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# DEVELOPING A SITE SPECIFIC UNDERSTANDING OF THE TOXICITY OF RARE EARTH ELEMENTS, CERIUM AND DYSPROSIUM, TO *DAPHNIA PULEX* AND *HYALELLA AZTECA*

by

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## Abstract

Rare earth elements (REEs) are a group of similarly reactive metals that are divided into two groups; the light (LREEs) and heavy (HREEs) rare earths. These metals are used in a variety of industries and are critical to the function of modern society. The demand for REEs is expected to increase and as a result new mining projects are being developed with a potential for future mining in Canada. The toxicological understanding of these metals in the aquatic environment is very limited. The objective of this research is to compare the toxicological effect of REEs to the freshwater invertebrates *Daphnia pulex* and *Hyalella azteca* and in the more sensitive *Hyalella* to understand the toxicity modifying influence of cationic competition ($Ca^{2+}$, $Mg^{2+}$, $Na^{+}$, $H^{+}$) and dissolved organic matter (DOM) complexation. In this study cerium (Ce; a light REE) and dysprosium (Dy; a heavy REE) were chosen as representative of the two REE groups. Standard methods (Environment Canada) were followed for testing and culture; acute Ce tests were performed at low hardness (12.5 mg $CaCO_3$ mg/L, pH 7.0, Ca 0.18, Na 0.14, Mg 0.14 (mM) for *D. pulex* and 32 mg $CaCO_3$/L, pH 7.2, Ca 0.1, Na 0.101, Mg 0.03 (mM) for *Hyalella* at 21$^{o}$C) while Dy tests were completed at intermediate hardness (62.5 mg $CaCO_3$ mg/L) adjusted to pH 7.8 with Ca at 0.5, Na 0.5, Mg 0.125 (mM) and 23$^{o}$C for both organisms. Acute toxicity tests were performed using <24 h old neonates for 48 h in the case of *Daphnia* and with 2-9 d old offspring for 96 h tests with *Hyalella*. Dissolved concentrations (0.45 µm filtered) for Ce and Dy were lower than total (unfiltered) which demonstrated precipitation over the course of the exposures. As a result, the LC50s based on end concentrations provided the most conservative estimates of toxicity. *Daphnia* was identified as the more resilient organism to REE exposures and thus further testing with toxicity modifying factors was performed using *Hyalella*. The protective effect of cationic competition was tested with Ca (0.1 to 2.0 mM), Na (0.1 to 2.0 mM) and Mg (0.03 to 0.5 mM) and it was determined that Ca and Na (in the case of Dy exposures) were protective of acute REE toxicity while Mg had no effect. Moreover, tests where pH (6.5 - 8.0) was altered showed a protective effect of increased $H^+$ on acute Dy toxicity. The ability of dissolved organic matter (DOM) to complex Ce and Dy were tested with DOM additions (4 to 15 mg C/L) and showed a dose dependent decrease in LC50s. Biotic ligand based parameters (Log K values) were calculated based on free ion relationships using geochemical equilibrium software (WHAM ver. 7.02). The Log K values derived from $Ce^{3+}$ and $Dy^{3+}$ LC50 to *Hyalella* were calculated as 7.3 and 7.75, respectively. The protective influence of $Ca^{2+}$ was determined for $Ce^{3+}$ (Log K = 3.9) and $Dy^{3+}$ (Log K = 3.95) and in the case of $Na^+$ and $Dy^{3+}$ (Log K = 4.10). This study contributes data to the development of site specific water quality guidelines and criteria for Ce and Dy in freshwater.

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# CHAPTER 1

## 1.0 Introduction

### *1.1 Rare Earth Elements*

Rare earth elements (REEs) are a group of 17 similarly reactive metals that include 15 lanthanides, yttrium (Y) and scandium (Sc) (Table 1; Environment Canada, 2012). Yttrium (Y) and Scandium (Sc) are included since they share much of the same chemical properties as lanthanides (Hirano and Suzuki, 1996). Lanthanides are divided into two groups: the light rare earth elements (LREEs), lanthanum (La) to samarium (Sm), and heavy rare earth elements (HREEs; Table 1) comprised of europium (Eu) to lutetium (Lu) as well as Y (Environment Canada, 2012). Scandium is not classified as either a light or heavy REE but because it shares similar chemical properties to other lanthanides it is included as a REE (Environment Canada, 2012). Contrary to their name REEs are not rare; when compared to other metals, the soil REE concentrations are similar to iodine and cobalt and more abundant than those of mercury or silver (Environment Canada, 2012; Paul and Campbell, 2011). In general, the abundance of REEs decreases with increasing atomic number. Enriched deposits are limited and therefore there are a few areas where mining is economically viable particularly for HREEs (Paul and Campbell, 2011).

#### *1.1.1 Production and uses of Rare Earth Elements*

Uses and applications of REEs have resulted in increased demand in recent years and this has altered global production patterns. Until 1985 a single mine, the Mountain Pass mine (in California currently owned by Molycorp), was the primary producer of REEs around the world (Environment Canada, 2012; Paul and Campbell, 2011). However in the early 1990's there was a dramatic shift in production as a result of Chinese involvement in the market. As of 2007 approximately 95% of the global REE production has been from China (Environment Canada, 2012; Paul and Campbell, 2011). The Chinese dominance is believed to be the result of minimal labor costs, low requirements for mining startup investment, abundant high grade reserves, and lax environmental regulations (Paul and Campbell, 2011). Rare earth element production is driven by demand for uses in a variety of sectors; oil industry (petroleum refining catalysts), automotive industry (catalytic converters and permanent magnets), glass production (pigments in ceramic manufacturing, polishers and colorizers), agriculture (chemical supplements and fertilizer), electronic industry (high temperature superconductors and lasers) and health care (anticancer treatment; Ng et al., 2011). Rare earth elements are also used in military equipment ranging from night vision devices to missile guidance systems (Paul and Campbell, 2011**)**. This broad diversity of REEs uses and applications reflect their unique catalytic, magnetic and optical properties (Paul and Campbell, 2011). The electronics sector is the main user of REEs and within this sector the main industry that is predicted to drive future demand is the phosphorous industry from which efficient lighting technology is developed (Paul and Campbell, 2011).

Increasing uses and applications has increased demand. In 2008 the global demand for REE was 125 000 tones and by 2014 it is expected to exceed 200 000 tons annually (Paul and Campbell, 2011). In recent years, however, the Chinese government has dramatically restricted its lanthanide production. In 2010 the Chinese HREE exports decreased by 40% in comparison to the previous year, resulting in massive price increases (Paul and Campbell, 2011**)**. Due to the growing demand and restrictions in supply by 2014 it is expected that there will be a shortage of La, Y and Eu and a likely shortage of Nd, Dy, Tb and Pr. These shortages will likely affect numerous industries but would be more pronounced in the automobile and electronic sectors (United States Environmental Protection Agency (USEPA), 2012). As a result the price increases caused by supply-demand restrictions, there has been an increased activity in exploration and development of potential REE deposits. In Canada the most developed mining project is located at Thor Lake, Northwest Territories and is owned by Avalon Rare Metals Inc. (Environment Canada, 2012**)**.

### *1.1.2 Inputs of REEs into aquatic environment and need for regulation*

Due to the increased production and use of REEs in multiple industries, there is also a growing interest in understanding the potential impact of REEs in the environment especially aquatic systems. As with other naturally occurring potential contaminants, lanthanides can enter aquatic environments and cause impacts via improper practices during mining, recycling and disposal (USEPA, 2012). The growing interest in the potential impacts of REEs in the aquatic systems is due to the fact that there are no water quality guidelines by the Canadian Council of Ministries of the Environment (CCME) and this is also the case in the US where the EPA has not developed water quality criteria. There is a strong need to develop a better understanding of discharge limits and effect thresholds in aquatic systems. Since there are no water quality guidelines or criteria this reflects the fact that there are limited data available on the toxicity of REEs. There is even less known on the influence of potential toxicity modifying factors (TMFs).

## *1.2 Modifying factors for metal toxicity*

Toxicity modifying factors are factors that influence metal toxicity either by mitigating or increasing its effects. TMFs can be divided into two categories: biotic and abiotic factors (Wang, 1987). The biotic factors are related to the biology of the species being studied and are influenced by size, life stage, tolerance as well as adaptability of the organism (Wang, 1987). Abiotic factors are often related to water chemistry and include the influence of temperature, organic substances, inorganic ligands, pH, alkalinity, hardness, the presence of sediment (Wang, 1987) and dissolved organic matter (DOM, quantified as additions of dissolved organic carbon (DOC); Di Toro et al., 2001). The abiotic TMFs reduce metal toxicity by decreasing the bioavailability of the most toxic metal form, the free-metal ion (Campbell, 1995), which in the case of rare earths is represented as $REE^{3+}$ (Figure 1). Metal toxicity to aquatic organisms is influenced by pH, cationic competition, and complexation by inorganic and organic ligands (ie DOM) which influence the bioavailability of the free-metal ion.

### *1.2.1 pH*

Solution pH is one of the main modifying factors influencing metal toxicity (Schubauer-Berigan and Dierkes, 1993) by altering metal speciation and thus bioavailability to aquatic organisms (Campbell and Stokes, 1985). The most bioavailable and toxic form of metal species is the free-metal ion and which dominates at low pH (Campbell, 1995). In contrast, when pH is elevated there may be a decrease in toxicity because carbonates and hydroxides dominate at higher pH and may complex the majority of the free-metal ions (Meyer, 1999). However, for some metals (for instance Cu, Al, Hg, and Pb) decreased pH can also have a protective effect, mitigating toxicity because of decreased uptake. This is due to $H^+$ competition for the same toxicity-inducing sites between metal free-ions and protons (Meyer, 1999). Importantly, while toxicity can vary with pH, the pH level at which toxicity is highest depends on the metal. Different metals have various modes of toxicity and the free-metal ion is not necessarily the only bioavailable form.

### *1.2.2 Cationic competition*

Cationic competition results from the competitive binding between the free-metal ions and major cations ($Mg^{2+}$, $Ca^{2+}$, $Na^{+}$, $H^{+}$) at sites of uptake and/or toxicity on the biological membrane (Di Toro et al., 2001). For example the free-metal ion can cause toxicity by antagonistically binding to the ionoregulatory channels that are normally utilized by essential cations thus disrupting normal function (Paquin et al., 2002). For instance, Ag and Cu act as antagonists of Na while Cd, Zn, Pb and Co are antagonists of Ca and in the case of Ni the presumed antagonist is Mg (McGeer et al., 2011). For instance, Erickson et al. (1996) demonstrated that Cu is an antagonistic inhibitor of Na to fathead minnows, Clifford and McGeer (2010) confirmed that Cd and Ca antagonistically bind in *Daphnia pulex*, and in the same organism Kozlova et al. (2009) showed that Ni is an antagonist of Mg. All three of these studies also found that water hardness (Ca and Mg) had a protective effect (Erickson et al., 1996; Clifford and McGeer, 2010; Kozlova et al., 2009). Rare earth elements share similar physiochemical properties with Ca and are known to be used as Ca probes in cellular membranes (Palasz and Czekaj, 2000). Therefore it is possible that their mode of toxicity towards aquatic invertebrates could be related to disruption of Ca channels.

### *1.2.3 Toxicity mitigation by DOM*

Dissolved organic matter complexes with the free-metal ions and thereby reduces bioavailability (Di Toro et al., 2001). In freshwater systems DOM can vary in its composition and this can be important in its toxicity mitigation ability (Al-Reasi et al., 2011). Dissolved organic matter enters the freshwater environment from several sources including allochthonous (terrestrial), autochthonous (aquatic) and anthropogenic (Baken et al., 2011). Differences among input sources result in each aquatic environment having a unique composition (referred to as DOM quality). Dissolved organic matter can be characterized based on the presence of humic, fulvic and hydrophilic acids and these are often measured using UV absorbance and absorbance-to-fluorescence ratios (De Schamphelaere et al., 2004). Linkages between toxicity and UV absorbance have been shown by De Schamphelaere et al. (2004). A higher absorbance is associated with greater aromaticity which is indicative of elevated humic content. Dissolved organic matter with elevated humic content have stronger metal binding capacity. Higher humic content is found in allochthonous sources and this is important because site-specific differences in DOM quality may result in different toxicity mitigation capacities (Al-Reasi et al., 2011). For example the binding strength and complexation capacity among DOM sources can vary up to one order of magnitude for Cu.

### *1.3 Biotic ligand model (BLM)*

The biotic ligand model (BLM) is a tool for predicting metal toxicity in aquatic environments. The model predicts toxicity based on the interaction of the free-metal ions with the biotic ligand and it accounts for the influence of TMFs such as competing cations, and complexation with inorganic and organic ligands (Di Toro et al., 2001; Niyogi and Wood, 2004; Paquin et al., 2000; Paquin et al., 2002). Complexation reduces toxicity by binding chemical ligands to the free-metal ion thereby reducing bioavailability towards the organism while cationic competition reduces toxicity by antagonistically competing with the free-metal ions for uptake at the site of action (referred to as the biotic ligand; Di Toro et al., 2001; Paquin et al., 2000; Paquin et al., 2002; Niyogi and Wood, 2004; Figure 1). The BLM simultaneously considers the effects of TMFs within a geochemical equilibrium context to predict metal speciation and subsequently toxicity to specific organisms. Mortality is predicted when the concentration of the metal bound to the biotic ligand surpasses the threshold accumulation for that organism. This threshold is defined as the accumulation (at the biotic ligand) that is associated with 50% effect, for the lethality end point the LA50. The practical application of the BLM is to predict metal toxicity on a site-specific basis, as a function of local water chemistry conditions (Paquin et al., 2000). This approach can also be applied for developing water quality criteria/guidelines and in this case sensitive organisms are used so that the resulting policies are protective of all species at a given location. Currently BLMs have been developed for Cu, Ag, Zn and Ni (Niyogi and Wood, 2004), however no BLM has been developed for any of the REEs. There is limited data available on the effects of TMFs towards REE toxicity and no data indicating whether REEs would follow BLM predictions. The only study available is the survey of 63 elements by Borgmann et al. (2005a) which showed that for some REEs toxicity in hard water was less than in soft water.

