of Ni^{2+} changes, particularly with the formation of carbonate and hydroxide species. As well, protons, if they compete for Ni^{2+} uptake sites, can be a factor in altering the expression of toxicity as pH changes. Some investigators have examined the effect of pH on Ni toxicity and have obtained different results. Reader et al. (1989) and Pyle et al. (2002) agree that as pH increases, especially between 7.0 and 8.4, toxicity decreases. However, other studies report no effect of pH on Ni toxicity (Van

Sprang and Janssen 2001; Hoang et al. 2004). Indeed an increase in Ni toxicity with an increase in pH has been reported in *Ceriodaphnia dubia (*Schubauer-Berigan et al., 1993; Parametrix Inc., 2004), and in *Daphnia magna* (Deleebeeck et al., 2007b). Therefore, the effect of pH on Ni toxicity is complex and requires further study in order to develop a mechanistic understanding.

1.2.5 Soft vs. hard water.

As was discussed above, water hardness usually plays an important role in the process of metal bioavailability. Hard water is highly protective against the majority of trace metals, including nickel. This is because of the high concentrations of competitive ions (Ca and Mg). In addition, hard water contains high concentrations of many inorganic ions capable of forming metal complexes. On the other hand, soft water lacks competitive factors so generally the bioavailability of metals is greater. Deleebeeck et al. (2007a,b) highlight the importance of evaluation of separate influence of Ca and Mg on nickel toxicity. Several authors have focused attention on consideration of the Ca: Mg ratio in metal toxicity studies (Allen and Hansen, 1996; Welsh et al., 2000; Naddy et al., 2002). They found that Ca and Mg may affect metal toxicity differently. This conclusion coupled with the fact that the Ca:Mg ratio in standard laboratory-reconstituted waters often differs from the ratio in natural surface waters (Naddy et al., 2002) makes the consideration of the influence of ratio on metal toxicity important.

Most studies on Ni toxicity have been performed in hard or moderately hard water. However, many Canadian and European fresh waters are classified as soft. Therefore, understanding the fate of metals in soft water is relevant and needs further investigation.

1.3 Biotic Ligand Models

1.3.1 Principles and goals of development

As was illustrated above, the understanding of all processes that can affect metal toxicity in natural waters is complex. Integration of individual effects into a comprehensive model is even more complex. However, recently the biotic ligand model (BLM) has been developed to accomplish this. It quantitatively evaluates and predicts metal bioavailability at the site of action (the BL). The BLM has been developed to predict toxicity to a number of metals on a site-specific (water chemistry) basis and has been applied in regulations for site-specific ambient water quality criteria (AWQC). The development of this mechanistic model evolved from an understanding of toxic mechanisms combined with the use of geochemical speciation models (e.g. MINEQL, WHAM), thereby incorporating biological surface interactions. The first BLMs were calculated very recently and were based on metal binding constants determined for fish gills (USEPA, 1999; McGeer et al., 2000; Di Toro et al., 2000; 2001; Santore et al., 2001).

Biotic ligand models are based on geochemical equilibrium modelling principles, and inputs are the concentration of metal and ligands as well as the associated stability constants (K) for each reaction. (McGeer et al., 2000; Smith et al., 2002; Adams and Kramer, 1999; Slaveykova and Wilkinson, 2005). BLM's are flexible, so all aspects of water chemistry that can affect metal toxicity can be included (Di Toro et al., 2001; Niyogi and Wood, 2004; Markich et al., 2006). The first step in building a BLM for a particular metal is to determine the factors that alter the expression of toxicity. These experiments usually use a simple, laboratory-designed water with low concentrations of

most components as the reference condition. Ideally, experiments determine the effects of added inorganic ligands (e.g. hydroxide, carbonate), organic ligands (organic matter and surfaces) and also competitive effects at the site of toxic action. These experiments also depend on understanding geochemical speciation, which can also be complex. For example, when considering Me^{2+}-NOM interactions, the concentrations of Ca^{2+} and Mg^{2+} need to be considered not only as competitors with metals at the biotic ligand (i.e. protective effects), but also because high Ca^{2+} and Mg^{2+} levels can interfere with and reduce metal binding to NOM (i.e. toxicity enhancing effects) (Mandal et al., 2002; Smith et al., 2002).

1.3.2. Biotic ligand model for Ni toxicity.

BLMs for nickel toxicity are in a state of development. The base for Ni BLM development is an existing acute gill-nickel accumulation model (Meyer et al., 1999). This study incorporates affinity constants for gill-binding and a wide range of water hardness but does not investigate the effect of pH and organic matter. This model was quite successfully adjusted for use in other fish species. It demonstrates that the mechanistic approach of the BLM framework is applicable to a wide range of water chemistries and organisms. Often BLMs that have been developed for one species are subsequently applied to a number of other species. However, as has been shown by Pane et al. (2003ab, 2004ab), nickel has a different mechanism of toxicity for different aquatic organisms. Therefore any BLM requires validation before extrapolation both to other water chemistries and to other species. Such a comprehensive modeling framework was recently carried out on fish (Hoang et al., 2004; Deleebeeck et al., 2007a) and *Daphnia magna* (Deleebeeck et al., 2007b). However, a number of questions remain unanswered,

including the influence of particular toxicity modifying factors on nickel toxicity and affinity constants for certain ligands.

1.4. Project objectives

The overall objective of this thesis was to study factors that affect the acute toxicity of Ni to *Daphnia pulex*. This species was chosen because it is a typical cladoceran of fresh waters, one which can be acclimated to very soft water and easily maintained under laboratory conditions. In controlled lab studies I tested a series of factors (Ca, Mg, Na, K, Cl, pH and two sources of NOM) for their toxicity modifying effects. *Daphnia pulex* was used as the test organism and my studies focused on impacts in very soft waters. Using these characterizations, the work will contribute towards the development of a BLM for the acute toxicity of Ni to *D. pulex* in very soft waters.

CHAPTER TWO

THE EFFECTS OF MODIFYING FACTORS ON THE ACUTE TOXICITY OF NI TO *DAPHNIA PULEX* IN RECONSTITUTED SOFT WATER

2.1 Introduction

Nickel (Ni) is an essential element but can also cause toxic impacts when above threshold concentrations (http://www.nickelinstitute.org/index.cfm/ci_id/100.htm: Muyssen et al., 2004). As an essential metal, Ni plays roles in lipid metabolism, hematopoiesis and other functions (Phipps at al., 2002; Anke et al., 1995; Nielsen, 1996). Essentiality has been reported for terrestrial organisms but is not well-investigated in aquatic organisms (Muyssen et al., 2004). Mining activities, the metal finishing and plating industry and other industrial sources can result in the contamination of aquatic environments with elevated levels of Ni. Normal background concentrations of Ni in aquatic ecosystems range from about 1 up to 10 μg/L and can reach up to 3,000 μg/L (Eisler, 1998). Understanding the level of Ni in the aquatic environment that may cause impacts can be challenging because of the interactions between natural background concentrations, essentiality and toxicity.

The aquatic toxicity of metals varies depending on the sensitivity of the organism and also, the environmental conditions. Water quality greatly affects the chemical speciation and toxicity of metals (Meyer et al., 1999; Di Toro et al., 2001; De Schamphelaere et al., 2002) because different forms of a metal have different bioavailabilities. The metabolic processes of an organism can play an important role in its sensitivity to metal toxicity and these can also be affected by environmental

circumstances (Bury et al., 1999; McGeer et al., 2000; Paquin et al., 2002; Niyogi and Wood, 2004). Therefore, the development of aquatic toxicity prediction models such as the biotic ligand model (BLM, McGeer et al., 2000; Di Toro, 2001; Santore et al., 2001) that account for the bioavailability and toxicity of a metal under varied water chemistry is highly advantageous. The BLM connects the influence of water chemistry on the fate of metal in the environment, its bioavailability to live organisms, and physiological mechanisms of toxicity. Mechanisms of Ni toxicity are not as well understood in comparison to other divalent metals; however the study of Pane et al (2003) linked the impacts of Ni to *Daphnia magna* in hard water to disruption of Mg balance.

Existing BLMs for nickel have been developed for fish (Meyer et al, 1999; Wu et al., 2003; De Schamphelaere et al., 2004a; Hoang et al., 2004). However, there are very few studies applying the BLM approach to the impacts of Ni on invertebrates (Keithly et al., 2004; Pane et al., 2003; Deleebeeck et al., 2007) and all these studies were done in hard or moderately hard water. The bioavailability of metal cations is elevated in soft water (Galvez and Wood, 1997; Wood et al., 1998; Meyer et al., 1999; Taylor et al., 2000; Di Toro et al., 2001) and since many Canadian and European fresh waters are classified as soft, the investigation of metal toxicity in soft water is important. Understanding the impacts of Ni in soft waters and establishing a BLM framework for these conditions would contribute towards establishing site-specific thresholds for Ni.

The development of a Ni BLM for *Daphnia pulex* in soft water is relevant because of the potential for elevated bioavailability of metals in naturally occurring soft water. In this study, the effects of metal-toxicity modifying factors on Ni toxicity to *D. pulex* in soft water were investigated. An artificial reconstituted soft water (RSW) was used for culture and testing. The goal of this research work was to understand the effect of toxicity modifying factors without the co-influence of other parameters. To clarify the

relationship between toxicity modifying factors and Ni toxicity, about 50 bioassays in RSW were performed using *D. pulex*. In these experiments, the general approach was to experimentally vary one parameter per series, while holding all other parameters constant. This thereby allowed quantification of the degree of protection as a function of the concentration of the parameter in question. In turn, such regression relationships could be used in the future to generate constants for a BLM for *D. pulex* in soft water.

2.2 Materials and Methods

2.2.1 Acute toxicity methods for Daphnia pulex

The test species, *Daphnia pulex,* was obtained from Aquatic Research Organisms (Hampton, NH). Acute 48-h assays were conducted according to a standard Biological Test Method from Environment Canada for *Daphnia* (Reference Method EPS 1/RM/11). The 48h EC50 was the endpoint for toxicity bioassays and represented the waterborne Ni concentration at which 50% of the immobilized neonates were observed. For each test at least 7 nickel concentrations were examined using two replicates for each concentration with 10 neonates per replicate.

The culture of daphnids was pre-acclimated, first to a standard US EPA soft water and then to a modified RSW with a lower concentration of Mg ions (see paragraph 2.2.2). Adults were acclimated for at least 30 days prior to starting tests, which were done using neonates born to these adults. In accordance with the test method, neonates were always under 24 h of age when tests were initiated and they were from the second (or later) batch of offspring produced by a given group of adults.