### *1.4 Sensitive organisms*

Invertebrates are often used to generate toxicological data and BLMs because they can be sensitive to environmental contaminants. In addition to its sensitivity other important factors to consider when selecting an organism for study are its ecological significance, its distribution, the existence of standard testing methods, the ease of culturing, and its ability to survive in most sensitive environmental conditions. *Hyalella azteca* and *Daphnia pulex* are some of the most commonly used invertebrates for assessing metal toxicity that meet all of the considerations stated above.

### *1.4.1 Hyalella azteca*

*Hyalella azteca* is small sediment burrowing detritivore and an epibenthic amphipod that is found in freshwater bodies including streams, ponds and lakes of North and South America (Borgmann et al., 2005b; Environment Canada, 2013; De March, 1977). There is noticeable sexual dimorphism between males and females. The males are larger than the females and possess a large second gnathopod. Mating occurs when the male uses the second gnathopod to adhere to the female in a union called amplexus. The fertilized eggs and hatching neonates are contained within the marsupium for a period of seven to eight days and are released once the female molts. The average brood size per female is 18 neonates while larger females produce bigger broods. Reproduction takes place sexually after each molt (Environment Canada, 2013).

*H. azteca* is a suitable model organism for conducting toxicity testing because of its wide geographic distribution throughout North America, sensitivity towards contaminants, ease of culturing and ecological significance (Borgmann et al., 2005b; Environment Canada, 2013). Additionally, standard biological test methods are available in assessing metal toxicity (Environment Canada, 2013). More importantly, the organism is capable of thriving in soft waters (low hardness) representative of the Canadian Boreal Shield lakes that are associated with heightened toxicity due to increased metal bioavailability.

### *1.4.2 Daphnia pulex*

*Daphnia pulex*, commonly known as the 'water flea', is a small planktonic crustacean that is distributed throughout the world's freshwater streams, rivers and ponds (Environment Canada, 1996). The organism belongs to the order of Cladocera that is characterized by having a translucent shell called a carapace (Hebert, 1978). During normal environmental conditions reproduction occurs asexually though the process called parthenogenesis in which the female produces identical female clones. During periods of stress, involving high population density and food shortage, the organism reverts to sexual reproduction (Stollewerk, 2010). The eggs are produced in the ovaries and are released by the oviduct into the brood chambers where they mature in one to three days. The neonates reach reproductive maturity within ten days and are reproductively viable for five weeks (Environment Canada, 1996). *Daphnia* is a primary consumer that filter-feeds on organic material and is a dominant herbivore (Hebert, 1978; Environment Canada, 1996). Ecologically, the organism is at the bottom of the food chain and is a food source for aquatic invertebrates as well as various fish species (Hebert, 1978; Environment Canada, 1996).

The organism is ideal for soft water toxicity testing since it can be easily cultured in the laboratory, is sensitive to metals, has a short life-cycle, is ecologically significant and can be grown in soft waters that increase metal bioavailability (Environment Canada, 1996).

### *1.5 Previous aquatic toxicity studies with REEs*

There exists a gap in understanding how REEs affect aquatic organisms. Currently there are a limited number of toxicity tests conducted with lanthanides and no clear understanding of how toxicity is modulated in natural waters. Out of the few published studies the most notable ones were conducted with algae (Tai et al., 2010; Hao et al., 1997), sea urchin (Oral et al., 2010), a microcosm of algae, protozoa, and bacteria (Fuma et al., 2005), *Hyalella* (Borgmann et al., 2005a), and *Daphnia* (Barry and Meehan, 2000; King et al., 2003). These works, while relatively limited, offer some insight into REEs toxicity, particularly with respect to prioritizing further studies.

### *1.5.1 REE comparison studies*

Tai et al. (2010) examined the toxicity of 13 lanthanides towards the marine algae *Skeletonema costatum*. Individual lanthanides had the same 50% effect concentration (EC50) of 29 µM for the whole series and mixtures had the same toxicological effect as individual elements indicating that algal cells do not discriminate between individual lanthanides. However, there were cases, for examples with Y and Sc where EC50 values varied (Tai et al., 2010). This suggests that lanthanides as a chemical group behave similarly to single celled algae in comparison to other REEs. In contrast, closely related lanthanides respond differently in multicellular organisms such as the sea urchin. Oral et al. (2010) examined the toxicity of La and Ce towards the sea urchin (*Echinus melo*) embryos using development and survival as test endpoints. At the highest concentration of 10 µM for both metals, toxicity was more pronounced for Ce than La since Ce-exposed embryos perished while the La-exposed ones survived but with defects (Oral et al., 2010).

### *1.5.2 TMFs and REE toxicity*

There is limited information available on REE toxicity as it relates to TMFs such as pH, cationic competition, and organic ligand complexation. Fuma et al. (2005) confirmed that for REEs the free-metal ion is the metal species responsible for inducing toxicity and that its presence is influenced by pH fluctuations. The toxicity of Dy was assessed in a microcosm with species which included algae (*Euglena gracilis),* protozoa (*Tetrahymena thermophile)* and bacteria (*Escherichia coli).* The study revealed that the pure culture organisms were up to two times more sensitive than their microcosm counterparts. The decreased Dy toxicity within the microcosm is believed to occur due to the lower concentration of $Dy^{3+}$ that resulted from increased complexation with organic matter and hydroxides at elevated pH (Fuma et al., 2005).

Barry and Meehan (2010) conducted acute and chronic toxicity La studies with *Daphnia carinata* using three different media that covered a range of water hardnesses. When the water hardness in the media increased from 22 to 160 mg $CaCO_3$/L the acute EC50 values increased from 43.2 to 1180 µg La/L. Since Ca and La have physicochemical similarities they may behave similarly in biological systems and it was argued that low Ca would result in decreased competition among the two metals, greater La uptake and increased toxicity (Barry and Meehan, 2010). Since the remaining REEs share much of the same chemical properties with La they may behave similarly to increases of Ca in solution.

Hao et al. (1997) performed toxicity testing with La, Gd and Y on the alga *Chlorella vulgaris* Beijerinck in an attempt to see the influence of organic ligands on bioavailability. The addition of citrate, nitrilotriacetic acid (NTA) or ethylenediaminetetraacetic acid (EDTA) resulted in reducing the bioconcentration factor compared to solution without complexing ligands thus demonstrating that the biotic ligand principle of complexation may apply to La, Gd and Y (Hao et al., 1997).

Environmental cases where metal toxicity is heightened often have low levels of TMFs. In Canada such conditions are representative of Boreal Shield waters which are low in hardness. Currently, there are two published studies which assess the toxicity of all the REEs in the water chemistry conditions representative of Boreal Shield soft waters. These studies use two sensitive freshwater invertebrates *Daphnia pulex* and *Hyalella azteca* (King et al., 2003; Borgmann et al., 2005a) since they are capable in thriving under conditions of low hardness. King et al. (2003)

compared the toxicity of light and heavy REEs to *Daphnia pulex* in soft waters of 12 mg $CaCO_3$/L. It was determined that the EC50 values exceeded the Canadian Domestic Substances criteria of 1 mg/L for the majority of the metals except for Nd, Lu and Yb. This study indicates that there is a need to establish concrete endpoints using *D. pulex* for the metals in which no EC50 is established. Moreover, there is a need to use a more sensitive organism in order to accurately assess lanthanide toxicity in extremely soft waters. Similarly, Borgmann et al. (2005a) assessed the toxicity of REEs using a more sensitive organisms *Hyalella azteca* in soft waters of 18 mg $CaCO_3$/L. This study established EC50 values for all of the lanthanides and determined that some of the most toxic REEs from both the light and heavy groups were Cerium (Ce) and Dysprosium (Dy), respectively. Since Ce and Dy were determined to be relatively toxic metals in their respective groups their toxicological profile in the environmentally sensitive conditions is of interest.

### *1.6 Cerium and Dysprosium speciation*

In the aqueous environment REEs readily complex common anions such as fluorides ($F^-$), sulfates ($SO_4^{2-}$), phosphates ($PO_4^{3-}$), hydroxides ($OH^-$), carbonates ($CO_3^{2-}$), silicates ($SiO_4^{4-}$) and NOM; with carbonates and organic complexes being the most dominant forms of REE species (Janssen and Verweiji, 2003; Moermond et al., 2001; Sneller et al., 2000). In some instances the free-metal ion and sulfates can be included as the main REE fractions. Data obtained from the groundwater sites located in Vierlingsbeek, Netherlands demonstrates this notion since the predominant forms of REE species were $REE^{3+}$, $REE(SO_4^+)$, $REE(CO_3^+)$ and REE(DOC) **(**Janssen and Verweiji, 2003). The persistence of the free-metal ion in natural environments is of particular interest since this form is believed to be most bioavailable and thus most toxic.

The notion that the free-metal ion is the most bioavailable species appears to hold true for REEs. Hao et al. (1997) confirmed that the most bioavailable form of these metals to algae *Chlorella vulgaris* Beijerinck is the free-metal ion and ranked the relative bioavailability of the dissolved metal fractions to be highest for the $REE^{3+}$ and the lowest for the inorganic and organic complexes (Hao et al., 1997). Moreover, Moermond et al. (2001) suggested that the main bioavailable forms of lanthanides in estuaries are $REE^{3+}$ and REE (OH). Since REEs are divided into light and heavy, Ce and Dy are of particular concern since they have been shown to exhibit higher toxicity in their respective groups to a sensitive amphipod *Hyalella azteca* (Borgmann et al., 2005a); thus the persistence of these free-metal ions is of interest.

Cerium has two oxidation states: $Ce^{3+}$ and $Ce^{4+}$ (Table 1). In anoxic environments Ce can exist as the soluble, and thus more bioavailable $Ce^{3+}$, which in oxic environments can be oxidized to the insoluble $Ce^{4+}$ with the formation of $CeO_2$ (Ng et al., 2011). Dysprosium does not have a second oxidation state and thus $Dy^{3+}$ (Table 1) is believed to be the most bioavailable metal form (Ng et al., 2011). The abundance of these free-metal ions in aquatic environments can be estimated with the use of geochemical modeling programs.

In aqueous solutions, metal speciation can be predicted with the use of geochemical equilibrium software which predicts metal speciation across a wide range of natural water chemistry parameters. One of the most advanced modeling packages is the Windermere Humic Aqueous Model (WHAM; model 7.02; Tipping et al., 2011) which takes into account lanthanide inorganic and organic complexation. Since Ce and Dy are the most relevant lanthanides, their speciation can be modeled using this software.

Modeling metal speciation using WHAM7 can be applied to predict various fractions of aqueous metal and to identify the most abundant species. Based on rearing and testing water chemistry (Table 2) and using equivalent Ce and Dy concentration (1mM) the most prevalent species (as predicted by WHAM7) are summarized in Table 3. Since $Ce^{3+}$ and $Dy^{3+}$ are the most relevant fractions investigating how variation in $H^+$ effects speciation and toxicity is of interest.

Variation in pH has a strong influence on $REE^{3+}$ speciation and thereby toxicity. As pH increases from 6.3 to 8.3 WHAM7 predicts a substantial decrease in $Ce^{3+}$ and $Dy^{3+}$ (Table 3; Figure 2). On the basis of % of total aqueous metal, pH increase lowers the free-metal ion fraction from 88 to 10% and 24 to 0.3% for Ce and Dy, respectively (Table 3). The decrease in $Ce^{3+}$ is offset with increasing carbonate formation which dominates Ce speciation at elevated pH and this is also true for Dy (Table 3; Figure 2). The elevated carbonate formation is supported by Sneller et al. (2000) and Moermond et al. (2001) who state that lanthanides in general predominantly form carbonate complexes at higher pH. Since lower pH produces more $Ce^{3+}$ and $Dy^{3+}$ the toxicity of these metals is expected to increase in these conditions.

The change in Ce and Dy speciation is not limited to pH alterations and WHAM7 can be used to predict $REE^{3+}$ speciation with the addition of other toxicity modifying factors such as salts of common cations and DOM. Changes in the salts of major cations are expected to change $REE^{3+}$ speciation through complexation of the free-metal ion with inorganic salt anions (Figure 1). For instance, increase in $NaHCO_3$ should decrease the presence of $REE^{3+}$ through inorganic complexation with the predominant $HCO_3^-$ anion at circumneutral pH. Furthermore, modeling Ce or Dy speciation with DOM additions above 4 mg C/L drastically decreased the presence of $Ce^{3+}$ and $Dy^{3+}$ in solution. Increase in DOM is known to complex metal thereby making it less bioavailable to the organism (Al-Reasi et al., 2011, Figure 1).

## 1.7 Objectives

The overall objective of this research is to contribute data towards the development of water quality guidelines and/or criteria that are protective of freshwater organisms in the presence of REEs. In order to fulfil this broad goal the purpose of this study was to generate toxicological data based on acute exposures of two REEs to common freshwater invertebrates and in the more sensitive organism assess how water chemistry affects toxicity. The two metals used in this study are Ce (a LREE) and Dy (a HREE): the focus of Chapter 2 and Chapter 3, respectively.

In both chapters the objectives of this research are to:

1) Compare the relative sensitivity of *Daphnia pulex* and *Hyalella azteca* to acute Ce or Dy exposures and then in the more sensitive organism:
2) Determine if changes in water chemistry (e.g. alteration of $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$, $H^{+}$ and DOM) follow BLM principles of cationic competition and DOM complexation and if they do:
3) Establish geochemical equilibrium binding parameters (e.g. Log K values) as a means to link speciation and REE toxicity.

### 1.8 Tables and Figures

Table 1. Rare Earth Element chemical properties and occurrence in the Earth's crust.

| Element[1] | Atomic number[1] | Atomic weight (g/mol)[1] | Light/ Heavy REE[2] | Oxidation state[1] | Ionic radius (pm)[1c] | Crustal abundance[3] |
|---|---|---|---|---|---|---|
| Lanthanum (La) | 57 | 138.91 | Light | $3^+$ | 118 | 30 ppm |
| Cerium (Ce) | 58 | 140.12 | | $3,4^+$ | 114 | 60 ppm |
| Praseodymium (Pr) | 59 | 140.91 | | $3^+$ | 114 | 8.2 ppm |
| Neodymium (Nd) | 60 | 144.24 | | $3^+$ | 112 | 28 ppm |
| Promethium (Pm) | 61 | 145 | | $3^+$ | 110 | [b] |
| Samarium (Sm) | 62 | 150.36 | | $2,3^+$ | 109 | 6 ppm |
| Europium (Eu) | 63 | 151.96 | | $2,3^+$ | 107 | 1.2 ppm |
| Gadolinium (Gd) | 64 | 157.25 | Heavy | $3^+$ | 106 | 5.4 ppm |
| Terbium (Tb) | 65 | 158.93 | | $3^+$ | 104 | 0.9 ppm |
| Dysprosium (Dy) | 66 | 162.50 | | $3^+$ | 103 | 3 ppm |
| Holmium (Ho) | 67 | 164.93 | | $3^+$ | 102 | 1.2 ppm |
| Erbium (Er) | 68 | 167.26 | | $3^+$ | 100 | 2.8 ppm |
| Thulium (Tm) | 69 | 168.93 | | $3^+$ | 99 | 0.48 ppm |
| Ytterbium (Yb) | 70 | 173.04 | | $2,3^+$ | 98 | 3 ppm |
| Lutetium (Lu) | 71 | 174.98 | | $3^+$ | 97 | 0.5 ppm |
| Yttrium (Y) | 39 | 88.91 | | $3^+$ | 102 | 33 ppm |
| Scandium (Sc) | 21 | 44.96 | [a] | $3^+$ | 87 | 22 ppm |
| Magnesium (Mg) | 12 | 24.30 | - | $2^+$ | 89 | 2.33 % |
| Calcium (Ca) | 20 | 40.08 | - | $2^+$ | 112 | 4.18 % |
| Sodium (Na) | 11 | 22.99 | - | $1^+$ | 118 | 2.36 % |
| Potassium (K) | 19 | 39.10 | - | $1^+$ | 151 | 2.09 % |

[1] - (Lide, 2010). [2] - (Environment Canada, 2012). [3] - (Taylor, 1964).

[a] - Scandium is classified as one of the rare earth elements but is not considered as light or heavy (Environment Canada, 2012). [b] - Promethium is not naturally present in the earth's crust since is a byproduct of uranium decay (Ng et al., 2011). [c] - Data derived from coordination number 8.

Table 2. Base water chemistry from culture and test media. Tests with Ce and Dy were performed in two different mediums.

| Components | Ce | Dy | units |
|---|---|---|---|
| $CaCl_2$ | 0.1 | 0.5 | mM |
| $MgSO_4$ | 0.025 | 0.125 | |
| KCl | 0.005 | 0.025 | |
| $NaHCO_3$ | 0.1 | 0.5 | |
| NaBr | 0.001 | 0.005 | |
| pH | 7.3± 0.1 | 7.7± 0.1 | |
| DOC | 0.03 | 0.4 | mg C/L |
| Hardness | 12.5 | 62.5 | mg $CaCO_3$/L |
| Temperature | 21± 1 | 23± 1 | $^o$C |

Table 3. Ce and Dy speciation given as the % of the total concentration (1 mM) as predicted by WHAM7 (ver. 7.0.2) and based on Table 2 water chemistry with colloidal DOC (modeled 10% humic (HA) and 90% fulvic acid (FA)) and atmospheric pressure (1 atm).

| Ce speciation | pH |  |  | Dy speciation | pH |  |  |
|---|---|---|---|---|---|---|---|
|  | 6.3 | 7.3 | 8.3 |  | 6.3 | 7.3 | 8.3 |
| Ce[3+] | 88.7 | 53.7 | 10.3 | Dy[3+] | 24.4 | 4.2 | 0.3 |
| CeSO4[+] | 6.4 | 3.9 | 0.7 | DySO4[+] | 9.7 | 1.7 | 0.1 |
| CeCO3[+] | 3.3 | 37.8 | 79.0 | DyCO3[+] | 12.1 | 38.3 | 27.3 |
| CeHCO3[2+] | 0.7 | 0.8 | 0.2 | DyHCO3[2+] | 1.3 | 0.4 | 0.0 |
| Ce(CO3)2[-] | 0.0 | 0.1 | 3.2 | Dy(CO3)2[-] | 0.1 | 3.3 | 25.2 |
| CeOH[2+] | 0.5 | 3.2 | 6.1 | DyOH[2+] | 0.8 | 1.4 | 0.9 |
| HA - Ce | 0.0 | 0.0 | 0.0 | HA - Dy | 4.5 | 4.5 | 4.0 |
| FA - Ce | 0.4 | 0.4 | 0.4 | FA - Dy | 47.0 | 46.2 | 42.1 |

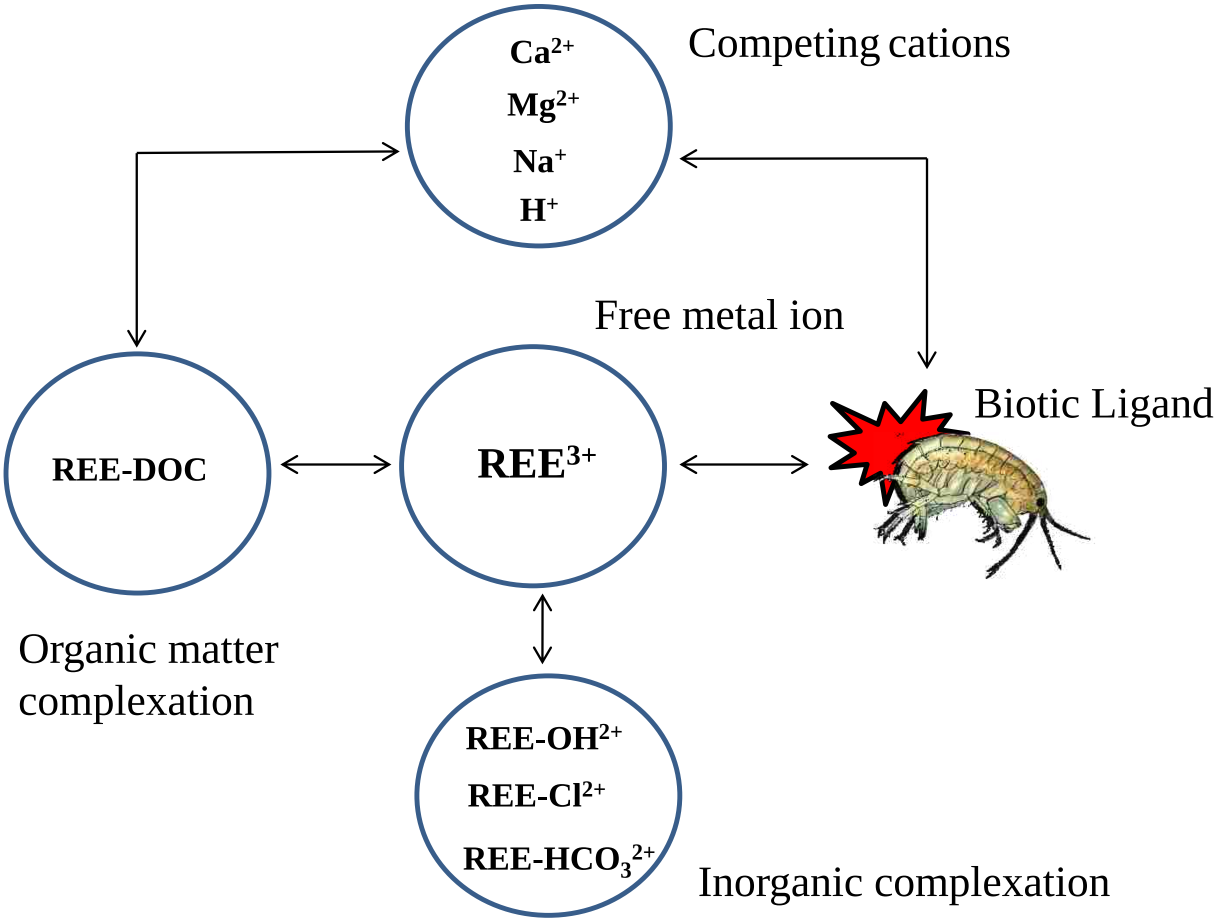

Figure 1. Conceptual representation of the Biotic Ligand Model (modified from Di Toro et al., 2001; Paquin et al., 2002). Metal toxicity is predicted based on the free-metal ion ($REE^{3+}$) bioavailability to the organism as the parameters of water chemistry (cationic competition, inorganic and organic complexation) change.

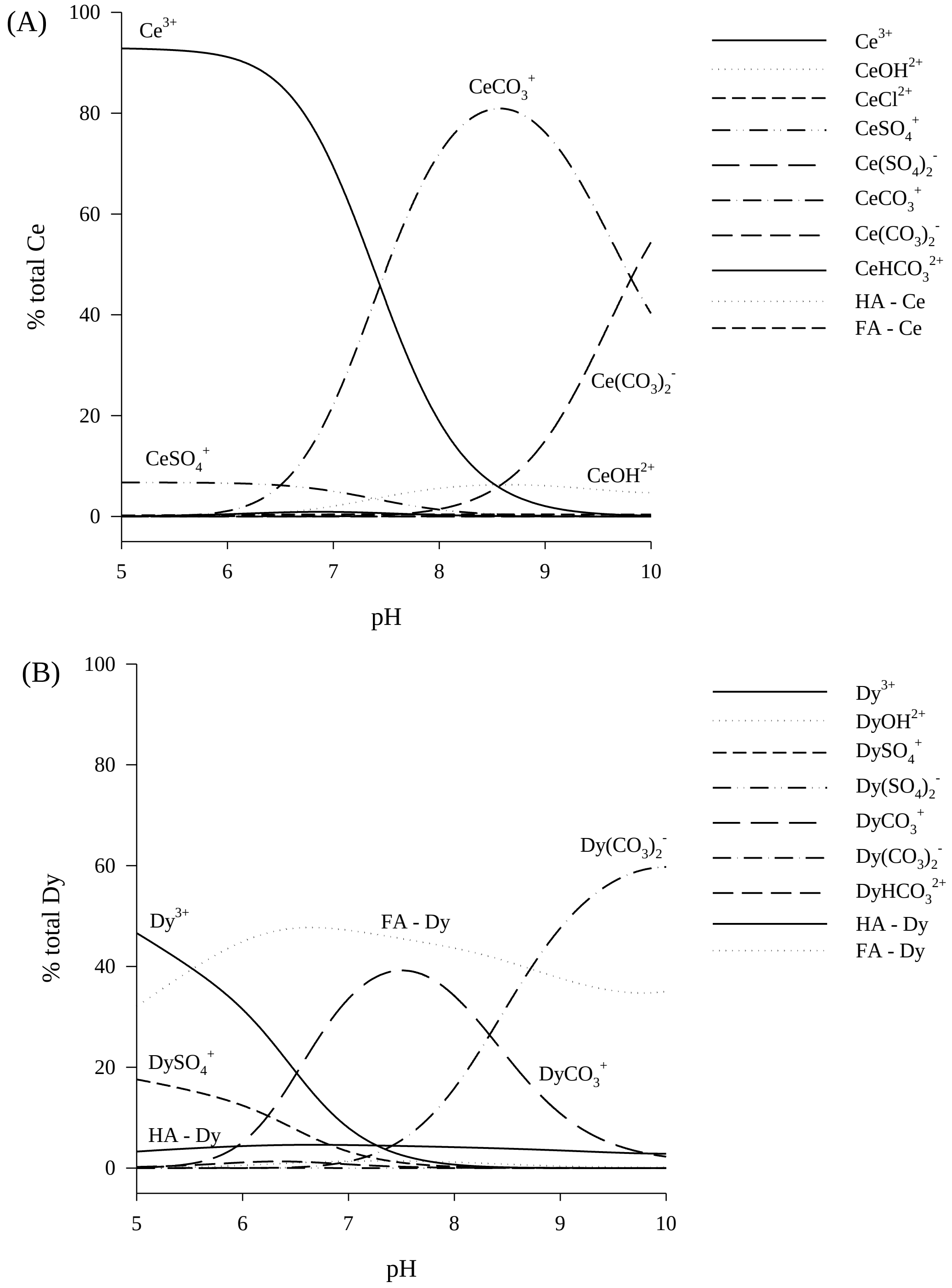

Figure 2. Rare earth element speciation as % of total Ce (A) or Dy (B) and function of pH in *Hyalella* base medium. Modeled (WHAM ver. 7.02) based on Table 2 water chemistry with REEs (1 mM), colloidal DOC (10% humic) and pressure (1 atm).

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# CHAPTER 2

# Toxicity modifying effects of cationic competition and dissolved organic matter complexation on acute Cerium toxicity to *Hyalella azteca*.

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## Abstract

The toxicological understanding of rare earth elements (REEs) in the aquatic environment is very limited but of increasing concern as the use of these metals continues to grow. The objective of this research is to compare the toxicological effect of the REE Cerium (Ce) to the freshwater invertebrates *Daphnia pulex* and *Hyalella azteca* and in the more sensitive organism, understand the toxicity modifying influence of Ca, Na, Mg and dissolved organic matter (DOM). Standard methods (Environment Canada) were followed for testing and culture: for *D. pulex* (32 mg $CaCO_3$ mg/L) at pH 7.0 with Ca at 0.18, Na 0.14, Mg 0.14 (mM) and $21^{o}$C while for *H. azteca* (12.5 mg $CaCO_3$ mg/L) at pH 7.1 with Ca at 0.1, Na 0.1, Mg 0.03 (mM) and $21^{o}$C. Acute toxicity tests were done with <24 h old neonates for 48 h in the case of *Daphnia* and with 2-9 d old offspring for 96 h tests with *Hyalella*. The potential protective effect of cationic competition was tested with Ca (0.1 to 2.0 mM), Na (0.1 to 2.0 mM) and Mg (0.03 to 0.5 mM). The effect of White River DOM complexation was tested with dissolved organic carbon (DOC) at nominal 6 and 12 mg C/L. Dissolved Ce concentrations were lower than total (unfiltered) indicating precipitation, particularly at higher concentrations. Acute toxicity of Ce to *H. azteca* and *D. pulex* revealed *Hyalella* to be 11 times more sensitive than *Daphnia* in hardness matched soft water. Additions of Ca but not Na and Mg provided significant protection against Ce toxicity to *Hyalella*. DOM also mitigated Ce toxicity in a concentration dependent matter. Biotic ligand based parameters (Log K values) were calculated based on free ion relationships as determined by geochemical equilibrium modeling software (WHAM ver. 7.02) and based on initial dissolved Ce concentrations. The log K value for $Ce^{3+}$ toxicity to *Hyalella* was 7.3 while the protective influence of Ca was 3.9. This study contributes data towards the development of site specific water quality guidelines and criteria for Ce and offers insight into the complex bio-geochemical nature of this element.