The culture and test beakers were maintained at 20.5 ±1.5 °C. Photoperiod was fixed at 16 h of light and 8 h of dark. The culture medium was renewed daily. A mixture

of two algae species, *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* (the ratio 1:3 respectively), along with a yeast, cerophyll and trout feed suspension (YCT, from Aquatic Research Organisms, Hampton, NH) was fed to the daphnia cultures. At approximately the same time each day, 8 ml of the algae mix (35 x 10^6 cells/ml) and 4 ml of YCT were added to each 800 ml culture beaker. Culture beakers were not aerated, as aeration drives animals to the surface (Baudouin and Scoppa, 1974).

2.2.2 Water chemistry of culture media

Culturing and testing of *D. pulex* in soft RSW was done using the standard EPA soft water but with a modified Ca:Mg ratio. The standard EPA soft water (EPA/600/4-90/027F) has a Ca:Mg ratio of 1 (in mg/L) and this was modified by lowering the concentration of Mg. RSW for culture and testing had a 2-to-1 Ca:Mg ratio (in mg/L). This modified ratio was more representative of soft waters found in central Canada and northern Europe (Table 1). Several authors have focused attention on the importance of Ca:Mg ratios and the influence that modification of this ratio has on metal toxicity (Welsh et al., 2000a; Naddy et al., 2002). In Table 1, the physico-chemical parameters for Canadian waters are tabulated from American Water Works Association (AWWA, 1964) as well as Hare and Tessier (1998). For European countries (except Norway) data were taken from the European Environment Agency (http://reports.eea.europa.eu). For Norway, the data from Wright and Henriksen (1978) were used.

The RSW for culture and testing of *D. pulex* was made as required, stored in a 50L polyethylene carboy at 20.5 ±1.5°C, and aerated continuously. Dissolved oxygen (% saturation), pH, conductivity (μS/cm^2) and temperature (^{o}C) were measured on a regular basis. The nominal concentrations of major ions for RSW are shown in Table 2. Different volumes of stock solutions of $CaSO_4$, $MgSO_4$, $NaHCO_3$, and KCl were added to

Table 1: Physico-chemical parameters of several natural soft waters from Canada and Europe (in mg/L). The overall averages of European and Canadian fresh waters are given. Of note is the Ca : Mg ratio that is typically found.

Region	Source	pH	Na	K	Ca	Mg	Ca : Mg ratio
Canada	Great Whate R.[1], QC	5.9	0.6	0.3	1.5	0.3	5:1
	Ottawa R.[1], ON	7.0	4.0	4.9	8.8	2.1	4:1
	Assiniboine R. [1],Man	8.8	56	8.2	84	41	2:1
	Rideau R. [1], ON	7.7	4.0	4.9	39	11	3.5:1
	Oldman R.[1], AB	8.2	9.6	1.0	37.0	14	2.5:1
	Milk R..[1], AB	8.2	35	2.1	49.0	24	2:1
	Muskoka Lakes[2], ON	6.1	n/a	n/a	2.5	0.8	3:1
	Sudbury Lakes[2], ON	5.7	n/a	n/a	5.6	1.7	3:1
	Rouyn-Noranda Lakes[2], QC	6.9	n/a	n/a	7.3	2.7	2.5:1
	Average Canada	*6.5*	*2.0*	*1.5*	*4.5*	*1.2*	*4:1*
Scandinavian	Sweden[4]	5.5	7.9	6.2	2.5	0.48	5:1
	Norway[3]	5.5	2.7	0.2	1.7	0.84	2:1
	Belgian[4]	5.9	6.4	1.8	2.55	0.9	2.8:1
	Average N. Europe	*5.7*	*5.7*	*2.7*	*2.2*	*0.74*	*3:1*

1. adapted from American Water Works Association (AWWA, 1964).
2. adapted from Hare and Tessier (1998); an average of several lakes of each region is used.
3. data from Wright and Henriksen (1978)
4. data taken from European Environment Agency (http://reports.eea.europa.eu).

Table 2: Comparison of the US EPA standard soft water with the reconstituted soft water (RSW) used for culture and toxicity testing with *Daphnia pulex*. The nominal concentrations of the main ions are shown in mg/L (and mM). The total hardness values were calculated from Ca and Mg measurements. The right hand column shows the range of water chemistry for the parameter (lowest - highest) during Ni toxicity tests.

parameter	EPA Standard Reconstituted Soft Water	RSW used for culture and testing	range used in the Ni toxicity tests
pH	7.80	7.85	5.5 – 8.50
Alkalinity, as mg/L $CaCO_3$	-----	28.0	0.50 – 60.0 (0.01-0.97)
Ca as mg/L (mM)	6.98 (0.17)	6.98 (0.17)	0.80 – 60.0 (0.02-1.25)
Mg as mg/L (mM)	6.00 (0.25)	3.37 (0.14)	0.25 – 35.0 (0.02-1.03)
Na as mg/L (mM)	13.14 (0.57)	13.14 (0.57)	0.45–25.0 (0.02-1.09)
K as mg/L (mM)	1.05 (0.027)	1.05 (0.027)	0.20 – 30.0 (0.005-0.78)
DOM as mg/L	0.30	0.15	0.15 – 35.0
Hardness, mg/L $CaCO_3$	42	31.5	3 – 294

double de-ionized water for RSW preparation. As well, for culture water a mixture of vitamins containing B_1, B_7, and B_{12} in equal proportion was added at a total concentration of 0.1 mg/L. RSW in culture beakers was renewed daily.

Ion concentrations and hardness in RSW culture water were tested at regular intervals throughout the study. Hardness was calculated using the formula from Standard Methods for the Examination of Water and Wastewater (Method 2340B):

$$2.497*Ca + 4.118*Mg = \text{Hardness (mg/L as } CaCO_3) \qquad \textbf{Equation 1}$$

where Ca and Mg are the concentrations in the aquatic medium, in mg/L. The calculated hardness values were verified using a commercial hardness kit (La Motte Chemical Products, Chestertown, MD).

2.2.3 Test water chemistry and experimental design

For each experimental set of treatments, one water chemistry variable was varied and all others were kept constant. The chemistry variables tested included Ca (added as $CaSO_4$), Mg (added as $MgSO_4$), Na (added as NaCl) and K (added as KCl). The combined effect of pH, alkalinity, and Na on Ni toxicity was tested via the addition of $NaHCO_3$, while pH and alkalinity effects independent of Na were tested by additions of $NaHCO_3$ with NaCl added to maintain constant Na levels. Finally the effect of pH was tested by using the organic buffer 3-morpholinepropanesulfonic acid (MOPS, 750 mg/L or 3.6 mM, (Fisher Scientific Inc.) with small amounts of 1N HCl or NaOH to adjust pH). The effect of dissolved organic matter (DOM) was tested using two natural organic matter (NOM) sources, Suwannee River NOM (SNOM) and Nordic Reservoir NOM (NRNOM). Both NOMs were obtained from the International Humic Substances Society (St. Paul , MN).

Table 3 gives the nominal water chemistries used in the study. For each combination of water chemistry, a full 48h EC50 test was conducted, and for each of these, a minimum of 7 different Ni concentrations were used.

Toxicity test solutions were made using a 100 mg/L stock solution of $NiSO_4$ in double de-ionized water. Intermediate concentration solutions were made from the stock solution and were diluted into test solutions. For all test solutions, the concentrations of all components except for those of the factor(s) being tested were set at the concentration found in RSW. Test solutions were prepared a few hours before initiating the toxicity test except for pH tests where solutions were prepared and equilibrated for 1 d prior. The pH, conductivity, dissolved oxygen and temperature of test media measurements were taken before and after each test and met test acceptability requirements (Reference Method EPS 1/RM/11). In addition to the test parameters outlined in Table 3, a small number of additional toxicity tests were conducted to better understand some of the culturing influences. Additional Ni toxicity tests were done to compare EPA standard soft water vs. RSW as well as to test whether the provision of a vitamin mixture was important.

2.2.4 Water chemistry measurements

Water samples of approximately 10 ml were taken from each toxicity test solution at 0 and 48 h and saved for analysis. At each time, samples were filtered (0.45 μm Acrodisc HT Tuffryn Membrane, PALL New York) and at some of the times, unfiltered samples were collected for comparison. Samples for Ni and ion analysis were acidified to 1% using 16N HNO_3 while samples for measurement of DOM were not acidified, but were stored at 4 ^{o}C. Similar samples were collected from the culture medium on a regular basis. Measurements of temperature and pH were taken for all tests using a meter

PHM290 Meter Lab (Radiometer, Copenhagen, Denmark) with a pH electrode pHC2701 specifically designed for soft water. Conductivity was measured using a conductivity meter (Orion model 1230, ORION Research, Inc., Beverly MA) and dissolved oxygen was checked with an IonCheck 20 meter (Radiometer).

Measurements of Ni, Ca, Mg, Na, K, and Cl in water samples were done by the Analytical Service Group of CANMET MMSL (Natural Resources Canada) using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Varian Inc, Walnut Creek, CA). In these measurements, the detection limit for Ni measurement was 0.009 mg/L (0.15 μM). Dissolved organic matter (DOM) was determined using a total organic carbon analyzer (TOC-VCPH/CPN, Shimadzu, Columbia, MD). Alkalinity was measured using an automated titrator (Metrohm 751 GPD Titrino, Westbury, N.Y.). Colour of dissolved natural organic matter solutions was assessed using a spectrophotometer (752W UV-Visible Spectrophotometer, Beckman Coulter, Mississauga, ON).

2.2.5 Statistics

The 48h EC50 values for Ni toxicity were calculated using Comprehensive Environmental Toxicity Information System (CETIS), 2001-2002 Tidepool Scientific Software. Observed percent of immobilized neonates after 48 h at each measured Ni concentration along with measured (rather than nominal) water chemistry values were used as inputs for the calculation of EC50 Ni values. A difference between two EC50 values was considered significant when the 95% confidence intervals did not overlap.

Table 3: Test solution chemistry for Ni toxicity tests in soft water. Concentrations are in mM except for pH, and for dissolved organic matter (DOM) and alkalinity which are in mg/L. The test media were prepared by adding different amounts of $CaSO_4$, $MgSO_4$, $NaHCO_3$, KCl, and dissolved organic matter. The test series labeled pH1 varied both pH and Na concentration, the series pH2 was similar but used NaCl to keep a constant Na, and pH3 used the organic buffer MOPS. Tests characterizing the effect of Suwannee River natural organic matter on Ni toxicity are labelled as SRNOM while those with Nordic Reservoir natural organic matter are NRNOM. The data are nominal except for pH, DOM, and alkalinity in pH sets. The concentration of one tested parameter (in bold) in any set was varied while other physico-chemical characteristics were kept constant.

	Ca	Mg	Na	Cl	SO_4	K	HCO_3	pH	DOM	Alkalinity
Ca	**0.02**	0.14	0.57	0.027	*0.16*	0.027	0.57	7.85	0.15	28
Ca	**0.05**	0.14	0.57	0.027	*0.19*	0.027	0.57	7.85	0.15	28
Ca	**0.1**	0.14	0.57	0.027	*0.24*	0.027	0.57	7.85	0.15	28
Ca	**0.175**	0.14	0.57	0.027	*0.32*	0.027	0.57	7.85	0.15	28
Ca	**0.37**	0.14	0.57	0.027	*0.51*	0.027	0.57	7.85	0.15	28
Ca	**0.87**	0.14	0.57	0.027	*1.01*	0.027	0.57	7.85	0.15	28
Ca	**1.25**	0.14	0.57	0.027	*1.39*	0.027	0.57	7.85	0.15	28
Ca	**1.47**	0.14	0.57	0.027	*1.61*	0.027	0.57	7.85	0.15	28
Mg	0.17	**0.01**	0.57	0.027	*0.18*	0.027	0.57	7.85	0.15	28
Mg	0.17	**0.04**	0.57	0.027	*0.21*	0.027	0.57	7.85	0.15	28
Mg	0.17	**0.29**	0.57	0.027	*0.46*	0.027	0.57	7.85	0.15	28
Mg	0.17	**0.49**	0.57	0.027	*0.66*	0.027	0.57	7.85	0.15	28
Mg	0.17	**0.82**	0.57	0.027	*0.99*	0.027	0.57	7.85	0.15	28
Mg	0.17	**1.03**	0.57	0.027	*1.2*	0.027	0.57	7.85	0.15	28
Mg	0.17	**1.44**	0.57	0.027	*1.61*	0.027	0.57	7.85	0.15	28
K	0.17	0.14	0.57	*0.005*	0.31	**0.005**	0.57	7.85	0.15	28
K	0.17	0.14	0.57	*0.12*	0.31	**0.12**	0.57	7.85	0.15	28
K	0.17	0.14	0.57	*0.25*	0.31	**0.25**	0.57	7.85	0.15	28
K	0.17	0.14	0.57	*0.45*	0.31	**0.45**	0.57	7.85	0.15	28
K	0.17	0.14	0.57	*0.78*	0.31	**0.78**	0.57	7.85	0.15	28
Na	0.17	0.14	**0.20**	0.027	0.31	0.027	*0.015*	*6.30*	0.15	*0.75*
Na	0.17	0.14	**0.50**	0.027	0.31	0.027	*0.015*	*6.71*	0.15	*0.75*
Na	0.17	0.14	**1.00**	0.027	0.31	0.027	*0.015*	*6.81*	0.15	*0.75*

Table 3 cont'd

	Ca	Mg	Na	Cl	SO_4	K	HCO_3	pH	DOM	Alkalinity
pH1	0.17	0.14	**0.02**	0.027	0.31	0.027	*0.02*	*5.6*	0.15	*1*
pH1	0.17	0.14	**0.20**	0.027	0.31	0.027	*0.20*	*7.6*	0.15	*10*
pH1	0.17	0.14	**0.87**	0.027	0.31	0.027	*0.87*	*7.9*	0.15	*42*
pH1	0.17	0.14	**1.00**	0.027	0.31	0.027	*1.00*	*8*	0.15	*52*
pH2	0.17	0.14	0.57	*0.6*	0.31	0.027	*0*	**5.6**	0.15	*0*
pH2	0.17	0.14	0.57	*0.58*	0.31	0.027	*0.015*	**6.3**	0.15	*1*
pH2	0.17	0.14	0.57	*0.51*	0.31	0.027	*0.09*	**7.1**	0.15	*4*
pH2	0.17	0.14	0.57	*0.26*	0.31	0.027	*0.33*	**7.6**	0.15	*16*
pH2	0.17	0.14	0.57	*0.027*	0.31	0.027	*1.99*	**8.4**	0.15	*97*
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	**5.6**	0.15	28
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	**6.3**	0.15	28
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	**7.0**	0.15	28
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	**7.5**	0.15	28
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	**8.0**	0.15	28
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	**8.3**	0.15	28
SRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**0.5**	28
SRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**10**	28
SRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**19.8**	28
SRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**29.3**	28
SRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**41**	28
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**1.53**	28
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**2.84**	28
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**9.8**	28
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**16.5**	28
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**22.9**	28
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	**35.3**	28

2.3. Results

2.3.1 Water chemistry

The results of chemical analyses of the RSW used for culturing showed that measured concentrations matched nominal concentrations (Table 4). Nominal and measured Ni concentrations in test solutions are presented in Tables 5 and 6. Measured total and dissolved Ni concentrations at 0 h and 48 h were within 4% of nominal concentrations (Table 6). Filtered (dissolved metal) and unfiltered (total metal) samples were measured for Ni (as well as other cations) and demonstrated that dissolved Ni dominated (Tables 5 and 6). The results of pH measurement are shown in Figure 3. Dissolved oxygen and temperature of both culture and tests media always met test acceptability requirements (Reference Method EPS 1/RM/11). Temperature was always ±1.5°C of 20.5°C and dissolved oxygen was between 65% and 90 % saturation.

2.3.2 Nickel toxicity in RSW

A series of toxicity tests was done in EPA soft water and then also in RSW to compare different Ni salts and also to evaluate the effects of having vitamins added to the water. Two Ni toxicity tests were done with EPA soft water (no added vitamins), one with $NiSO_4$ and the other with $Ni(Cl)_2$ and the EC50 values were 30 μM (95% CI 23-38) and 32 μM (95% CI 27-38) respectively. In RSW, the EC50 for $NiSO_4$ was 16.4 μM (CI of 14.1-18.4) with added vitamins and 16.9 μM (CI of 14.0-18.5) without vitamins. A test done in similar water with $Ni(Cl)_2$ (and no vitamins) yielded an EC50 of 15.9 μM (13.6-18.2). A comparison of 48h EC50 values for EPA and RSW soft waters is presented in Fig. 1 where 48h EC50 values are the average of two and three experiments respectively.

Table 4: Comparison of nominal and measured concentrations (in mM) of ions in the RSW culture water. In this table, measured concentrations are the calculated averages of dissolved ion concentrations of 5 single measurements of each ion. Dissolved concentration was obtained by filtration through a 0.45 μm membrane filter. Nominal hardness was calculated using equation 1.

	Ca	**Mg**	**K**	**Na**	**Hardness, mg/L as CaCO3**
Nominal, mM	0.17	0.14	0.027	0.57	31.5
Measured,mM	0.17	0.15	0.030	0.58	32.0
% recovery	100	107	111	102	102

Table 5: Comparison of nominal and measured total and dissolved concentrations of Ca, Mg and K ions from test solutions. Measured values are an average of 4 sample measurements. Samples for total and dissolved concentrations determination were taken before testing and additional samples for dissolved concentration were taken at test completion (48 h). Percent recovery was calculated for each measured value as a proportion of the nominal concentration and therefore, recovery is presented as the average of 4 calculated values as well. For dissolved concentrations, samples were filtered through 0.45 μm filters.

		Measured					
	Nominal Conc.	**Unfiltered (total) conc. at 0 h**		**Filtered (dissolved) conc. at 0 h**		**Filtered (dissolved) conc. at 48 h**	
	mM	**mM**	**%recovery**	**mM**	**%recovery**	**mM**	**%recovery**
Ca	0.020	0.020	100	NA	NA	0.020	100
	0.049	0.049	100	0.048	98	NA	NA
	0.097	0.094	97	NA	NA	0.095	98
	0.367	0.343	93	0.333	91	NA	NA
	0.860	0.800	93	NA	NA	NA	NA
	1.470	1.450	97	NA	NA	NA	NA
Mg	0.01	0.010	100	0.104	104	0.105	105
	0.04	0.040	100	NA	NA	0.041	102
	0.30	0.290	99	0.300	100	0.3	100
	0.80	0.780	98	NA	NA	0.8	100
	1.00	0.980	98	NA	NA	1.0	100
K	0.006	0.008	133	0.008	133	0.095	158
	0.020	0.021	105	NA	NA	0.023	115
	0.120	0.120	100	0.124	103	0.129	107
	0.250	0.240	96	0.240	96	NA	NA
	0.780	0.780	100	NA	NA	0.780	100

Table 6: Comparison of nominal and measured total and dissolved concentrations of Ni in test solutions. Measured values are an average of 20 sample measurements. Samples for determination of total and dissolved concentrations were taken before testing and additional samples for dissolved concentration were taken at test completion (48 h). Percent recovery was calculated for each measured value as a proportion of the nominal concentration and therefore, recovery is presented as the average of n=20 calculated values as well. For dissolved concentrations, samples were filtered through 0.45 μm filters.

Nominal	**Measured**					
	Unfiltered (total) conc. at 0 h		**Filtered (dissolved) conc. at 0 h**		**Filtered (dissolved) conc. at 48 h**	
μM	**μM**	**%recovery**	**μM**	**%recovery**	**μM**	**%recovery**
0	<0.15	n/a	<0.15	n/a	10	n/a
10	10	100	9.5	96	10	100
20	20	100	19	97	20	100
25	25	100	23	98	27	101
30	30	100	29	97	30	100
39	40	102	39	100	40	102
60	60	100	58	99	60	100
82	83	101	80	97	81	99
100	102	101	103	102	NA	NA

values marked "<" were found to be below the detection limit of 0.15 μM.