Keywords: REEs; *Hyalella azteca*; TMFs; BLM; WQC; risk assessment

## 2.0 Introduction

Rare earth elements (REE), are a group of 17 similarly reactive metals that include 15 lanthanides as well as yttrium and scandium (Environment Canada, 2012; Migaszewski and Galuszka, 2015). These metals are divided into two distinct groups: the light rare earth (LREE) and heavy rare earth (HREE) elements. The more abundant LREEs are comprised of lanthanum to samarium while the less abundant HREEs consist of europium to lutetium in addition to yttrium (Environment Canada, 2012; Tyler, 2004; Migaszewski and Galuszka, 2015). Contrary to their name, REEs are not rare, but are named for limited areas where enriched deposits can be economically mined. In comparison to other metals, REEs have soil concentrations similar to iodine and cobalt and greater than those of mercury or silver (Environment Canada, 2012; Paul and Campbell, 2011).

Rare earth element industrial applications are diverse, reflecting their unique set of catalytic, magnetic and optical properties (Paul and Campbell, 2011). Cerium (Ce), classified as a LREE, is the most abundant rare earth found in mineral deposits (Dahle and Arai, 2015). Compounds consisting of Ce based salts and oxides have been used in various applications (Dahle and Arai, 2015). For instance, Ce salts have been used to relive vomiting, decrease blood clotting, treat topical burns, enhance alloy oxidative resistance and mediate organic synthesis reactions (Dahle and Arai, 2015). Whereas Ce oxides, such as cerium dioxide ($CeO_2$) nanoparticles, have been used to catalyze chemical reactions, manufacture solid oxide fuel cells, polish glass and absorb UV radiation (Dahle and Arai, 2015). The diversity in Ce uses is reflective of this metals distinct chemistry.

In comparison to other REEs, Ce and Eu are unique to the group since they have two oxidation states. Cerium can exist in two forms $Ce^{3+}$ and $Ce^{4+}$. Under conditions of elevated redox potential (Eh) $Ce^{3+}$ tends to oxidize to $Ce^{4+}$ and form the insoluble $CeO_2$. In freshwaters of moderate Eh and pH values Ce is predicted to precipitate as $CeO_2$ (Migaszewski and Galuszka, 2015) and this has been speculated to be the primary process for the occurrence of negative Ce anomalies (Seto and Akagi, 2008).

Rare earth elements occur at relatively low dissolved concentrations in freshwaters (usually ng/L to µg/L, Leybourne and Johannesson, 2008; Verplanck et al., 2001) and in most cases the dissolved Ce concentration is highest. Water samples from mining sites where REE concentrations were expected to be low had Ce concentrations documented below 161 µg/L (Verplanck et al., 2001) but for some mining projects Ce concentration has been recorded as high as 6270 µg/L in the surrounding groundwater (Miekeley et al., 1992). The toxic effects of Ce on aquatic organisms are not well understood. Currently, there are no water quality guidelines in Canada and this is also the case in the United States where there are no water quality criteria. The lack of regulatory development for Ce (or any REEs) in aquatic systems is reflective of the limited number of aquatic toxicity studies, especially those looking at the influence of water chemistry on Ce toxicity.

Out of the few published REE toxicity studies the most notable were performed with algae (EL-Akl et al., 2015; Tai et al., 2010; Hao et al., 1997; Yang et al., 2014; Zhao and Wilkinson, 2015), sea urchin (Oral et al., 2010), a microcosm of algae, protozoa, and bacteria (Fuma et al., 2005), *Daphnia* (Barry and Meehan, 2000) and *Hyalella* (Borgmann et al., 2005). These studies although conducted with different REEs under various chemical conditions, demonstrate that toxicity is influenced by water chemistry. For example, Barry and Meehan

(2010) conducted acute La exposures to *Daphnia carinata* and showed that increased water hardness ameliorates toxicity. They reasoned that the chemical similarity of $La^{3+}$ to $Ca^{2+}$ reduce toxicity through the inhibition of many Ca dependent biological systems and that elevated Ca concentrations were protective of La toxicity through cationic competition. Recently, study by EL-Akl et al. (2015) examined Ce uptake by algae and demonstrated decrease in uptake with increasing dissolved organic matter (DOM). The study of Borgmann et al. (2005) performed acute Ce exposures to *Hyalella azteca* and showed that the LC50 in soft water was lower than in intermediate hardness water. These studies indicate that water chemistry can influence Ce toxicity and stress the need for further investigations of Ce effects in aquatic settings.

It is well known that water chemistry can alter the acute toxicity of metals through the influence of toxicity modifying factors (TMFs; Paquin et al., 2002). The TMFs affect metal toxicity by either binding to the free-metal ion and/or antagonistically competing with the free-metal ion for uptake at the site of toxic action (Di Toro et al., 2001). The free-metal ion is believed to be the most toxic metal form (Campbell, 1995) but it is unclear whether the trivalent Ce ion (e.g. $Ce^{3+}$, Migaszewski and Galuszka, 2015) also induces toxicity. The influence of water chemistry on metal toxicity can be predicted with the use of geochemical equilibrium based model such as the biotic ligand model (BLM).

The BLM is the most advanced method to comprehensively account the influence of water chemistry on metal toxicity. Biotic ligand model predicts toxicity based on the interaction of the free-metal ions with the site of toxic action called the biotic ligand. This model accounts for the influence of TMFs such as competing cations, and complexation with inorganic and organic ligands (Di Toro et al., 2001; Niyogi and Wood, 2004; Paquin et al., 2000; Paquin et al., 2002). The BLM simultaneously accounts the influence of TMFs within a geochemical equilibrium framework to predict metal speciation and toxicity to specific organisms. Mortality is predicted when the concentration of the metal bound to the biotic ligand exceeds the threshold accumulation for that particular organism. This threshold is defined as the accumulation (at the biotic ligand) that is associated with 50% effect, for the lethality end point called the LA50. Biotic ligand model can be used to predict metal toxicity on a site-specific basis, as a function of local water chemistry parameters (Paquin et al., 2000).

The objective of this study was to develop data on the acute toxicity of Ce to sensitive aquatic invertebrates and to understand how water chemistry influences Ce toxicity. The initial step in this study was to compare the sensitivities to Ce between *Daphnia pulex* and *Hyalella azteca* and in the more sensitive organisms apply the BLM approach through the systematic investigation of potential TMFs, including $Ca^{2+}$, $Na^{+}$, $Mg^{2+}$ and DOM.

## 2.1 Materials and Methods

### *2.1.1 Invertebrate culturing*

#### *2.1.1.1 Hyalella azteca*

*Hyalella* cultures were acquired in 2010 from Hannah Lake near Sudbury Ontario and cultured at Wilfrid Laurier University. The collected organisms were identified morphologically (Pennak, 1978) and genetically as *H. azteca* belonging to the inner Sudbury clade **(**Babin-Fenske et al., 2012). Procedures for culturing *Hyalella* followed Standardized Biological Test Methods EPS 1/RM/33 2nd edition (Environment Canada, 2013). Culturing solution, called reconstituted soft water (RSW) was designed to mimic Boreal Shield soft waters (Borgman et al., 2005) and was based on a 90% dilution of the standardized aquatic medium for *Hyalella* growth and reproduction (Borgmann, 1996). RSW was made with analytical grade salts (Sigma Aldrich, Mississauga, ON) of $CaCl_{2,}$ $NaHCO_3$, $MgSO_4$, KCl and NaBr which when diluted with deionized water (18 Mohm, Milli-Q A30, Millipore Corporation, Fisher Scientific Nepean, ON) were at nominal concentrations of 100, 100, 25, 5 and 1 µM, respectively. Culture medium was aerated and had a hardness of 12.5 mg $CaCO_3$/L with solution pH 7.1 ± 0.1 (measured using Radiometer PHM240 meter equipped with pHC2701 electrode (ATI Scientific, Mississauga, ON)).

Cultures were maintained in 2 L polypropylene beakers (VITLAB® Griffin, VWR International, Mississauga, ON) with approximately 60 adult organisms in at least 1.5 L of RSW. Beakers were covered with a plastic lid and a water change was done weekly using 650 and 275 µm mesh to separate adults from neonates. After each water change a new piece cotton gauze of approximately 5 by 5 cm was placed in solution to act as substrate (Borgmann et al., 1989). *Hyalella* cultures were fed 5 mg ground fish flakes (TetraMin, Tetra Holding (US) Inc., Blacksburg, VA) three times weekly, on non-consecutive days. Neonates used for toxicity testing were collected (into separate glass beaker containing RSW but no food) during water change and were 2 to 9 d of age at the beginning of tests. Cultures were kept at 21 ± 1ºC with light intensity 500-1000 lux and photoperiod 16 h light: 8 h dark.

### *2.1.1.2 Daphnia pulex*

*Daphnia pulex* was acquired from Aquatic Research Organisms Inc. (Hampton, NH) and cultured in 1 L glass beaker. Procedures for culturing *D. pulex* followed standard methods (Environment Canada, 1996). Culturing was carried out in artificial soft water (ASW) which was made with analytical grade salts (Sigma Aldrich, Mississauga, ON) of $CaSO_4$, $NaHCO_3$, $MgSO_4$ and KCl which when diluted with deionized water (18 Mohm, Milli-Q A30, Millipore Corporation, Fisher Scientific Nepean, ON) were at nominal concentrations of 180, 140, 140 and 30 µM, respectively. Culture medium was aerated and had a hardness of 32 mg $CaCO_3$/L and solution pH of 7.0 ± 0.1. Water changes were carried out daily in order to separate neonates and adults. *Daphnia* feeding followed each water change and consisted of 10 mL of algae (30% *Chlorella vulgaris* and 70% *Pseudokirchneriella subcapitata*) and 5 ml of a yeast, cerophyl and trout chow mix (YCT, Aquatic Research Inc., Hampton, NH). Temperature, lighting and photoperiod were the same as for *Hyalella* cultures. *Daphnia* toxicity tests were carried out without food using neonates less than 24 h old.

### *2.1.2 Acute toxicity testing with Ce*

Acute toxicity tests for *Hyalella* (96 h) and *Daphnia* (48 h) were carried out following standard methods (Environment Canada, 2013 and 1996, respectively). In brief, Ce exposure solutions were prepared in duplicate by adding the appropriate amount of Ce stock solution generated from a 1 g/L atomic absorption standard (Inorganic Ventures, Christiansburg, VA) to culture medium (RSW for *Hyalella* and ASW for *Daphnia*) and then allowing 24 h of equilibration prior to test start. To allow for direct comparison of the two organisms, side-by-side tests were conducted, first in their respective rearing media and then in hardness matched conditions (Ca and Mg additions to RSW to match 32 mg $CaCO_3$/L ASW). These tests revealed *Daphnia* as the more resistant organism and therefore subsequent TMF tests were performed with the more sensitive *Hyalella*.

#### ***2.1.2.1 Tests with TMFs***

A series of tests were done for TMFs, beginning with the culture medium (RSW) and then in a progressive manner systematically modifying concentrations in order to understand how Ca, Mg, Na and DOM influence acute Ce toxicity to *Hyalella*. In each test series one chemical parameter was varied while keeping others constant. Test were done in duplicate in 400 mL polyethylene beakers with 240 mL of exposure solution. Test solutions were equilibrated for 24 h before the test started and during this time gauze pieces (5 cm x 5 cm) were also equilibrated but in a separate plastic beaker with the test medium. Exposures were initiated by adding the gauze and then 10 *Hyalella* 2-9 d of age to each beaker. Beakers were covered and held in the same environment as cultures (described above) without food for 96 h.

Acute Ce exposures used concentrations ranging from 0.04 - 66 µM with the exact range in any test dependent on the degree of toxicity modification anticipated by the TMF. The culture medium was altered by sequentially changing the concentration of the selected TMF while keeping others constant. Ca was added as $CaCl_2$, Mg as $MgSO_4$ and Na as NaCl. The pH in experimental solutions were stabilized using 3.6 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (De Schamphelaere et al., 2004) with the additions of KOH. Each test included two controls, the modified media either with or without added MOPS. The control with MOPS ensured that the buffer was not a contributing factor to toxicity and were at the same levels as the highest exposure concentration. The effect of DOM on Ce toxicity was evaluated by adding natural organic matter (previously collected as concentrate using reverse osmosis) from a site near White River, Ontario (WR-DOM) at either 6 or 12 mg C/L. Collection of DOM was performed based on the method described by Gheorghiu et al. (2010). Exposure water chemistry is summarized in Table 2.

#### *2.1.2.2 Ce sampling and characterization*

Water samples were collected shortly after solutions were made and when exposures ended (96 h) in order to characterize Ce content. For each sampling time and each exposure concentration, a pair of 10 mL samples were collected, one unfiltered and the other filtered (pre-rinsed 0.45 µm, Acrodisc HT tuffryn membrane, Pall Corporation, MI). Samples were stored in 15 mL centrifuge tubes (Celltreat, Mandel Scientific, Guelph, ON) and preserved with 2% v/v 16 N $HNO_3$ (trace metals grade, Fisher Scientific, Nepean, ON) before being analyzed. Additional samples (50 mL) from tests containing DOM were collected and 0.45 µm filtered for subsequent measurement of dissolved organic carbon (DOC) at the start or end of tests. DOC samples were stored at 4°C and not acidified before being measured with the total organic carbon analyzer (TOC-LCPH, Shimadzu, Mandel Scientific, Guelph, ON). Background DOC concentration in RSW was 0.272 mg C/L.

Ce concentrations were determined for unfiltered (total Ce: $Ce\text{-}_T$) and filtered (dissolved Ce: $Ce\text{-}_D$) samples (from the initial and final sampling period) using inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 8000, PerkinElmer Inc., Woodbridge, ON). Analysis parameters and wavelengths followed manufacturer recommendations. Quality assurance procedures for Ce measurements involved analysis of reference standards (Standard 26, Inorganic Ventures Inc., Christiansburg, VA) and reagent blanks (2% $HNO_3$) throughout each run. The average measured concentrations for Ce in controls (RSW medium and sampled from initial timeframe) for Ce-$_T$ was 2.8 µg/L (stdev= 3.0, n=9) and for Ce-$_D$ was 2.3 µg/L (stdev =1.3, n=15).

### *2.1.2.3 Calculations and statistics*

Measured Ce-$_T$ and Ce-$_D$ were compared by paired T-test assuming unequal variances. Ce concentration resulting in 50% mortality (LC50) was calculated based on nominal (Ce-$_N$) and measured (Ce-$_T$ and Ce-$_D$) exposure concentrations from the initial and final (96 h) sampling period with the Comprehensive Environmental Toxicity Information System software (CETIS, Tidepool Software, 2005) using the trimmed Spearman Karber method (Hamilton et al., 1977). Significant differences in LC50s were determined using the Litchfield and Wilcoxon method (1949, Environment Canada, 2005).

The $Ce^{3+}$ concentrations were estimated from LC50 values (Ce-$_D$ basis) using the Windermere Humic-Aqueous Model (WHAM ver. 7.02., Tipping et al., 2011) and nominal water chemistry (Table 2). DOC entered into WHAM was assumed to be in colloidal phase and at 90% fulvic and 10% humic acid (Santore et al., 2001). Speciation data from WHAM was used to develop stability constants for competitive interaction of cations ($Ca^{2+}$) on $Ce^{3+}$ toxicity. These were calculated according to the method developed by De Schamphelaere and Janssen (2002). Briefly, linear regression analysis of free cationic activities of Ca on $Ce^{3+}$ LC50 were performed to derive the slope and intercept value and an estimate of Log K Ca-$_{BL}$ developed (De Schamphelaere and Janssen, 2002). The conditional equilibrium constant used to quantify $Ce^{3+}$

binding to the biotic ligand (Log K Ce-$_{BL}$) was derived as the negative log of the intercept from the cation regression relationships of $Ce^{3+}$ on $Ca^{2+}$ (De Schamphelaere and Janssen, 2002).

## 2.2 Results

### *2.2.1 Exposure characterization*

Both Ce-$_{T}$ and Ce-$_{D}$ were measured and the overall results indicate increased precipitation at higher concentrations as well as during the course of the exposure (Figure 1, Supplementary data Table S1). The Ce-$_{D}$ concentrations from tests with added DOM indicated less precipitation (Figure 1). Ce-$_{T}$ concentrations measured immediately after solution preparation were closer to nominal concentrations in comparison to Ce-$_{D}$ from the same time period. These solutions were mixed before sampling and therefore could have contained precipitated Ce that was brought back into solution upon sample acidification. Dissolved concentrations in these initial samples of exposure solutions were 45% of total at the lowest exposure concentration and 58% at the highest (Figure 1). Measured Ce-$_{T}$ at the end of the test (collected without disturbing the solutions) were 5.8, 2.8, 9.5 and 33.3 fold less than initial Ce-$_{T}$ concentration in nominal 72, 384, 2304 and 5760 µg/L, respectively (Figure 1). After 96 h of exposure, the total and dissolved Ce concentrations were not significantly different for all concentrations (Figure 1). In tests where DOM was added Ce-$_{D}$ were much closer to the matched Ce-$_{T}$ and both were close to nominal concentrations indicating less precipitation (Supplementary data Table S1, Figure 1).

### *2.2.2 Species sensitivity to Ce*

Acute Ce exposure to *Daphnia pulex* and *Hyalella azteca* in side-by-side tests showed the latter organism to be more sensitive (Figure 2). It must be noted that test media were slightly different than their respective rearing media (*Daphnia* (ASW) and *Hyalella* (RSW)) and water hardness matched conditions (Figure 2). Therefore, testing to quantify the potential influence of TMFs was done using the more sensitive *Hyalella*.

### *2.2.3 Effect of cationic competition on Ce toxicity to Hyalella azteca*

Increases in Ca concentration significantly decreased acute Ce toxicity (Table 1, Figure 3A). Based on Ce-$_T$ from the initial measurements the LC50 increased 11 times over the range of added Ca (from 0.1 to 2 mM) and more distinctly a 16.7 fold increase based on Ce-$_D$. Increased Na, up to 1 mM, also significantly decreased Ce toxicity by a factor of 15 times based on measured Ce-$_T$ and 20.6 fold based on Ce-$_D$. Further Na increase to 2 mM did not provide protection. Expressing LC50s based on final Ce-$_D$ concentrations show that Na had no protective effect up to 1 mM and that further Na increase elevated toxicity (Table 1, Figure 3B). Tests with added Mg generally indicated no consistent protective effect (Table 1, Figure 3C). The linear regression for the relationships of $Ce^{3+}$ LC50 showed positive correlations to increasing $Ca^{2+}$ (Figure 3A).

### *2.2.4 Effect of dissolved organic matter complexation on Ce toxicity*

Addition of NOM to test solutions significantly decreased toxicity (Table 1, Figure 4). The LC50s based on initial Ce-$_T$ and Ce-$_D$ increased 29.9 and 61.4-fold respectively as DOC concentrations were increased 0.272 to 12.0 mg C/L (Table 1). There was a positive correlation in the linear relationship between DOC (mg C/L) and LC50 for Ce-$_D$ (Table 1, Figure 4A). Estimates of $Ce^{3+}$ LC50 values with DOC concentration were not correlated (Figure 4B).

### *2.2.5 Estimating Log K values*

Estimates of conditional equilibrium constants for the interaction of free ion activities of Ca and Ce on the biotic ligand (see Table 2) were derived from geochemical speciation modeling using WHAM and based on water chemistry as given in Table 1. The derived Log K value for Ce-$_{\text{BL-Ca}}$ was 3.9 while the interaction of Ce$^{3+}$ with the biotic ligand (Ce-$_{\text{BL}}$) was 7.3 (Table 2).

## 2.3 Discussion

In this study we were able to determine acute Ce toxicity in low hardness conditions to two sensitive freshwater invertebrate species and show that water chemistry can have a significant influence on toxic responses. Ce precipitated during the 96 h exposures and since dissolved concentrations tended to be lower at the end of the tests (Figure 1) we recommend that future LC50 calculations be based on end concentrations, but because our Ce data were complete from the initial timeframe the LC50s reported in this study are primarily based on initial concentrations. In the low hardness RSW medium, the solubility limit of Ce appeared to be less than 200 µg/L (Figure 1) and well above the LC50 for Ce-$_D$. In tests with added DOM the solubility of Ce was higher (Supplementary data Table S1, Figure 1). Acute toxicity of Ce to *H. azteca* and *D. pulex* was compared and in the hardness matched soft water the LC50, based on Ce-$_D$, showed *H. azteca* to be 11 times more sensitive than *D. pulex* (Figure 2). This may have been associated with the longer duration of the *Hyalella* standard test (96 h vs. 48 h for *Daphnia* spp.) in addition to inherent differences in sensitivity. Since, *H. azteca* was more sensitive, it was used for subsequent testing with toxicity modifying factors. Additions of Ca but not Na or Mg (Table 1, Figure 3) provided significant protection against acute Ce toxicity. Similarly, DOM also mitigated Ce toxicity over the range of DOC additions (Table 1).

### *2.3.1 Precipitation of Ce in solution*

Characterizing exposures proved challenging because of significant loss of Ce in all test solutions except for DOM trials (Table 1, Figure 1). Ce-$_D$ was much lower than Ce-$_T$ for all exposures and at higher concentrations this was more pronounced. Whether Ce adhered to the exposure container walls or precipitated was not explored in this study but Ce equilibration in solution appears not to be a rapid process since the initial Ce-$_D$ concentrations change substantially over the course of the exposures. Ce-$_T$ and Ce-$_D$ concentrations decreased dramatically between initial and final samples (Figure 1) and the difference increased with increasing concentrations. The difference in Ce-$_T$ can be explained by the fact that the initial solutions were stirred vigorously before sampling while final solutions were not. Therefore, initial Ce-$_T$ measurements undoubtedly contained precipitated forms of Ce. The most significant observation regarding Ce behavior in solution was the very low concentrations of Ce-$_D$ at the end of the test, which appear to plateau at approximately 200 µg/L in the RSW media (Figure 1). This approximate solubility limit in the culture and testing medium was above the LC50 for Ce-$_D$ (from 96 h) and often resulted in dramatic increases in mortality over a very narrow range of final Ce-$_D$ concentrations (Supplemental data Table S1).

Precipitation in aquatic toxicity tests has been demonstrated previously for REEs (e.g. Borgmann et al., 2005; Barry and Meehan, 2000). Few studies on REEs provide measured concentrations and those that do (Bowmer et al., 1993; Borgmann et al., 2005) generally agree with our observations that using nominal Ce concentrations to calculate endpoints underestimates toxicity (Gonzalez et al., 2014). In their study with La exposures to *Daphnia carinata*, Barry and Meehan (2000) noted precipitation and decided to use the mean of the nominal and measured concentrations at the end of the test in order to derive their EC50 values. We base our LC50 calculations on Ce-$_D$ concentrations from the initial timeframe since our data set was more complete from that sampling period but acknowledge that the measured concentrations after 96 h of exposure would represent the most conservative estimates of toxicity. In experiments where final concentrations were sampled we include calculated LC50s for Ce-$_T$ and Ce-$_D$ (Table 1). Toxicity calculations based on Ce-$_T$ were included to provide information for the Canadian regulatory context and Ce-$_N$ to illustrate the dramatic underestimation of toxicity if LC50s were based on nominal values. Given the differences between nominal and Ce-$_D$, the use of nominal concentrations in LC50 determination would dramatically underestimate toxicity.

The loss of Ce in test solutions increased with increased concentrations and this could have been due to adsorption and/or precipitation. Previously, Johannesson and Hendry (2000) indicated that the particular charge of the REE species is one of the factors controlling adsorption. For instance, the authors argue that the observed decrease in LREEs can result from adsorption to oppositely charged particulate or solid phases (Johannesson and Hendry, 2000). Thus adsorption to surfaces in our tests, such as the polyethylene beakers (Benes and Paulenova, 1973), could have enhanced the loss of Ce-$_D$ from solution. The low Ce concentrations in this study may also be due to precipitation. Ce is unique to REEs since it has two oxidation states ($Ce^{3+}$ and $Ce^{4+}$) which favor formation of insoluble $CeO_2$ in oxic conditions of moderate pH (Migaszewski and Galuszka, 2015). Since our exposures are representative of freshwater conditions it is likely that some Ce precipitated as solid $CeO_2$ especially above pH 7.0. As reviewed by Gonzalez et al. (2014) and demonstrated by Gonzalez et al. (2015), precipitation is a feature of most REE toxicity studies and this is because of phosphate and/or carbonate complexes which have low solubility in artificial media (Jiang and Ji, 2012). It seems likely that some of these processes contributed to the loss of Ce-$_D$ in solution. These results highlight the importance of exposure characterization of test solutions (Gonzalez et al., 2014) and stress the need for further understanding of how total and dissolved Ce effect toxicity.

### *2.3.2 Sensitivity difference to Ce toxicity between H. azteca and D. pulex*

Acute Ce exposures to *Daphnia pulex* (48 h) and *Hyalella azteca* (96 h) in conditions of matched water hardness demonstrate greater sensitivity in the latter organism (Figure 2). There are very few published studies on the toxicity of Ce salts (Gonzalez et al., 2014). One notable study is that of Borgmann et al. (2005) where the 7 d LC50 for Ce-$_D$ (samples collected at the end of the test) was 32 µg/L (95% CI of 14-70 µg/L) and this compares well to our results 16.7 µg/L (10.2-27.5) but because our LC50s are based on samples collected at the start of the test the 96 h Ce-$_D$ would likely be much lower. The Borgmann et al. (2005) study was performed in slightly harder water (hardness of 18 mg $CaCO_3$/L vs 12.5 for our RSW) and this may explain the lower toxicity value reported in our study. Borgmann et al. (2005) also conducted Ce tests in hard water (120 mg $CaCO_3$/L) and the reported 7 d LC50 value was 651 (521-813) µg/L. This value was based on nominal concentrations and our study indicates that the use of nominal values may be misleading, Borgmann et al. (2005) did demonstrate that increasing hardness reduces toxicity. The dramatic change in Ce concentration over the course of the 96 h illustrates the need for future investigations with REEs to use similar exposure length and sampling periods as this would allow for direct comparison.

### *2.3.3 Toxicity of Ce to H. azteca and the toxicity modifying effects of water chemistry*

The protective effect of waterborne Ca on Ce toxicity to *H. azteca* was demonstrated for Ce-$_T$ and Ce-$_D$ (Table 1, Figure 3A). Our results show that the protective effects of waterborne Ca are generally in agreement with the studies of Borgmann et al. (2005) and Barry and Meehan (2000) which indicated that REE toxicity was reduced as water hardness increased. For example, in the study of Borgmann et al. (2005) acute tests were performed in very soft and intermediate hardness water and demonstrated that exposures in soft water can increase Ce toxicity 5 fold. This change in toxicity cannot be attributed to Ca entirely since Borgmann et al. (2005) altered test solution chemistry by dilution of the hard water with deionized water. The protective effect of Ca on the uptake of REEs into algae has been previously demonstrated. Increasing Ca concentration (10,000 fold) resulted in a 30% reduction in uptake for La to the algal species *Scenedesmus pannonicus* (subsp. Berlin) but the same change had no effect on the fungus (*Aureobasidium pullulans*; Demon et al., 1989). More recently, EL-Akl et al. (2015) working with the freshwater algae (*Chlamydomonas reinhardtii*) showed a threefold decrease in Ce uptake as $Ca^{2+}$ increased from 0.01 to 1 mM but only at pH 5.0 and not 7.0 like our study. Although there are limited number of studies investigating competitive effects of REEs with Ca, our results with Ce support the hypothesis of a competitive interaction between Ce and Ca.