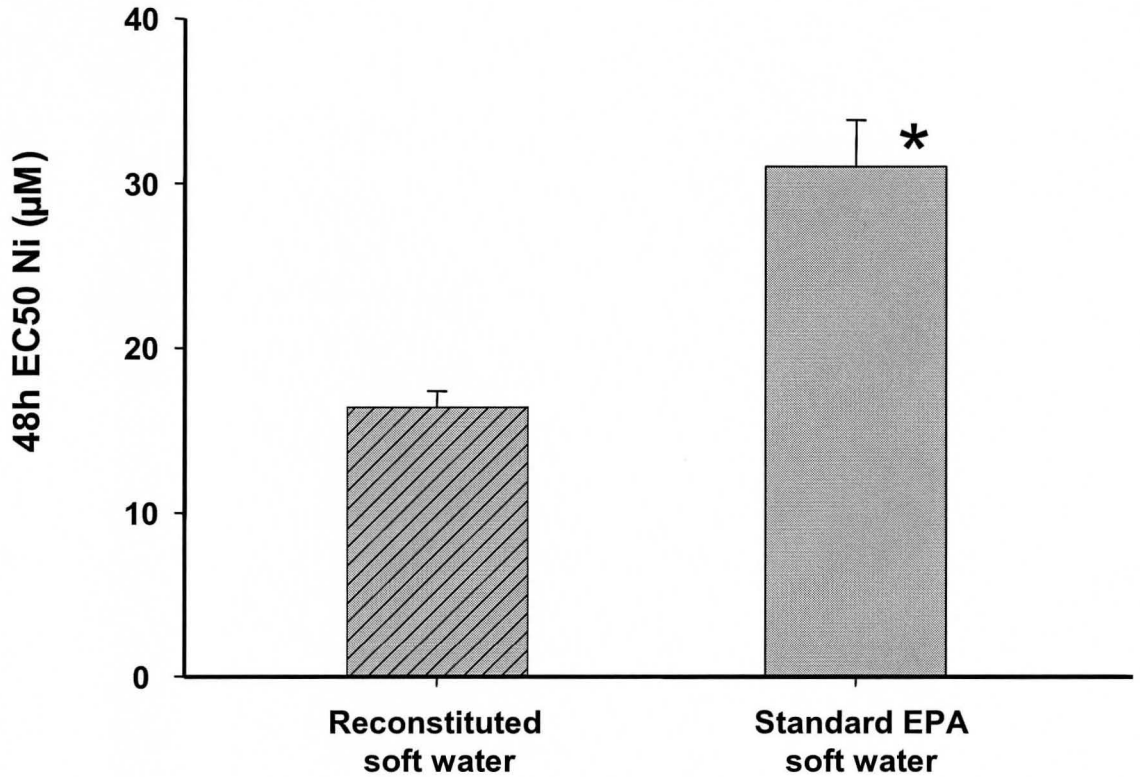
Table 7: Water chemistry used for toxicity tests and the resulting 48 h EC50 for Ni to *Daphnia pulex*. Water chemistry is shown as mM and Ni values are μM. For alkalinity and natural organic matter (NOM), the units are mg/L. The upper (UCL) and lower (LCL) 95% confidence limits of the EC50 values are included. Tests characterizing the effect of Suwannee River natural organic matter on Ni toxicity are labeled as SRNOM while those with Nordic Reservoir natural organic matter are NRNOM. The test series labeled pH1 varied both pH and Na concentration, the series pH2 was similar but used NaCl to keep a constant Na, and pH3 used the organic buffer MOPS. Within a test series, EC50 values labeled with the same letter are not significantly different from each other.

	Ca	Mg	Na	Cl	SO_4	K	HCO_3	pH	DOM	Alk	EC50	LCL	UCL
Ca	0.02	0.14	0.57	0.027	*0.16*	0.027	0.57	7.85	0.15	28	***7.5***^{a}	5	10
Ca	0.05	0.14	0.57	0.027	*0.19*	0.027	0.57	7.85	0.15	28	***17***^{b}	14	21
Ca	0.1	0.14	0.57	0.027	*0.24*	0.027	0.57	7.85	0.15	28	***22***^{b}	18	25
Ca	0.18	0.14	0.57	0.027	*0.32*	0.027	0.57	7.85	0.15	28	***16***^{b}	14	22
Ca	0.37	0.14	0.57	0.027	*0.51*	0.027	0.57	7.85	0.15	28	***46***^{c}	39	56
Ca	0.87	0.14	0.57	0.027	*1.01*	0.027	0.57	7.85	0.15	28	***92***^{d}	81	100
Ca	1.25	0.14	0.57	0.027	*1.39*	0.027	0.57	7.85	0.15	28	***89***^{d}	79	98
Ca	1.47	0.14	0.57	0.027	*1.61*	0.027	0.57	7.85	0.15	28	***132***^{e}	112	152
Mg	0.17	0.01	0.57	0.027	*0.18*	0.027	0.57	7.85	0.15	28	***28***^{a}	19	35
Mg	0.17	0.04	0.57	0.027	*0.21*	0.027	0.57	7.85	0.15	28	***30***^{a}	23	36
Mg	0.17	0.29	0.57	0.027	*0.46*	0.027	0.57	7.85	0.15	28	***40***^{ab}	32	52
Mg	0.17	0.49	0.57	0.027	*0.66*	0.027	0.57	7.85	0.15	28	***30***^{a}	26	38
Mg	0.17	0.82	0.57	0.027	*0.99*	0.027	0.57	7.85	0.15	28	***53***^{bc}	46	63
Mg	0.17	1.03	0.57	0.027	*1.2*	0.027	0.57	7.85	0.15	28	***51***^{bc}	44	56
Mg	0.17	1.44	0.57	0.027	*1.61*	0.027	0.57	7.85	0.15	28	***63***^{c}	55	71
Na	0.17	0.14	0.20	0.027	0.31	0.027	*0.015*	*6.30*	0.15	*0.8*	***26***^{a}	22	32
Na	0.17	0.14	0.50	0.027	0.31	0.027	*0.015*	*6.71*	0.15	*0.8*	***25***^{a}	20	28
Na	0.17	0.14	1.0	0.027	0.31	0.027	*0.015*	*6.81*	0.15	*0.8*	***26***^{a}	23	31
K	0.17	0.14	0.57	*0.005*	0.31	0.005	0.57	7.85	0.15	28	***32***^{a}	27	37
K	0.17	0.14	0.57	*0.12*	0.31	0.12	0.57	7.85	0.15	28	***42***^{a}	36	50
K	0.17	0.14	0.57	*0.25*	0.31	0.25	0.57	7.85	0.15	28	***33***^{a}	29	37
K	0.17	0.14	0.57	*0.45*	0.31	0.45	0.57	7.85	0.15	28	***34***^{a}	27	41
K	0.17	0.14	0.57	*0.78*	0.31	0.78	0.57	7.85	0.15	28	***33***^{a}	27	41

Table 7 cont'd

	Ca	Mg	Na	Cl	SO_4	K	HCO_3	pH	DOM	Alk	EC50	LCL	UCL
pH1	0.17	0.14	0.02	0.027	0.31	0.027	*0.02*	*5.6*	0.15	*1*	***22*** [a]	17	26
pH1	0.17	0.14	0.20	0.027	0.31	0.027	*0.20*	*7.6*	0.15	*10*	***26*** [a]	22	29
pH1	0.17	0.14	0.87	0.027	0.31	0.027	*0.87*	*7.9*	0.15	*42*	***24*** [a]	18	29
pH1	0.17	0.14	1.00	0.027	0.31	0.027	*1.00*	*8*	0.15	*52*	***23*** [a]	20	25
pH2	0.17	0.14	0.57	*0.6*	0.31	0.027	*0*	5.6	0.15	*0*	***24*** [a]	19	28
pH2	0.17	0.14	0.57	*0.58*	0.31	0.027	*0.015*	6.3	0.15	*1*	***22*** [a]	19	25
pH2	0.17	0.14	0.57	*0.51*	0.31	0.027	*0.09*	7.1	0.15	*4*	***26*** [ab]	21	33
pH2	0.17	0.14	0.57	*0.26*	0.31	0.027	*0.33*	7.6	0.15	*16*	***28*** [ab]	23	36
pH2	0.17	0.14	0.57	*0.027*	0.31	0.027	*1.99*	8.4	0.15	*97*	***37*** [b]	30	44
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	5.6	0.15	28	***26*** [a]	21	32
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	6.3	0.15	28	***24*** [a]	14	34
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.0	0.15	28	***37*** [a]	32	45
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.5	0.15	28	***54*** [b]	47	63
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	8.0	0.15	28	***68*** [b]	60	79
pH3	0.17	0.14	0.57	0.027	0.31	0.027	0.57	8.3	0.15	28	***67*** [b]	59	76
SRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	0.5	28	***17*** [a]	12	22
SRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	10	28	***50*** [b]	42	65
SRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	19.8	28	***48*** [b]	40	64
SRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	29.3	28	***42*** [b]	35	51
SRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	41	28	***55*** [b]	39	87
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	1.53	28	***25*** [a]	18	38
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	2.84	28	***28*** [a]	23	34
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	9.8	28	***52*** [b]	46	55
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	16.5	28	***89*** [c]	76	100
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	22.9	28	***86*** [c]	80	92
NRNOM	0.17	0.14	0.57	0.027	0.31	0.027	0.57	7.85	35.3	28	***120*** [d]	114	130

Figure 1. Comparison of Ni 48h EC50 values from *D. pulex* obtained using US EPA standard soft water and the reconstituted soft water (RSW) used for the culture and testing in this study. The primary difference between the two waters is that RSW had a Mg concentration that is half that of the EPA water. The mean of n=2 EC50 values are shown for EPA water and n=3 for RSW. The mean upper and lower 95% confidence limits are included. The means were significantly different.


48h EC50 Ni (µM)
40
30
20
10
0
*
Reconstituted soft water
Standard EPA soft water

2.3.3 The effects of Ca and Mg on Ni toxicity

An increase in Ca or Mg concentrations in test solutions resulted in a significant increase in the 48 h EC50 for Ni, in other words a decrease of Ni toxicity (Fig 2, Table 7). The protective effect of Ca was stronger than that of Mg (e.g. see slopes of regression lines in Fig 2). From Table 7, when Mg concentration increased from 0.01 to 1.44 mM, the 48h EC50 value increased by a factor of about 2.3. However, when Ca concentration increased from 0.02 to 1.25 mM, the 48h EC50 value increased by a factor of about 12. The regression of the EC50 for Ni vs. Ca waterborne concentration had a slope of 77 μM increase in Ni EC50 value for every 1 mM increase in Ca concentration (r^2=0.98) while for Mg, the comparable slope was 24 μM for every 1 mM increase in Mg concentration (r^2=0.89). Ca concentrations lower than in the culturing RSW resulted in significant increases of Ni toxicity. All EC50 values for Mg were higher than for RSW.

2.3.4 The effect of pH on Ni toxicity

There were 3 sets of pH tests completed during this study and the pattern of mortality differed amongst these tests. The tests series where pH was controlled with MOPS buffer (pH varied but not alkalinity) showed a significant reduction in Ni toxicity as pH increased (Fig 3). The EC50 value increased by 2.6 fold over the range from approximately pH 5.6 to pH 8.4 in these tests with MOPS. There were two series of tests where pH was controlled through the addition of $NaHCO_3$ and these showed either little or no effect of pH on Ni toxicity over a comparable range of pH values. For the $NaHCO_3$ set where Na concentration was controlled (pH, alkalinity, HCO_3^- and Cl^- varied), there was a small but significant increase in EC50 for Ni. In the other $NaHCO_3$ set, where Na concentration was not controlled (i.e. pH, alkalinity, HCO_3^-, and Na^+ varied) there was no

significant change in Ni EC50. Although the relationship of pH and Ni toxicity is shown in Fig 3 using linear regression the test results were perhaps not perfectly linear. In the pH tests with MOPS there was little change in EC50 at pH values of 7 and below, and a protective effect of increasing pH was seen only between pH 7.0 and 8.0 (Fig 3). Although less distinct, this trend was also observed in the second bicarbonate set.