In this study, we use WHAM for speciation modeling of measured $Ce_{-D}$ concentrations to estimate $Ce^{3+}$ content in solution. If $Ce^{3+}$ and $Ca^{2+}$ compete for uptake at the biotic ligand then the LC50 concentrations for $Ce^{3+}$ would have been positively correlated with $Ca^{2+}$ concentration and this was observed (Figure 3A). It was not surprising to observe the protective effect of Ca on Ce toxicity to *Hyalella* since lanthanides and Ca share similar chemical properties. For example, lanthanides share similar ionic radii with $Ca^{2+}$ (Hirano and Suzuki, 1996) and have been previously demonstrated to block Ca channels (Sandvig and Olsnes, 1982; Lansman, 1990). In this study, we demonstrate that Ce toxicity decreases with increasing waterborne Ca concentration.

Increased waterborne Na and Mg show no clear protective effect, interestingly LC50s based on initial $Ce_{-D}$ showed a significant reduction in toxicity with increasing Na and Mg (up to 0.1 mM) but when calculations were based on final $Ce_{-D}$ there was no change for Na (up to 1 mM) and Mg (Table 1). These results were expected since Ce does not share physiochemical properties with $Na^+$ or $Mg^{2+}$. Previous studies with Cd, Co, Cu, Pb, Ag and Zn show that acute metal toxicity is caused by inhibition of one of the major cation transporters. For example $Cd^{2+}$, $Co^{2+}$, $Pb^{2+}$ and $Zn^{2+}$ affect the Ca transporter and block $Ca^{2+}$ uptake while $Cu^{2+}$ and $Ag^+$ inhibit the Na transporter and impedes $Na^+$ and $Cl^-$ uptake across the fish gill (Niyogi and Wood, 2004). It is not known whether Ce interferes with the ion uptake process on the respiratory surface but this study provides evidence to suggesting that Ce affects Ca uptake.

Tests with White River DOM at nominal concentrations of 6 and 12 mg DOC/L demonstrated decrease in toxicity to *Hylaella* (Table 1, Figure 4). The chosen concentrations in our exposures are representative of Canadian Shield soft waters (David et al., 1997). The protective effects of DOM are well established for metals (Wood et al., 2011) and while little is known about how they influences REE toxicity in natural waters, at circumneutral pH the REEs are known to bind organic molecules (Tang and Johannesson, 2003; Johannesson et al., 2004). Our hypothesis was that while LC50 values for $Ce_{-D}$ increase with added DOC, when toxicity was expressed on $Ce^{3+}$ basis, the values would be similar across DOC trials. Indeed, the $Ce^{3+}$ LC50s were relatively consistent across DOC additions (Figure 4B) demonstrating that DOM complexes Ce and that $Ce^{3+}$ is associated with acute toxicity. Modeling of exposure chemistry for the lowest DOC concentration (nominal 6 mg C/L) indicated that 98.9% of $Ce_{-D}$ in solution was bound to humic and fulvic complexes. This suggests that small amounts of Ce are bioavailable (Supplemental data Table S2). Fuma et al. (2005) demonstrates that the decrease in Dy toxicity to microbial microcosm results from reduction in $Dy^{3+}$ through complexation with organic matter. Recently, EL-Akl et al. (2015) showed decreased Ce bioavailability to algae under increasing presence of Suwannee River DOM but also concluded that some REE-organic complexes may be bioavailable (Yang et al., 2014; Zhao and Wilkinson, 2015). The mitigating effect of NOM to Ce toxicity in our study is generally consistent with other REE investigations and indicates that Ce bioavailability is strongly influenced by DOM complexation.

### *2.3.4 BLM development for Ce*

This study illustrates that Ca and DOM can influence acute Ce toxicity and therefore we suggest that the BLM approach could be useful in estimating Ce toxicity on a site specific basis. Previous investigation has concluded that some REEs, such as Lu, also follow BLM principles (Weltje et al., 2004) and this is also the case with Ce. More recently, conditional equilibrium constants for REEs have been developed for algae (Yang et al., 2014; El-Akl et al., 2015) but no Log K values exist in determining Ce interaction with *Hyalella*. Our Ce-$_{BL}$ (Log K = 7.3, Table 2) correlate well with values from other REEs such as Ce-$_{BL}$ (7.3), Sm-$_{BL}$ (7.0), Eu-$_{BL}$ (7.0) and Tm-$_{BL}$ (7.1, Yang et al., 2014; El-Akl et al., 2015) and our calculation for Ca binding to the BL (3.9, Table 2) is similar to the value of 4.3 obtained by El-Akl et al. (2015) in Ce exposure to algae and different since their study only produced values at pH 5.0 and not 7.0. This is the first study where equilibrium binding constants for Ce and *Hyalella azeca* have been calculated and the data derived in this investigation are applicable for the development of BLM in very soft waters. This study calculates Ce binding constants based on the initial dissolved concentrations which are likely under protective of toxicity and future investigations should examine how sampling period influences acute Ce toxicity to *Hyalella*.

## 2.4 Conclusion

This study shows significantly influence of water chemistry on acute Ce toxicity to *Hyalella azteca*. Increased Ca and DOM decreased toxicity whereas Na and Mg had no clear effect. Data presented here is consistent with BLM principles since Ce toxicity correlates to the presence of $Ce^{3+}$. We have determined Log K values for the interaction of $Ce^{3+}$ on the biotic ligand as well as for the protective effect of Ca but these values were based on the dissolved concentrations from the initial sampling period and likely overestimate toxicity. We have also included regression constants based on end concentrations for some tests where sampling was performed. We recommend basing future tests on standardized exposure length and calculating LC50s on end concentrations as this will allow for direct comparison between treatments and permit lethal calculations to be based on the most conservative values.

## 2.5 Acknowledgements

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## 2.6 Tables and Figures

Table 1. Acute Ce exposures and the corresponding LC50s to *Hyalella* based on nominal (Ce-$_{N}$), total (Ce-$_{T}$) and dissolved (Ce-$_{D}$) values. Nominal water chemistry parameters (mM) except for pH, DOC (mg C/L) and for Ce-$_{N}$, Ce-$_{T}$ and Ce-$_{D}$ (µM). The $^{m}$ superscript represents MOPS addition and the LC50s labeled with the same letter are not significant in each test series.

|  | Ca | Na | Mg | DOC | pH | Initial |  |  | Final |  |
|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  |  | Ce-$_{N}$ | Ce-$_{T}$ | Ce-$_{D}$ | Ce-$_{T}$ | Ce-$_{D}$ |
| Base condition | **0.1** | **0.101** | **0.025** | **0.272** | 7.1 | 0.79$^{a}$ | 0.29$^{a}$ | 0.12$^{a}$ | - | - |
|  |  |  |  |  |  | (0.60-1.05) | (0.20-0.40) | (0.070-0.20) |  |  |
| Ca added | **0.5** | 0.101 | 0.025 | 0.272 | 7.0 | 3.48$^{b}$ | 2.18$^{b}$ | 0.99$^{b}$ | 0.3$^{a}$ | 0.23$^{a}$ |
|  |  |  |  |  |  | (2.30-5.29) | (1.51-3.17) | (0.68-1.45) | (0.20-0.46) | (0.16-0.33) |
|  | **1** | 0.101 | 0.025 | 0.272 | 7.2 | 3.22$^{b}$ | 2.07$^{b}$ | - | - | - |
|  |  |  |  |  |  | (1.18-8.78) | (1.60-2.68) |  |  |  |
|  | **2** | 0.101 | 0.025 | 0.272 | 7.2 | 5.07$^{b}$ | 3.17$^{b}$ | 2.00$^{c}$ | 1.2$^{b}$ | 1.01$^{b}$ |
|  |  |  |  |  |  | (3.54-7.26) | (2.10-4.77) | (1.33-3.00) | (0.75-1.90) | (0.65-1.55) |
| Na added | 0.1 | **0.5** | 0.025 | 0.272 | 7.2 | 2.67$^{b}$ | 1.83$^{b}$ | 0.96$^{b}$ | 0.28$^{a}$ | 0.33$^{a}$ |
|  |  |  |  |  |  | (1.39-5.16) | (0.95-3.50) | (0.53-1.74) | (0.14-0.57) | (0.18-0.62) |
|  | 0.1 | **1** | 0.025 | 0.272 | 7.3$^{m}$ | 6.14$^{b}$ | 4.36$^{b}$ | 2.46$^{b}$ | 0.33$^{ab}$ | 0.19$^{a}$ |
|  |  |  |  |  |  | (2.98-12.67) | (2.07-9.14) | (1.17-5.19) | (0.23-0.46) | (0.188-0.19) |
|  | 0.1 | **2** | 0.025 | 0.272 | 7.2$^{m}$ | 0.90$^{a}$ | 0.47$^{a}$ | 0.30$^{c}$ | 0.15$^{ac}$ | 0.09$^{b}$ |
|  |  |  |  |  |  | (0.47-1.72) | (0.23-0.95) | (0.15-0.61) | (0.09-0.23) | (0.07-0.12) |
| Mg added | 0.1 | 0.101 | **0.1** | 0.272 | 7.2$^{m}$ | 2.56$^{b}$ | 2.43$^{b}$ | 1.28$^{b}$ | 0.41$^{a}$ | 0.41$^{a}$ |
|  |  |  |  |  |  | (1.60-4.10) | (1.52-3.87) | (0.82-2.01) | (0.28-0.59) | (0.29-0.59) |
|  | 0.1 | 0.101 | **0.3** | 0.272 | 7.2$^{m}$ | 0.72$^{a}$ | 0.78$^{c}$ | 0.40$^{c}$ | 0.3$^{a}$ | 0.2$^{a}$ |
|  |  |  |  |  |  | (0.35-1.45) | (0.37-1.63) | (0.19-0.87) | (0.17-0.51) | (0.10-0.38) |
| DOC added | 0.1 | 0.101 | 0.025 | **6** | 7.2$^{m}$ | 6.69$^{b}$ | 6.31$^{b}$ | 6.08$^{b}$ | - | - |
|  |  |  |  |  |  | (4.09-10.95) | (3.81-10.46) | (3.77-9.81) |  |  |
|  | 0.1 | 0.101 | 0.025 | **12** | 7.2$^{m}$ | 8.12$^{b}$ | 7.99$^{b}$ | 7.32$^{b}$ | 6.83 | 6.96 |
|  |  |  |  |  |  | (7.79-8.47) | (7.61-8.39) | (7.03-7.63) | (6.55-7.11) | (6.69-7.23) |

Table 2. Linear regression coefficients calculated for the effect of $Ca^{2+}$, $Na^{+}$ and $Mg^{2+}$on acute $Ce^{3+}$ toxicity based on the dissolved data from initial and final time periods. The units of slope and intercept are on a molar basis.

| Relationship | Slope | Intercept | r | Derived Log K |
|---|---|---|---|---|
| Initial | | | | |
| $Ca^{2+}$ to $Ce^{3+}$ | $4.01x10^{-04}$ | $5.25x10^{-08}$ | 0.94 | 3.9 |
| $Na^{+}$ to $Ce^{3+}$ | $2.73x10^{-06}$ | $2.76x10^{-07}$ | 0.01 | NA |
| $Mg^{2+}$ to $Ce^{3+}$ | $-2.13x10^{-04}$ | $1.65x10^{-07}$ | 0.12 | NA |
| BL to $Ce^{3+}$ | - | - | - | 7.3 |
| Final | | | | |
| $Ca^{2+}$ to $Ce^{3+}$ | $2.37x10^{-04}$ | $-7.27x10^{-08}$ | 1.00 | NA |
| $Na^{+}$ to $Ce^{3+}$ | $-2.00x10^{-05}$ | $3.49x10^{-08}$ | 0.84 | NA |
| $Mg^{2+}$ to $Ce^{3+}$ | $-2.84x10^{-04}$ | $7.80x10^{-08}$ | 1.00 | NA |
| BL to $Ce^{3+}$ | - | - | - | NA |

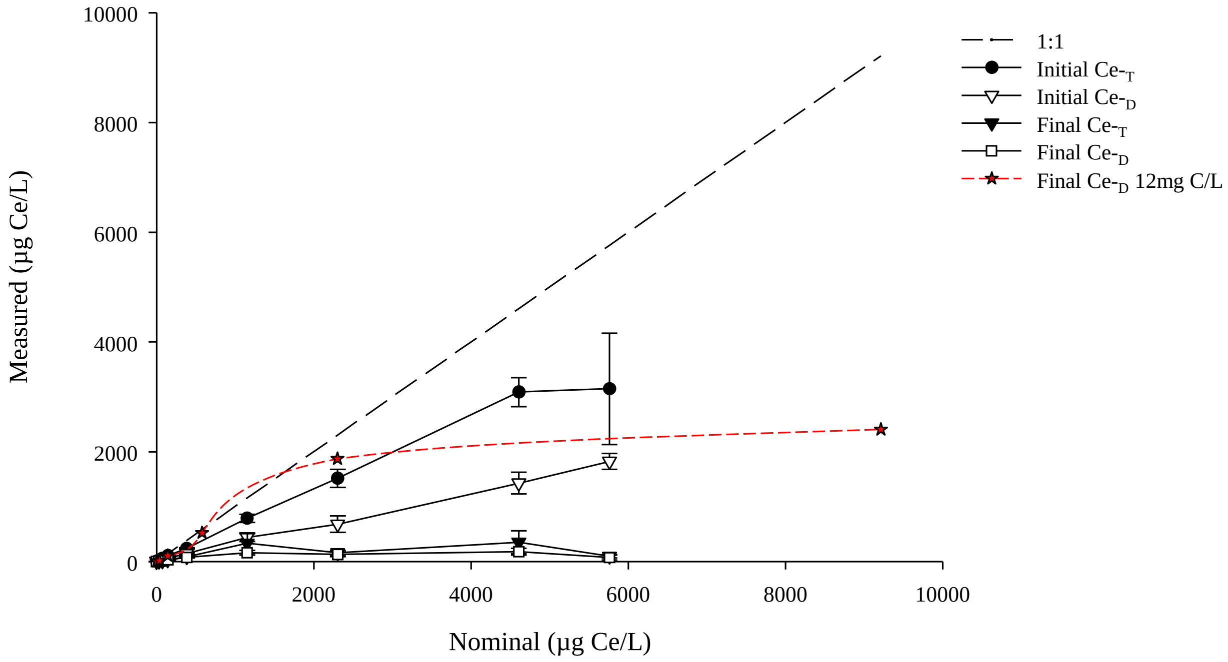


Figure 1. Comparison of nominal and measured Ce measurements for total (Ce-$_T$) and dissolved (Ce-$_D$) concentrations from the initial and final time periods. Tests involving cationic competition are summarized and exclude data from the 32 mg $CaCO_3$/L test, initial Ce-$_T$ tests <384 µg/L (underlined in table S1) and DOM alterations except for the 12 mg C/L final Ce-$_D$ data fitted to the simple spline curve (in red and showing stars as data points). Error bars represent standard error of the mean (n = 2 - 20).

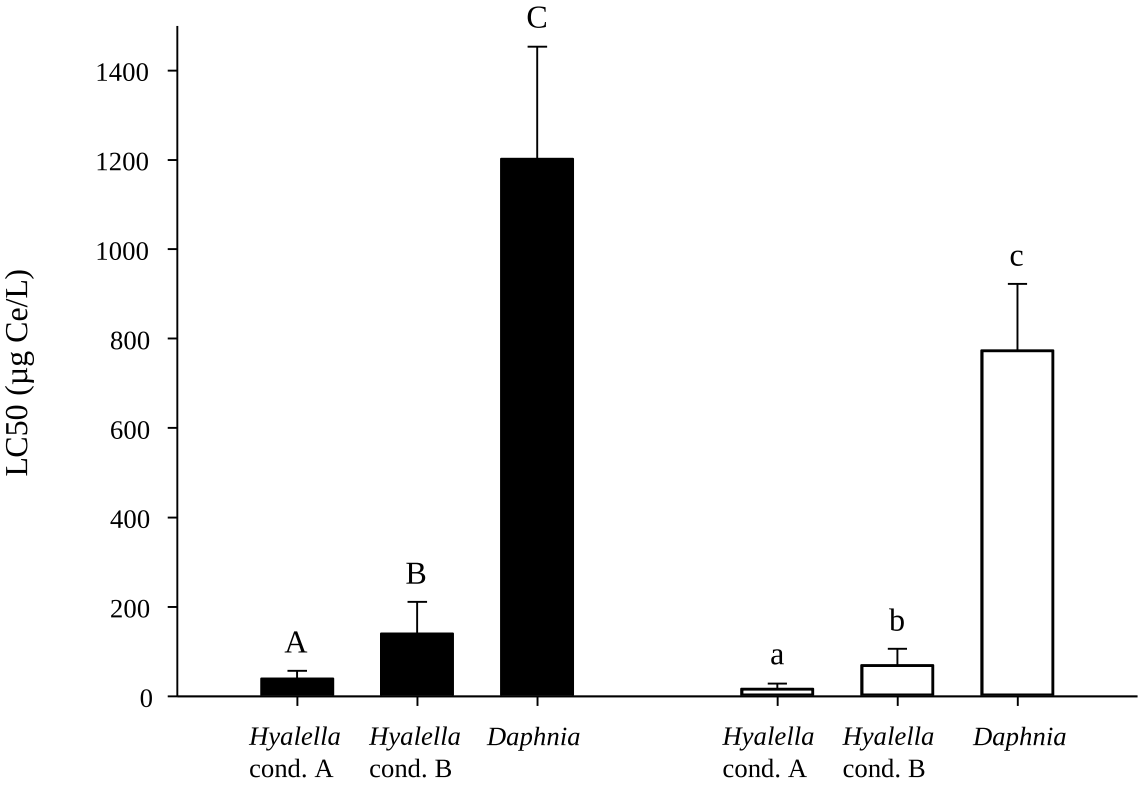
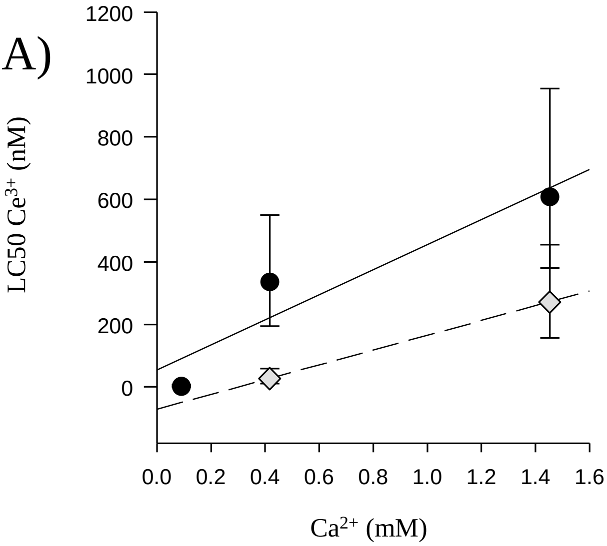


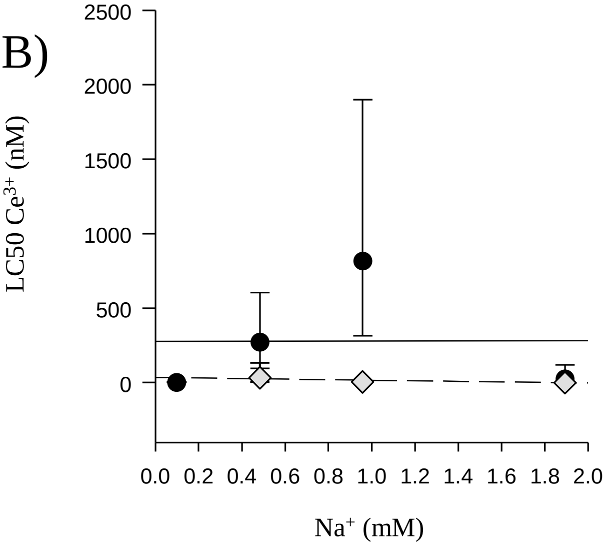
Figure 2. The LC50 values (with upper 95% confidence interval) for *Hyalella azteca* and *Daphnia pulex* exposure to Ce. The LC50 values are based on 48 h (*Daphnia*) and 96 h (*Hyalella*) tests and measured total ($Ce_{\text{-T}}$ black bars) and dissolved ($Ce_{\text{-D}}$ white bars) concentrations. Acute Ce tests with *Hyalella* were performed under two conditions: condition (A) exposures were performed in RSW water and in condition (B) the RSW modified to the water hardness of 32 mg $CaCO_3$/L found in the *Daphnia* test media. Different letters indicate LC50 values that are statistically different for either $Ce_{\text{-T}}$ or $Ce_{\text{-D}}$.

(A)



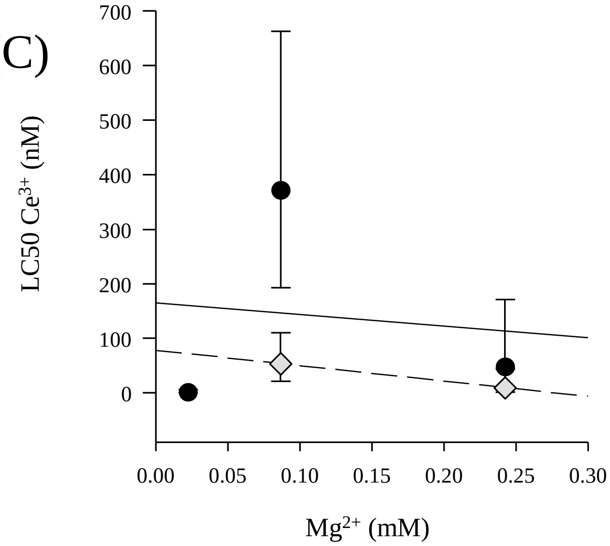
LC50 Ce$^{3+}$ (nM)
1200
1000
800
600
400
200
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Ca$^{2+}$ (mM)

(B)



LC50 Ce$^{3+}$ (nM)
2500
2000
1500
1000
500
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Na$^{+}$ (mM)

(C)



LC50 Ce$^{3+}$ (nM)
700
600
500
400
300
200
100
0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
Mg$^{2+}$ (mM)

Figure 3. The LC50 values (with 95% confidence interval) and the competitive effect of $Ca^{2+}$ (A), $Na^{+}$ (B) and $Mg^{2+}$ on $Ce^{3+}$ to *Hyalella azteca*. Linear regression line is shown and ion activities were calculated from measured dissolved Ce concentrations from the initial (black circles) and final (gray diamonds) sampling periods and modelled using WHAM 7 (ver. 7.02). See Table 2 for linear regression coefficients and associated Log K values.

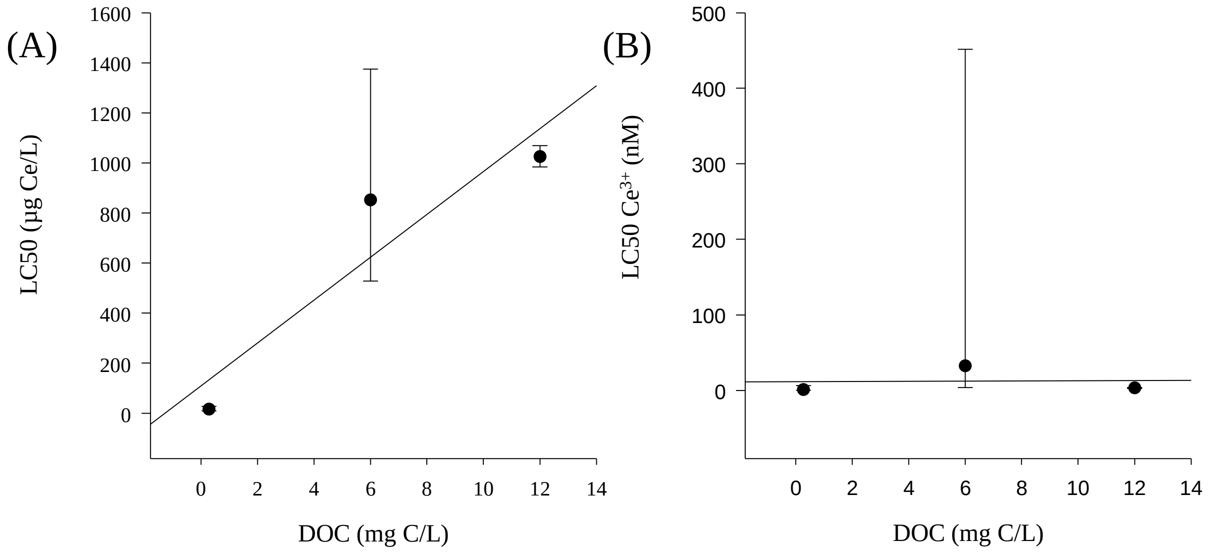


Figure 4. The LC50 values (with 95% confidence intervals) and the protective effect of DOM (quantified as DOC) on Ce toxicity to *H. azteca*. Both panels are from initial measured dissolved and (A) shows regression as a function of Ce toxicity while (B) as a function of $Ce^{3+}$ activity. Single source of DOM was used from White River (WR-DOM). Regression panel (A) LC50 $Ce\text{-}_D$ = 85.57 (WR-DOM) + 110.25 (r = 0.93) and panel (B) LC50 $Ce^{3+} = 1.29 * 10^{-10}$ (WR-DOM) + $1.15 * 10^{-8}$ (r = 0.04).