2.3.5 The influence of different NOM sources on Ni toxicity

In tests with addition of NOM, dissolved organic carbon concentrations ranged from 0.5 to 41 mg/L for Suwannee River NOM and from 1.5 to 35.3 mg/L for Nordic Reservoir NOM. In both cases the toxicity of Ni decreased as DOC increased (Fig 4), however there were significant differences between the two sources. The toxicity-reducing effect of Suwannee River NOM was much weaker than that of Nordic Reservoir NOM. The toxicity of Ni was reduced by about 3 fold over the range of concentrations of Suwannee River natural organic matter that were tested. For Nordic Reservoir natural organic matter a similar increase in DOC resulted in a 5 fold decrease in acute Ni toxicity (Fig. 4, Table 7). The effect of DOC from Nordic Reservoir was reasonably consistent across the tested concentrations while for Suwannee River, there was little increased protective effect at concentrations above 10 mg/L. In other words, Suwannee River NOM was protective up to 10 mg/L DOC with no further effect beyond that concentration (Fig 4).

The turbidity of the investigated organic matter was not measured but a distinct difference in the amount of suspended matter between the two sources was noted. Thus,

Fig 2. Effects of either Ca or Mg on the 48h EC50 value of Ni toxicity in very soft waters. The linear regression variables linking the effect of each factor (either Ca or Mg) are shown. The EC50 values are shown with the 95% confidence interval. Included on the graph is the 48h EC50 value in unmodified RSW, which is shown by the "**x**".

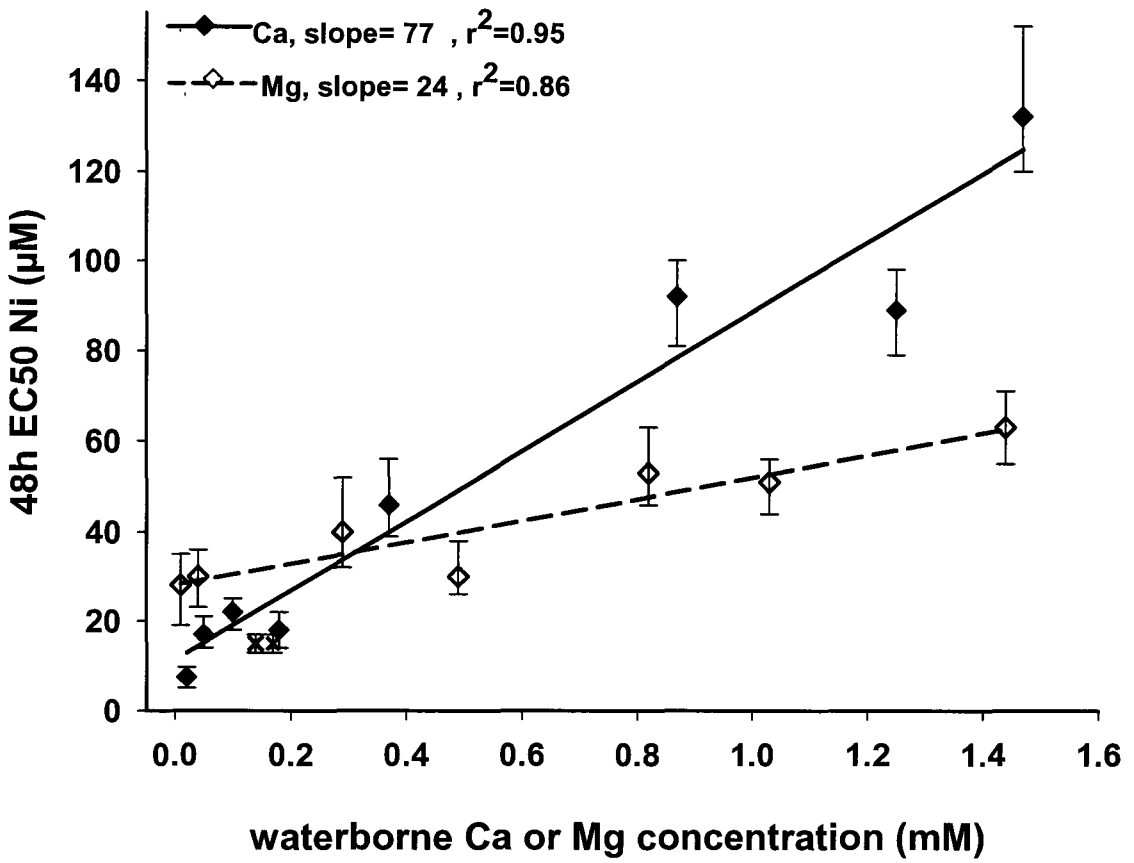

Ca, slope= 77 , r2=0.95
Mg, slope= 24 , r2=0.86
48h EC50 Ni (µM)
0
20
40
60
80
100
120
140
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
waterborne Ca or Mg concentration (mM)

Fig. 3. The effect of pH on the 48h EC50 value for Ni in very soft water. There were 3 sets of toxicity tests where different approaches were used to adjust water pH. In one set of tests, the organic buffer MOPS was used to adjust pH. In the other 2 sets, pH was adjusted using $NaHCO_3$ and for one of these (set 1) the Na content was held constant through adjustments with NaCl. In the other set (set 2) the Na content of the medium was allowed to vary. Error bars show the upper and lower 95% confidence intervals and the EC50 in RSW culture water is shown by the "**x**".

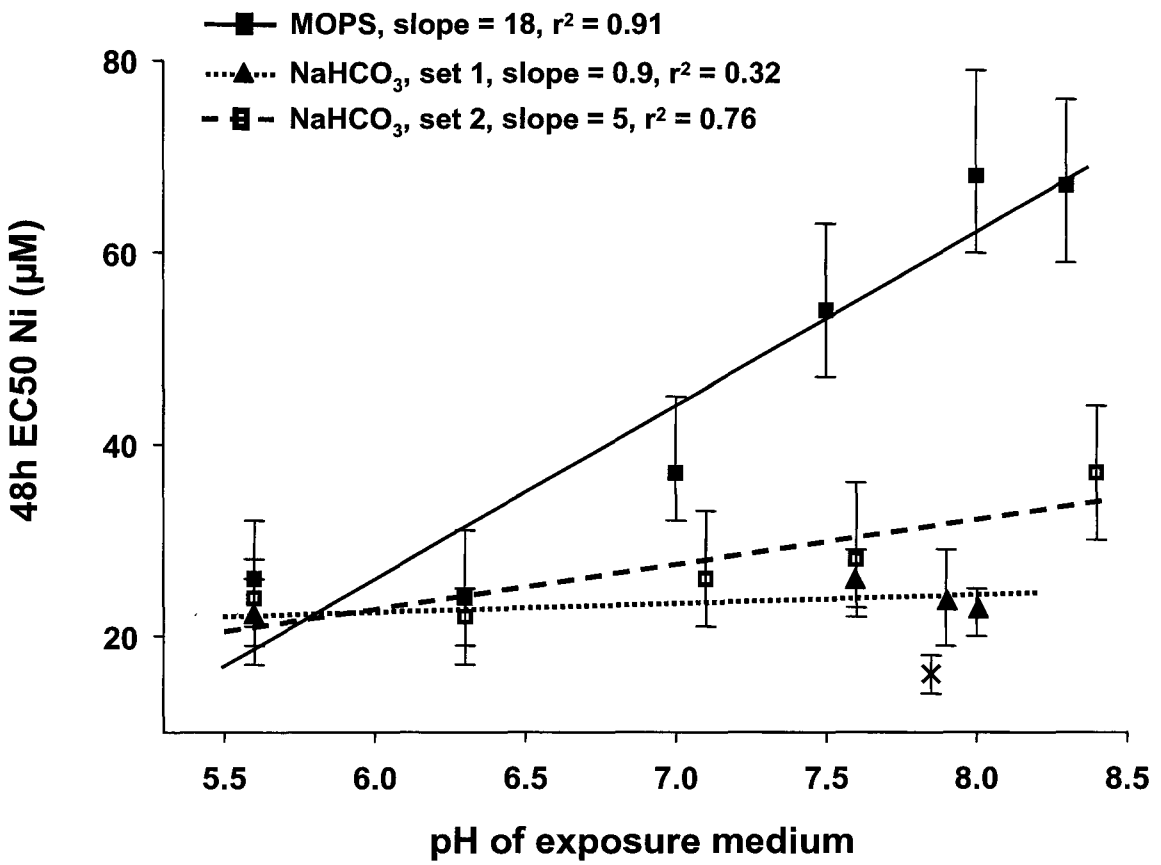

MOPS, slope = 18, r² = 0.91
NaHCO3, set 1, slope = 0.9, r² = 0.32
NaHCO3, set 2, slope = 5, r² = 0.76
48h EC50 Ni (µM)
80
60
40
20
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH of exposure medium

Fig 4. Protective effects of NOM on the 48h EC50 of Ni on *Daphnia pulex* in very soft waters. Two NOM sources were tested, Suwannee River and Nordic Reservoir. Best fit linear regression lines are shown. Error bars show the upper and lower 95% confidence intervals and the EC50 in RSW culture water is shown by the "**x**".

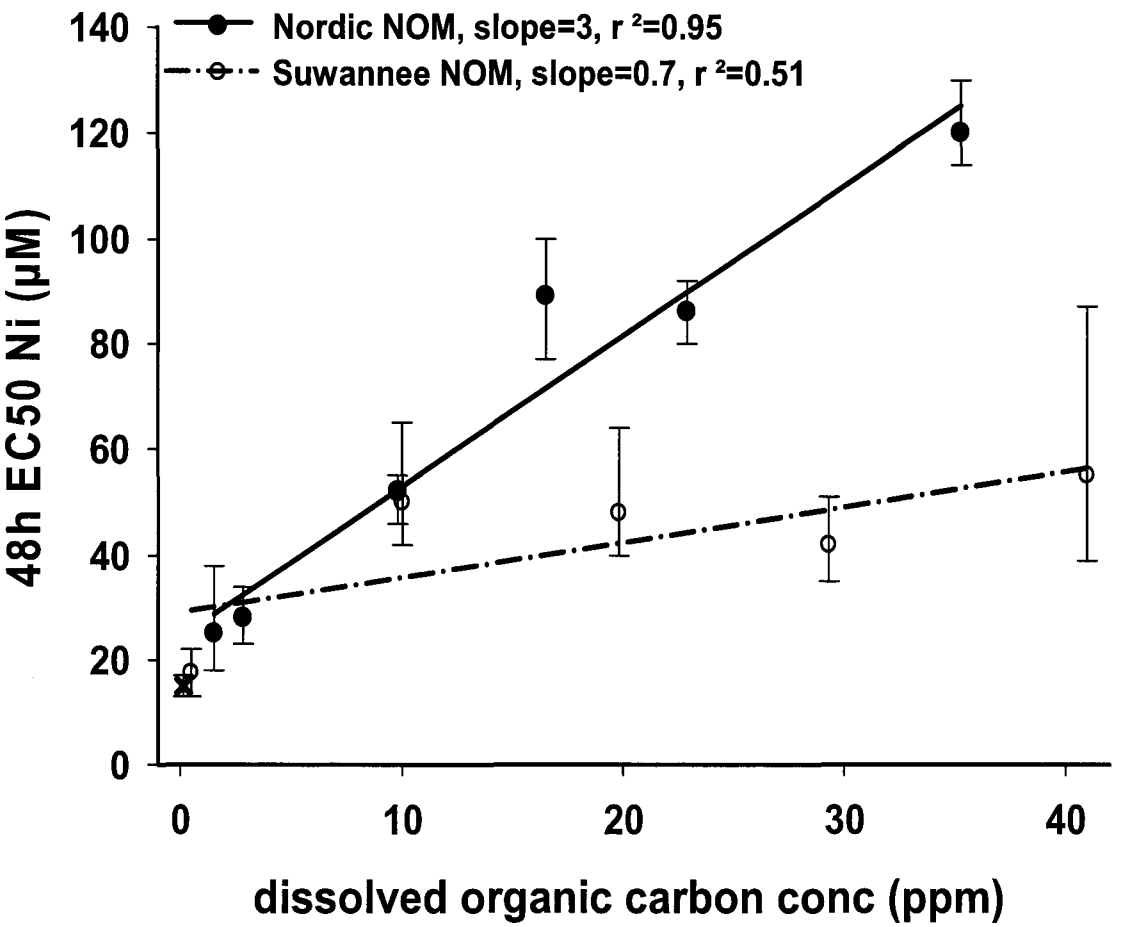

Nordic NOM, slope=3, r ²=0.95
Suwannee NOM, slope=0.7, r ²=0.51
48h EC50 Ni (µM)
140
120
100
80
60
40
20
0
0
10
20
30
40
dissolved organic carbon conc (ppm)

Table 8: Chemical characterizations of two sources of natural organic matter. The data in this table are adapted from the web site of the International Humic Substances Society (IHSS) (www.ihss.gatech.edu) except for solubility, conductivity and colour which were measured directly. The acronyms HA and FA refer to humic and fulvic acid respectively. For carbon distribution in mg/L, the peak areas from which these percentages are calculated were obtained from solid-state CPMAS ^{13}C NMR spectra.

Parameter	NOM Source		
	Suwannee River	Nordic Reservoir	
Measured characteristics			
Solubility, %	45	30	
Colour, tcu	1618	4072	
Conductivity, μS/cm	130	132	
Carbon distribution, %			
Carbonyl (220-190 mg/L)	8	8	
Carboxyl (190-165 mg/L)	20	21	
Aromatic (165-110 mg/L)	23	19	
Acetal (110-90 mg/L)	7	5	
Heteroaliphatic (90-60 mg/L)	15	16	
Aliphatic (60-0 mg/L)	27	31	
Elemental composition, % w/w			
Ash	7	41	
C	52.5	53.2	
H	4.19	5.67	
O	42.7	Nd	
N	1.1	1.1	
S	0.65	Nd	
P	0.02	Nd	
Acidic functional groups			
		HA	**FA**
n1	3.6	3.95	4.22
n2	1.19	1.0	1.1
Q1	10.5	12.95	10.32
Q2	2.61	1.49	1.64
LogK1	3.94	3.79	4.32
LogK2	9.74	9.67	9.89

Notes: Colour of both sources of natural organic matter was measured for the same concentration 10 mg/L of DOM taking in account the difference in solubility. Tcu is true colour units (from Ambient Water Quality Criteria for Colour in BC, 1997). For carbon distribution the table contains the electronically integrated peak area percentages for selected ranges of chemical shift. Ash is expressed as the % (w/w) of inorganic residue in a dry sample. The elemental composition in % (w/w) of a dry, ash-free sample. Q_1,Q_2 – Henderson-Hasselbalch coefficient of maximum charge density of the 2 classes of binding sites of NOM; LogK_1, Log K_2 - Log K value for proton binding by the 2 classes of binding sites of NOM; n1, n2 – empirical parameters that control the width (in log K) of the classes of proton binding sites.

Nordic Reservoir NOM has a greater amount of suspended matter, lower solubility, 6 times greater ash concentration and a higher colour in comparison to Suwannee River NOM (see Table 8 for some of the physico-chemical differences).

2.3.6 The effects of K and Cl on Ni toxicity

The toxicity tests with KCl did not show any change in 48 h EC50 as K (and Cl) concentration increased (Fig 5, Table 7). The overall average EC50 values for these tests was 35 μM Ni and individual test outcomes ranged from 32 to 42 μM.

2.3.7 The effect of Na on Ni toxicity

The relationship between Na ions and Ni toxicity was examined in two series of tests with different sources of Na ions (Fig 6). Both sets showed that Na did not influence the acute toxicity of Ni to *Daphnia pulex*. The first test series for Na used $NaHCO_3$ and in these tests pH, HCO_3^- and alkalinity also varied. The second Na series used NaCl to vary the Na concentration. In this test, pH varied a little but alkalinity did not (Table 7). The overall mean EC50 value for the NaCl set was 25.6 μM while for the $NaHCO_3$ it was 23.8 μM Ni.

2.4.8 The effect of alkalinity on Ni toxicity

The fact that alkalinity did not alter the acute toxicity to Ni is shown in Fig 7. However, the alkalinity experiments never varied alkalinity independently. Two series of tests with varying alkalinity were done, both using $NaHCO_3$, however in one the concentration of Na varied (as did pH) while in the other Na was held constant (but pH varied).

Fig. 5. Effect of K on the 48h EC50 of Ni to *Daphnia pulex* in soft water. K was added as KCl. Error bars represent the lower and upper 95% confidence intervals. Ni toxicity of the culture water RSW is marked as "**x**" on the graph.

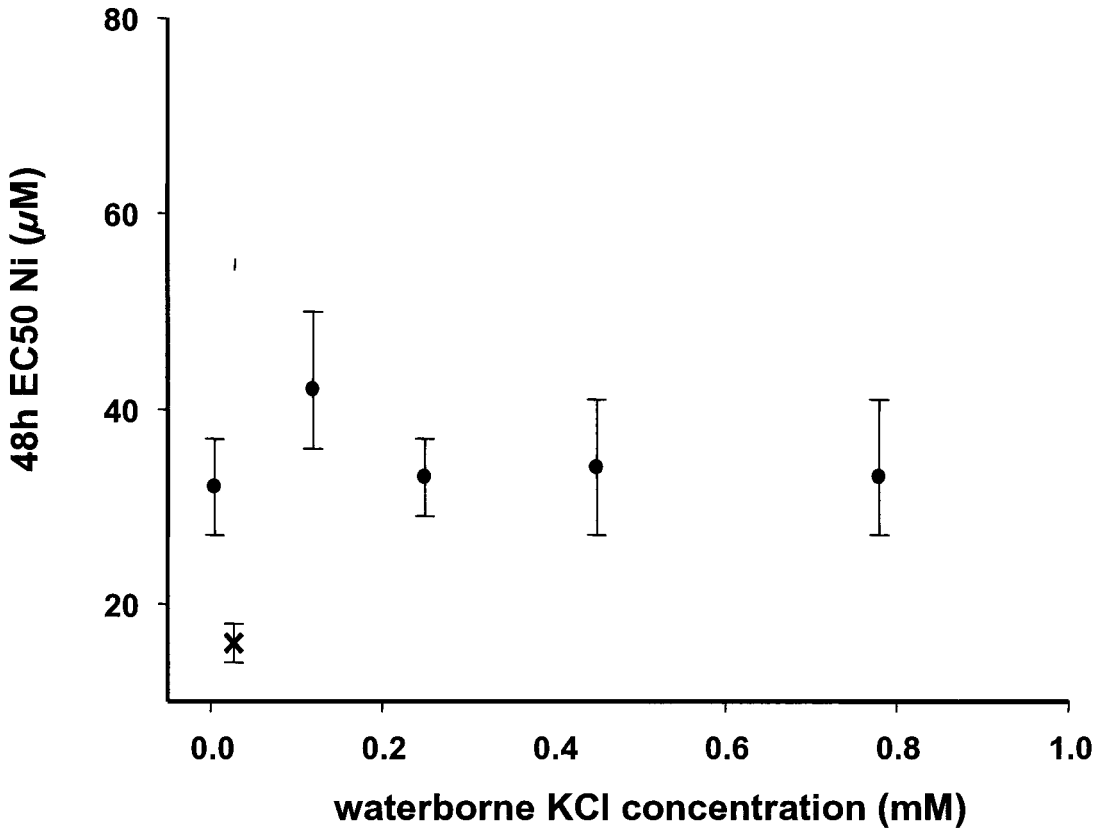

80
60
40
20
48h EC50 Ni (μM)
0.0
0.2
0.4
0.6
0.8
1.0
waterborne KCl concentration (mM)

Fig. 6. Influence of Na on the acute toxicity of Ni to *Daphnia pulex* in soft water. The 48 h EC50 values are shown with error bars representing the upper and lower 95% confidence intervals. There were two series of Na testing. In the first, $NaHCO_3$ was used to adjust Na levels (filled squares) and in the other, NaCl was used (open triangles). Ni toxicity of culture RSW is marked as "**x**" on the graph.

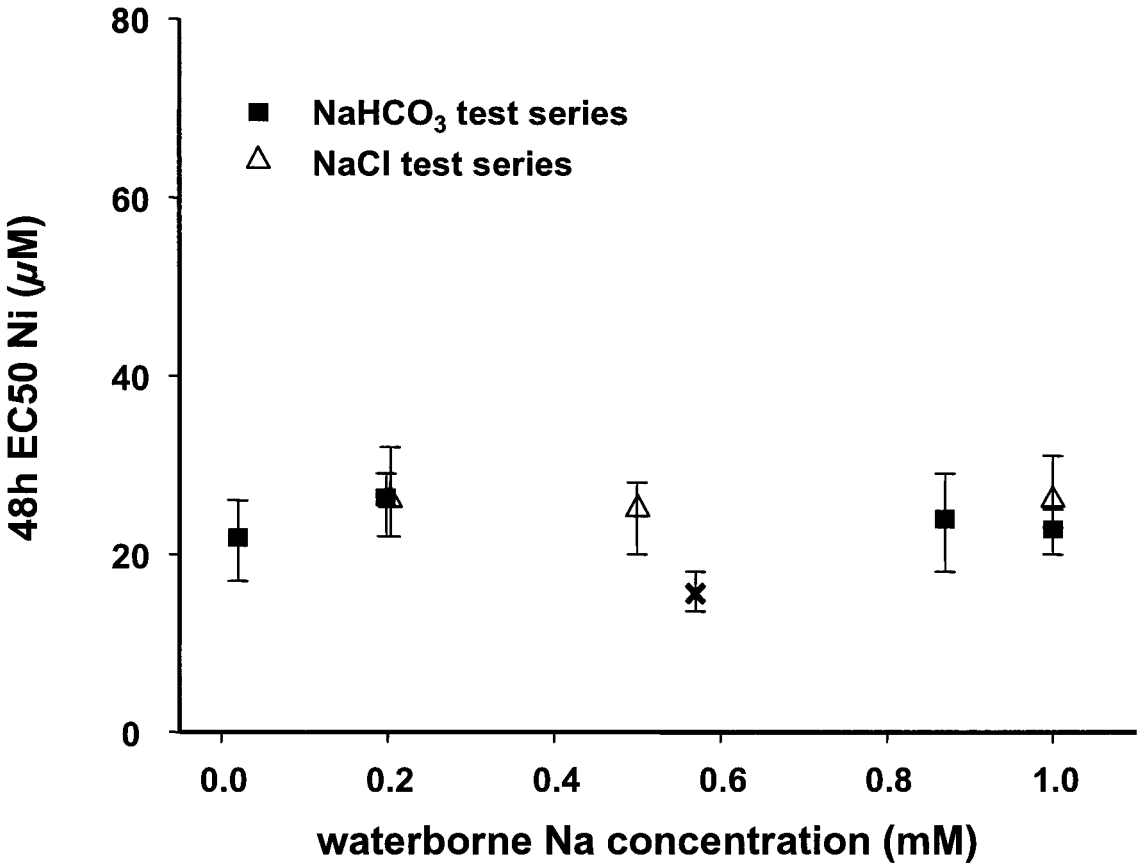
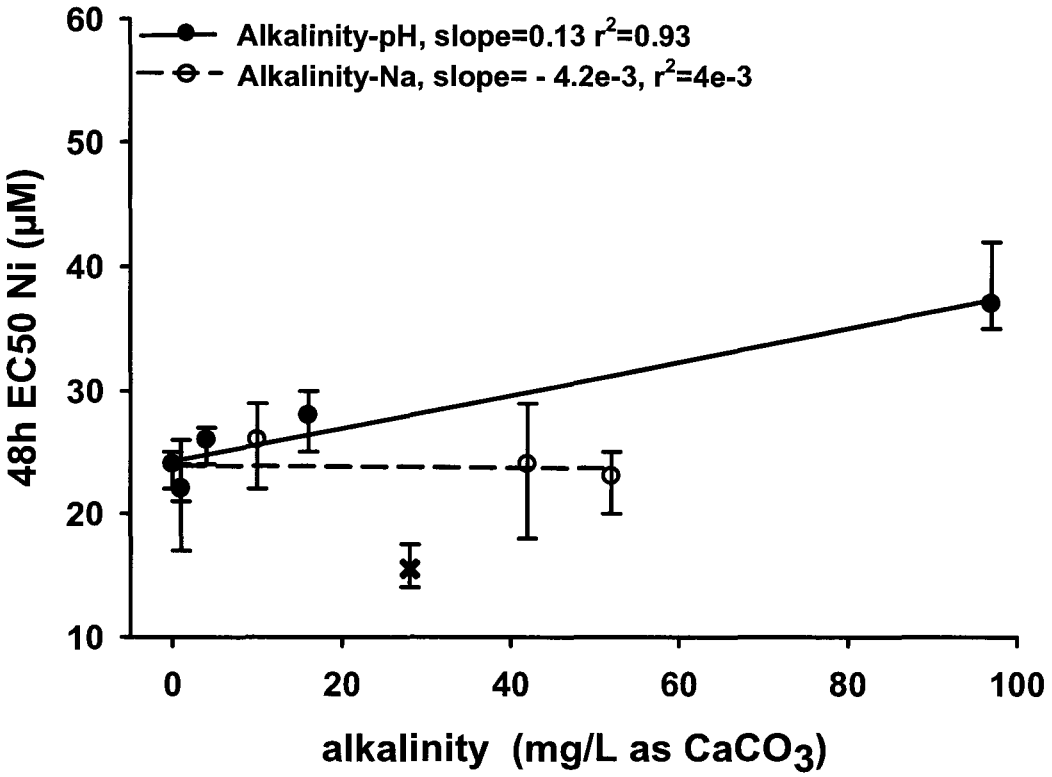

80
60
40
20
0
48h EC50 Ni (μM)
NaHCO3 test series
NaCl test series
0.0
0.2
0.4
0.6
0.8
1.0
waterborne Na concentration (mM)

Fig 7. The effect of alkalinity on the acute toxicity of Ni to *Daphnia pulex* in soft water. Alkalinity was not manipulated independently and therefore different test series were conducted. In the Na alkalinity set, the combined effect of pH, alkalinity, and Na on Ni toxicity was tested. In the pH alkalinity test series, Na was kept constant but pH, HCO_3^-, and alkalinity all varied. The EC50 values are shown with the upper and lower 95% confidence intervals. Ni toxicity of culture RSW is marked as "**x**" on the graph.


Alkalinity-pH, slope=0.13 r2=0.93
Alkalinity-Na, slope= - 4.2e-3, r2=4e-3
48h EC50 Ni (µM)
60
50
40
30
20
10
0
20
40
60
80
100
alkalinity (mg/L as CaCO3)

2.4 Discussion

2.4.1. Water chemistry measurements

The results of the chemistry analyses demonstrated that a consistent culture medium was used throughout this study as there was very little variation over time (Table 4). The dissolved oxygen, pH and temperature measurements of the culture medium were stable and met the requirements of the standard methods from Environment Canada, EPS 1/RM/11 (Environment Canada 1990). An important feature of this study was that the *Daphnia pulex* were cultured in very soft water and therefore, at least to some degree, acclimated to the underlying chemistry present during toxicity tests. Usually, researchers of metal toxicity in very soft waters have not pre-acclimated their organisms to soft water conditions (Keithly et al., 2003; Bury et al., 1999). For example, a recent characterization of the effects of Ni on *D. magna* in very soft water has been reported, but the culture of test organisms was maintained in hard water prior to testing (Deleebeeck et al., (2007b). However, several authors did pre-acclimate the test organisms prior to testing, but did not study the influence of pre-acclimation (Bianchini et al., 2002; Pane et al., 2003). The same is true in our study. The effect of dramatic changes in water chemistry on the responses of *D. pulex* during toxicity tests has not been studied in detail but results with other aquatic organisms have shown that transfer to very soft water has a significant physiological effect. For example, reproduction (but not survival) in *Ceriodaphnia dubia* is dramatically impaired by step changes in water Ca content (M. Schwartz, Natural Resources Canada, Ottawa. pers. comm.). The study of Taylor et al (2001) with rainbow trout (*Oncorhynchus mykiss*) illustrated that the physiological acclimation to very soft water can take many weeks. Therefore the pre-acclimation to

very soft water may have helped to alleviate any additional stresses that could have sensitized the daphnia to Ni.

Standard methodologies for the culture and testing of daphnia do not usually include vitamins in the culture water. The addition of vitamins to culture media should have a positive effect on adult daphnids and the health of their neonates, as well as on reproduction (Keating, 1985). A multi-vitamin mixture was added to cultures and although their effect was not characterized (e.g. on longevity or reproduction), the effect of having vitamins in the toxicity test medium was assessed. The results from the toxicity tests with and without vitamins were very similar, indicating that the vitamin mix did not influence the acute toxicity of Ni.

2.4.2. Nickel toxicity in RSW

In some of the initial Ni toxicity tests conducted in this study, different Ni salts, namely $NiSO_4$ and $Ni(Cl)_2$, were found to produce similar 48 h EC50 values. This similarity between Ni salts occurred in both standard EPA soft water as well as in the RSW used in this study. However there were significant differences between the EC50 values calculated from tests with RSW and tests with standard EPA soft water. The EC50 values calculated from tests using RSW water were about half of the EC50 values of tests using EPA soft water (Fig 1). This illustrates the effect of reducing Mg ion concentration from 0.25 mM to 0.14 mM. RSW chemistry, particularly the Ca:Mg ratio of 2:1, was considered more representative of natural waters (Table 1) than the standard EPA soft water with its ratio of 1:1. The increased toxicity of Ni in RSW water provided some initial data illustrating the effect of Mg on Ni toxicity.

2.4.3. Influence of hardness cations on nickel toxicity

Both Ca and Mg protected *Daphnia pulex* against the toxic effects of Ni (Fig 2). This protective effect is an example of cationic competition where Ca^{2+} and Mg^{2+} compete with Ni^{2+} for binding on the biotic ligand (Table 3; Fig 2). According to biotic ligand theory, cations compete for trans-membrane uptake sites because of inherent similarities such as ionic diameter and charge (Chapman et al., 1980; Schubauer-Berigan et al., 1993; Meyer et al., 1999). These uptake sites were first described for Cu uptake on the gills of fish (Playle et al., 1993; Welsh et al., 2000b; Di Toro et al 2001) and although the specific site of uptake in *D. pulex* is uncertain, it is clear that competitive effects occur. Our results suggest that Ni uptake is via Ca and/or Mg pathways. The fact that both Ca and Mg were found to be protective against Ni toxicity confirms the conclusion of other investigators that hardness is the main toxicity modifying factor (Chapman et al., 1980; Pane et al., 2003, 2004; Long et al., 2004; Keithly et al., 2004; Hoang et al., 2004).

In addition to the common Ca/Mg pathway discussed above, Ni^{2+} uptake very likely occurred via the Mg^{2+} - HCO_3^- co-transport pathway (Gunther et al., 1986). This theory is supported by the similarity of the ionic radii (Ni-0.066 and Mg-0.069 nm; Weast, 1973). The study of Pane et al (2003) illustrated that the impact of Ni is via a Mg uptake mechanism. From this information it was expected that increasing Mg would have the strongest toxicity reducing effect. But, in fact the toxicity testing results show a much greater effect of Ca ions compared to Mg ions (Fig 2). The slope of regression for the toxicity reducing effect of Ca is about 3 times greater than that of Mg (77 and 24 μM Ni per mM of either Ca or Mg, respectively). Even very small changes in Ca content had a toxicity mitigating effect. (Table 3; Fig.2). The relationship between Ca and Mg

concentration and Ni toxicity observed in this study is very close to that obtained by Deleebeeck et al (2007b) using *D. magna*. That study also observed a stronger effect for Ca compared to Mg. Deleebeeck et al (2007b) hypothesized that both Mg and Ca are taken up via the same pathway (Snavely et al 1991). The stronger protective effect of Ca may be a result of the role Ca has in regulation of membrane permeability (McWilliams, 1983; Hunn, 1985). It is possible that Ni has several mechanisms of competition with Ca include a substitution in annexin proteins of phospholipid membranes channels (see paragraph. 1.1.4). The proteins of voltage-gated membrane channels have much higher selectivity to Ca than to Mg ions (Melgunov et al., 2000). The Irving-Williams order of cation binding to ligands also suggests that Mg protection will be weaker than that of Ca (Schwartz and Playle, 2001).

2.4.4 The effect of pH on nickel toxicity

Three series of toxicity tests were conducted to assess the influence of pH on Ni toxicity. In general there was little change in Ni toxicity between water pH values of 5.5 and 7.0 and it was only at higher pH values that Ni EC50s increased. Therefore it was only on the basic side of the pH scale that Ni toxicity was significantly reduced. The test series using the inorganic buffer $NaHCO_3$ to vary pH were difficult to interpret because not only pH, but also alkalinity and either Na^+ or Cl^- concentrations varied. The $NaHCO_3$ test series where Na^+ increased as pH increased showed no significant change in Ni toxicity between pH 5.6 and 8.0. The $NaHCO_3$ tests where Na^+ was held constant but Cl^- increased as pH increased, showed a significant decrease of Ni toxicity at higher pH. However this toxicity-pH relationship was weak, based on only one EC50 value at pH 8.4

which was significantly different from EC50 values at pH 5.6 and 6.3. Independent tests with NaCl showed that Na^+ and Cl^- did not significantly influence Ni toxicity suggesting that any effects observed in the $NaHCO_3$ tests at pH 8.4 appear to be due to the combined effects of pH and alkalinity. However, these NaCl tests were done at pH values below 7 where Ni toxicity would not be expected to vary with pH.

The pH tests with the organic buffer MOPS varied the pH independently from other variables (i.e. Na^+, Cl^- and alkalinity were essentially constant) and showed a stronger toxicity- reducing effect as pH increased. As an organic buffer, MOPS is considered ideal for testing the effects of pH on metal toxicity because it does not complex metals in solution (Kandegedara and Rorabacher, 1999; De Schamphelaere and Janssen, 2004b). According to BLM theory, effects of pH on metal toxicity can be related to either competitive interaction by protons or alterations in the metal complexation capacity in solution. Competitive interactions between Ni^+ and H^+ can be ruled out because toxicity was highest at lower pHs. As solution pH increases, the concentration of hydroxide and bicarbonate ions increases. Both HCO_3^- and OH^- have a high affinity for Ni (Tipping, 1994). However, the increased complexation of Ni^{2+} to HCO_3^- as pH increased did not reduce toxicity because in the first set of pH experiments, both HCO_3^- and pH increased but toxicity was unchanged. A similar pattern (i.e. increasing HCO_3^- and pH with no change in Ni toxicity) occurred in the second pH set, except at the very highest HCO_3^- concentration and pH where there was a significant decrease in toxicity (Fig. 3). It is possible that changes in media pH could have had a physiological effect on the daphnia and this may have influenced their sensitivity to Ni. Physiological effects are not accounted for in the BLM approach. Why variations in pH

and alkalinity together did not alter Ni toxicity but pH change alone did (MOPS treatment) remains to be studied.

Other studies examining the effect of pH on Ni toxicity have variable outcomes. For example, some studies are similar to these results in showing that as pH increases (especially between 7.0 and 8.4), toxicity decreases (Reader et al.,1989; Pyle et al, 2002). However, other studies report no effect of pH on Ni toxicity (Van Sprang and Janssen 2001; Hoang et al. 2004). At least two studies on the effects of Ni to *Ceriodaphnia dubia*, reported an increase in Ni toxicity when pH increased (Schubauer-Berigan et al., 1993; Parametrix Inc., 2004). The study of Deleebeeck et al. (2007) reported no effect on Ni toxicity to *D. magna* up to pH 7.5 but did show increased toxicity between pH 7.5 and 8.1. Clearly the effect of pH on Ni toxicity is complex and requires further study in order to develop a mechanistic understanding.

2.4.5. The influence of NOM on Ni toxicity.

The tests with SRNOM and NRNOM illustrated that Ni toxicity was reduced in the presence of organic matter. Also, these tests clearly demonstrated that different sources of NOM have different toxicity-mitigating capacities for Ni. NRNOM had a much higher protective capacity than SRNOM. The reduction of toxicity was expected because organic matter complexes with free metal ions to reduce their bioavailability (Weber, 1988; Livens, 1991; Campbell et al., 1997; Glover and Wood, 2005). The study of Schwartz et al. (2004) showed that the relative protective effect of natural organic matter sources on metal toxicity was directly related to solution colour (specific absorbance capacity at 340 nm). Darker NOM sources provided a strong protective

effect. NRNOM solutions were much darker than solutions of SRNOM of the same dissolved organic carbon (DOC) concentration (Table 8), therefore these results support the conclusions of Schwartz et al. (2004) and illustrate that the specific absorbance capacity principle can be extended to Ni. A reduction of Ni toxicity in the presence of elevated DOC has been shown previously in the fathead minnow, *Pimephales promelas* (Wu et al., 2003; Deleebeeck et al, 2007a) and *D. magna* (Deleebeeck et al., 2007b). However, in these studies the toxicity reducing impact of Ni-DOC binding was relatively low compared to what might be seen for other metals. For example, silver exhibits a higher ability to complex DOC that results in significant reduction of toxicity for juvenile rainbow trout (Bury et al., 1999). The ability of copper to bind to different sources of natural DOM varies but always results in significant reductions in toxicity to rainbow trout (Schwartz et al., 2004). The study with Ni performed by Deleebeeck et al. (2007a) found that only 8-20% of the total dissolved Ni was complexed by DOC in tests with rainbow trout (*Oncorhynchus mykiss*) and fathead minnow (*Pimephales promelas*). Approximately the same result was obtained in the study of Wu et al. (2003) with fathead minnow. The study of Wu et al (2003) explained that the reduced influence of DOC for Ni (compared to other metals) was likely due to the relatively high EC50 concentrations coupled with the relatively low binding constants of Ni-DOC complexes.

2.4.6. The influence of Na, K or Cl ions on Ni toxicity

As covered previously in the pH discussion, Na and Cl did not alter Ni toxicity to *D. pulex*. Tests with KCl also showed no change in Ni toxicity. These results are similar to

those of Wu et al. (2003) and Pane et al. (2003; 2004). This indicates that Ni is not taken up via mechanisms involving these ions (Deleebeeck et al., 2007b).

2.4.7. The relationship between alkalinity and Ni toxicity.

Several studies with other species have indicated that increase of alkalinity results in decrease of Ni toxicity due to formation of carbonate complexes and this decreases the bioavailability of Ni (Wu et al., 2003; Hoang et al., 2004). Although the tests in this study varied pH and Na, as well as alkalinity, the present results with *Daphnia pulex* seem to differ from those studies, in showing no significant protective effect of alkalinity.

CHAPTER THREE

CONCLUSIONS

These experiments demonstrated the modifying effects that water chemistry and natural organic matter have on the acute toxicity of Ni to *Daphnia pulex*. The test conditions and as well as the water chemistry that was measured on a regular basis, followed the standard methods from Environment Canada, EPS 1/RM/11 (Environment Canada 1990) and throughout this research, the results of the chemistry measurements always met standard test acceptability requirements. Measured concentrations of the major ions of RSW along with Ni concentrations in the test solutions matched nominal concentrations. This allows us to be confident in the results of the performed bioassays. An important feature of this study was that the *Daphnia pulex* were pre-acclimated to the chemistry of very soft water present during toxicity tests. According to previous studies with fish and invertebrates, pre-acclimation helps to avoid additional stress and therefore any negative physiologic effects on test organisms.

Analysis of the results from toxicity tests done with and without an added mixture vitamins revealed that the vitamin mix used in this study did not influence either the water chemistry or the health of the neonate daphnia in acute Ni toxicity tests.

Another important feature of this research is the modification of the Ca: Mg ratio in the recommended standard EPA soft water, to 2: 1. This was done by lowering the Mg concentration. This combination of hardness ions is more representative of soft waters found in central Canada and northern Europe. The change in Ca:Mg ratio resulted in a significant increase of Ni toxicity, illustrating the importance of Mg ions, hardness of water, and probably the ratio itself.

It was expected that both of the hardness ions Ca and Mg would show a major protective effect against Ni toxicity. We can conclude with confidence that competitive effects for binding on the biotic ligand occur. There was a greater effect from Ca possibly because Ca not only competes with Ni for binding, but also regulates membrane permeability. The exact mechanism of competition is uncertain. Our results suggest that Ni uptake is via Ca and/or Mg pathways. This conclusion is supported by the fact that competitive interaction was not observed for Na, Cl or K. It is also possible that Mg competes with Ni for the Mg/CO_3^- co-transport pathway. The difference in the protective effect of Ca might be due to different or additional competition with Ni in binding to Ca-liposome complexes.

In a series of tests, different methods for manipulating water pH were assessed to understand the effects of pH and alkalinity on acute Ni toxicity. The pH results were equivocal. Increases of pH alone (alkalinity was constant) had a significant toxicity-reducing effect. This was demonstrated by the set prepared with organic buffer MOPS. The only difference between the two sets with inorganic buffer was that in one, Na concentration were held essentially stable, however the results obtained were different. Simultaneous adjustments of pH and alkalinity had little impact on the response to Ni. The response to Ni seemed to vary little at pH values below 7.0 and mitigation of toxicity was strongest as water pH value increased from 7.0 to 8.4.

The results of this study support the theory that mechanisms through which pH alters acute Ni toxicity are related to either competitive interaction with protons and/or alterations in the metal complexation capacity in solution. Clearly the effect of pH on Ni

toxicity is complex and requires further study in order to develop a mechanistic understanding.

Tests with NOM sources showed that as DOC concentrations increased, Ni toxicity decreased, probably due to Ni complexation. The results also show apparent differences in the protective effects of different NOM sources. Nordic Reservoir NOM has a 4 fold greater effect than that of Suwannee River NOM. These tests clearly demonstrate the importance of understanding the chemistry of natural OM mitigating capacities for Ni. In addition, our study supports earlier findings that darker coloured NOM sources provide a stronger protective effect against metal toxicity.

As a general conclusion, the presented research demonstrates that the effect of alterations in water chemistry were as predicted within the context of the biotic ligand approach. The data provide information necessary to develop a BLM for the acute effects of Ni in soft water.

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