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## 2.8 Supplementary data

Table S1. Exposure chemistry with mortalities for all *H. azteca* tests. Measured values for nominal, Ce-$_T$ (T) and Ce-$_D$ (D) in µg Ce/L and mortality are averages of two replicates per concentration. Underlined tests are concentrations sampled from 0 h.

| Test type: | MOPS/NO MOPS | Valid Test | Nominal | Initial | | Final | | Initial | Final | % mortality |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | T | D | T | D | D/T% | D/T% | |
| RSW trial 1 | NO MOPS | YES | 0 | 2.7 | 3.6 | - | - | NA | - | 5 |
| | | | 8 | 2.2 | 2.1 | - | - | 95.0 | - | 25 |
| | | | 16 | 3.5 | 3.4 | - | - | 96.5 | - | 5 |
| | | | 32 | 14.3 | 3.1 | - | - | 21.9 | - | 25 |
| | | | 64 | 35.4 | 12.0 | - | - | 33.9 | - | 20 |
| | | | 128 | 79.3 | 50.6 | - | - | 63.8 | - | 55 |
| | | | 256 | 163.8 | 129.8 | - | - | 79.2 | - | 60 |
| RSW trial 2 | NO MOPS | YES | 0 | 3.2 | 2.6 | - | - | NA | - | 0 |
| | | | 8 | 4.6 | 2.6 | - | - | 58.0 | - | 15 |
| | | | 16 | 4.5 | 2.8 | - | - | 63.2 | - | 15 |
| | | | 32 | 8.4 | 3.5 | - | - | 42.4 | - | 30 |
| | | | 64 | 24.6 | 4.9 | - | - | 20.1 | - | 50 |
| | | | 128 | 48.9 | 25.0 | - | - | 51.2 | - | 65 |
| | | | 256 | 156.3 | 126.5 | - | - | 80.9 | - | 70 |
| | | | 512 | 317.6 | 279.0 | - | - | 87.8 | - | 65 |
| RSW trial 3 | NO MOPS | NO | 0 | 3.1 | 3.0 | - | - | NA | - | 25 |
| | | | 12 | 3.8 | 2.3 | - | - | 61.4 | - | 25 |
| | | | 24 | 8.2 | 6.3 | - | - | 77.3 | - | 40 |
| | | | 48 | 19.2 | 16.2 | - | - | 84.5 | - | 45 |
| | | | 96 | 60.7 | 54.0 | - | - | 89.0 | - | 70 |
| | | | 192 | 119.9 | 110.2 | - | - | 91.9 | - | 90 |
| | | | 384 | 291.4 | 257.1 | - | - | 88.2 | - | 100 |
| | | | 768 | 621.9 | 549.2 | - | - | 88.3 | - | 95 |
| RSW trial 4 | NO MOPS | NO | 0 | 0.5 | 0.5 | - | - | NA | - | 45 |
| | | | 6 | 0.7 | 3.6 | - | - | 505.5 | - | 15 |
| | | | 30 | 14.6 | 10.6 | - | - | 72.4 | - | 65 |
| | | | 72 | 42.0 | 39.1 | - | - | 93.1 | - | 35 |
| | | | 144 | 91.9 | 94.5 | - | - | 102.8 | - | 75 |
| | | | 384 | 305.3 | 306.2 | - | - | 100.3 | - | 100 |
| | | | 1152 | 1030.1 | 1019.7 | - | - | 99.0 | - | 100 |
| RSW trial 5 | NO MOPS | YES | 0 | 1.6 | 2.6 | - | - | NA | - | 10 |
| | | | 6 | 2.0 | 1.8 | - | - | 91.1 | - | 25 |
| | | | 30 | 7.3 | 2.9 | - | - | 39.4 | - | 23.81 |
| | | | 72 | 12.4 | 6.1 | - | - | 49.0 | - | 40 |
| | | | 144 | 43.6 | 28.7 | - | - | 65.8 | - | 75 |
| | | | 384 | 97.7 | 106.2 | - | - | 108.7 | - | 90 |
| | | | 1152 | 338.2 | 272.2 | - | - | 80.5 | - | 95 |

## Table S1. Continued.

| Test type: | MOPS/NO MOPS | Valid Test | Nominal | Initial | | Final | | Initial | Final | % mortality |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | T | D | T | D | D/T% | D/T% | |
| RSW | MOPS | NO | 0 | 1.2 | 2.0 | 1.2 | 1.7 | NA | NA | 10 |
| | | | 0+MOPS | 2.0 | 0.9 | 0.9 | 1.2 | NA | NA | 100 |
| | | | 30 | 23.0 | 14.5 | 3.6 | 3.1 | 63.1 | 87.1 | 10 |
| | | | 72 | 59.0 | 25.4 | 3.1 | 2.8 | 43.0 | 92.1 | 25 |
| | | | 144 | 99.7 | 41.3 | 14.8 | 11.5 | 41.5 | 77.4 | 50 |
| | | | 384 | 292.5 | 100.7 | 30.2 | 19.1 | 34.4 | 63.4 | 60 |
| | | | 1152 | 788.1 | 244.5 | 35.8 | 20.3 | 31.0 | 56.7 | 85 |
| | | | 2304 | 1562.3 | 336.0 | 47.7 | 19.9 | 21.5 | 41.7 | 100 |
| 32 mg $CaCO_3$/L | MOPS | YES | 0 | 1.7 | 2.2 | 1.2 | 0.7 | NA | NA | 20 |
| | | | 0+MOPS | 2.0 | 1.3 | 2.1 | 1.6 | NA | NA | 10 |
| | | | 36 | 36.0 | 16.0 | 4.9 | 4.3 | 44.3 | 88.1 | 15 |
| | | | 72 | 77.3 | 41.0 | 13.1 | 12.2 | 53.1 | 93.6 | 50 |
| | | | 288 | 276.6 | 136.4 | 115.5 | 111.8 | 49.3 | 96.8 | 70 |
| | | | 1152 | 1022.5 | 532.3 | 225.0 | 241.7 | 52.1 | 107.4 | 95 |
| | | | 4608 | 3245.9 | 1436.7 | 137.9 | 128.6 | 44.3 | 93.3 | 100 |
| 1mM $CaCl_2$ | NO MOPS | YES | 0 | 3.3 | 4.1 | - | - | NA | - | 15 |
| | | | 0+Ca | 1.5 | 2.4 | - | - | NA | - | 10 |
| | | | 6 | 1.5 | 0.0 | - | - | 0.0 | - | 30 |
| | | | 30 | 7.9 | 6.5 | - | - | 82.1 | - | 20 |
| | | | 72 | 28.4 | 25.3 | - | - | 89.0 | - | 55 |
| | | | 144 | 40.5 | 36.1 | - | - | 89.1 | - | 40 |
| | | | 384 | 212.3 | 178.2 | - | - | 84.0 | - | 40 |
| | | | 1152 | 357.2 | 265.1 | - | - | 74.2 | - | 70 |
| 1mM $CaCl_2$ | NO MOPS | NO | 0 | 2.1 | 2.7 | - | - | NA | - | 5 |
| | | | 0+Ca | 2.0 | 1.6 | - | - | NA | - | 90 |
| | | | 30 | 13.6 | 2.5 | - | - | 18.7 | - | 60 |
| | | | 72 | 33.0 | 23.3 | - | - | 70.7 | - | 5 |
| | | | 144 | 48.5 | 37.5 | - | - | 77.2 | - | 40 |
| | | | 384 | 156.5 | 128.8 | - | - | 82.3 | - | 60 |
| | | | 1152 | 351.3 | 270.9 | - | - | 77.1 | - | 80 |
| | | | 2304 | 597.9 | 403.5 | - | - | 67.5 | - | 100 |
| | | | 4608 | 1178.7 | 342.7 | - | - | 29.1 | - | 100 |
| 1mM $CaCl_2$ | NO MOPS | NO | 0 | 2.1 | 1.4 | 1.7 | 2.4 | NA | NA | 15 |
| | | | 0+Ca | 0.8 | 1.2 | 1.6 | 0.7 | NA | NA | 35 |
| | | | 30 | 26.1 | 21.2 | 2.9 | 2.5 | 81.0 | 87.7 | 15 |
| | | | 72 | 48.0 | 39.9 | 17.8 | 18.1 | 83.3 | 101.4 | 25 |
| | | | 144 | 94.8 | 79.3 | 47.0 | 44.0 | 83.6 | 93.7 | 20 |
| | | | 384 | 230.4 | 204.1 | 115.5 | 103.0 | 88.6 | 89.2 | 45 |
| | | | 1152 | 783.8 | 660.1 | 171.8 | 149.9 | 84.2 | 87.3 | 80.95 |
| | | | 2304 | 1565.6 | 1392.6 | 185.7 | 152.4 | 88.9 | 82.1 | 100 |
| | | | 4608 | 3082.1 | 2479.3 | 304.2 | 255.4 | 80.4 | 84.0 | 100 |
| 1mM $CaCl_2$ | NO MOPS | NO | 0 | 2.4 | 0.0 | - | - | NA | - | 50 |
| | | | 0+Ca | 1.4 | 1.2 | - | - | NA | - | 45 |
| | | | 30 | 22.9 | 3.4 | - | - | 14.7 | - | 45 |
| | | | 384 | 211.1 | 30.1 | - | - | 14.2 | - | 45 |
| | | | 768 | 444.7 | 33.2 | - | - | 7.5 | - | 35 |
| | | | 1152 | 614.0 | 43.0 | - | - | 7.0 | - | 40 |
| | | | 1728 | 927.8 | 57.9 | - | - | 6.2 | - | 80 |
| | | | 2304 | 1185.8 | 80.0 | - | - | 6.7 | - | 85 |

## Table S1. Continued.

| Test type: | MOPS/NO MOPS | Valid Test | Nominal | Initial | | Final | | Initial | Final | % mortality |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | T | D | T | D | D/T% | D/T% | |
| 0.5mM CaCl$_2$ | NO MOPS | YES | 0 | 10.1 | 4.4 | 1.0 | 0.8 | NA | NA | 20 |
| | | | 0+Ca | 4.7 | 3.0 | 2.9 | 0.0 | NA | NA | 10 |
| | | | 30 | 30.8 | 7.7 | 3.4 | 4.1 | 25.2 | 119.3 | 15 |
| | | | 72 | 55.4 | 29.6 | 6.0 | 5.4 | 53.4 | 89.8 | 20 |
| | | | 144 | 97.2 | 46.2 | 9.7 | 9.1 | 47.5 | 93.8 | 40 |
| | | | 384 | 237.3 | 102.4 | 51.7 | 42.8 | 43.2 | 82.8 | 45 |
| | | | 1152 | 696.1 | 291.7 | 109.8 | 73.2 | 41.9 | 66.6 | 60 |
| | | | 2304 | 1169.7 | 572.8 | 152.0 | 98.8 | 49.0 | 65.0 | 95.24 |
| 2mM CaCl$_2$ | NO MOPS | NO | 0 | 2.0 | 2.9 | 3.0 | 1.9 | NA | NA | 35 |
| | | | 0+Ca | 1.4 | 2.2 | 1.8 | 1.3 | NA | NA | 50 |
| | | | 30 | 34.0 | 4.8 | 2.8 | 2.0 | 14.1 | 70.7 | 20 |
| | | | 144 | 104.2 | 24.7 | 5.1 | 4.8 | 23.7 | 93.4 | 15 |
| | | | 384 | 260.4 | 46.5 | 22.9 | 7.0 | 17.9 | 30.5 | 25 |
| | | | 1152 | 810.9 | 118.5 | 38.8 | 7.3 | 14.6 | 18.7 | 50 |
| | | | 2304 | 1538.6 | 224.8 | 53.3 | 8.4 | 14.6 | 15.8 | 90 |
| | | | 4608 | 3180.9 | 414.6 | 66.8 | 29.3 | 13.0 | 43.8 | 100 |
| 2mM CaCl$_2$ | NO MOPS | YES | 0 | 3.3 | 2.3 | 1.3 | 1.6 | NA | NA | 5 |
| | | | 0+Ca | 2.3 | 0.7 | 0.9 | 0.2 | NA | NA | 10 |
| | | | 30 | 11.1 | 7.4 | 2.6 | 2.0 | 67.1 | 75.2 | 0 |
| | | | 384 | 247.5 | 147.0 | 133.2 | 136.8 | 59.4 | 102.7 | 25 |
| | | | 768 | 508.3 | 327.5 | 231.5 | 230.5 | 64.4 | 99.6 | 65 |
| | | | 1152 | 738.7 | 521.4 | 198.5 | 183.6 | 70.6 | 92.5 | 50 |
| | | | 1728 | 1143.6 | 771.2 | 238.6 | 214.5 | 67.4 | 89.9 | 75 |
| | | | 4608 | 3388.9 | 1880.4 | 2408.1 | 697.9 | 55.5 | 29.0 | 100 |
| 0.1mM MgSO$_4$ | MOPS | YES | 0+Mg | 2.8 | 3.0 | 2.4 | 2.3 | NA | NA | 30 |
| | | | 0+Mg+MOPS | 2.9 | 3.1 | 1.7 | 2.6 | NA | NA | 20 |
| | | | 30 | 26.3 | 11.5 | 3.4 | 3.8 | 43.9 | 113.8 | 0 |
| | | | 72 | 72.8 | 38.3 | 20.4 | 19.0 | 52.6 | 93.3 | 30 |
| | | | 144 | 134.0 | 91.2 | 40.0 | 44.7 | 68.1 | 111.8 | 35 |
| | | | 1152 | 1117.8 | 519.1 | 159.3 | 166.6 | 46.4 | 104.6 | 75 |
| | | | 2304 | 2057.7 | 1002.0 | 122.0 | 109.1 | 48.7 | 89.4 | 95 |
| 0.3mM MgSO$_4$ | MOPS | YES | 0+Mg | 1.3 | 1.9 | 2.8 | 2.0 | NA | NA | 15 |
| | | | 0+Mg+MOPS | 2.7 | 1.6 | 3.2 | 1.8 | NA | NA | 10 |
| | | | 30 | 29.2 | 15.5 | 11.4 | 6.2 | 53.2 | 54.5 | 30 |
| | | | 144 | 167.4 | 81.3 | 78.4 | 58.4 | 48.6 | 74.5 | 65 |
| | | | 1152 | 1253.6 | 846.8 | 2021.3 | 446.9 | 67.5 | 22.1 | 90 |
| | | | 2304 | 2261.4 | 962.3 | 188.1 | 177.1 | 42.6 | 94.2 | 100 |
| | | | 4608 | 4534.9 | 1941.3 | 128.3 | 112.6 | 42.8 | 87.8 | 100 |

## Table S1. Continued.

| Test type: | MOPS/NO MOPS | Valid Test | Nominal | Initial | | Final | | Initial | Final | % mortality |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | T | D | T | D | D/T% | D/T% | |
| 0.5mM NaCl | NO MOPS | YES | 0 | 0.0 | 1.5 | 0.9 | 1.4 | NA | NA | 45 |
| | | | 0+Na | 1.2 | 0.8 | 1.5 | 1.2 | NA | NA | 5 |
| | | | 30 | 14.7 | 7.3 | 3.2 | 2.3 | 49.8 | 70.6 | 15.0 |
| | | | 72 | 46.1 | 33.9 | 8.2 | 6.1 | 73.5 | 74.4 | 40.0 |
| | | | 144 | 107.1 | 66.8 | 21.1 | 18.3 | 62.4 | 86.8 | 65.0 |
| | | | 384 | 294.9 | 176.7 | 140.1 | 122.0 | 59.9 | 87.1 | 60.0 |
| | | | 1152 | 1071.9 | 593.2 | 306.9 | 275.8 | 55.3 | 89.9 | 66.67 |
| | | | 2304 | 1698.6 | 1133.7 | 370.9 | 340.7 | 66.7 | 91.9 | 75 |
| 0.5mM NaCl | NO MOPS | NO | 0 | 0.7 | 0.4 | 0.0 | 0.6 | NA | NA | 20 |
| | | | 0+Na | 1.0 | 0.3 | 1.1 | 0.2 | NA | NA | 30 |
| | | | 30 | 9.9 | 6.2 | 2.0 | 1.5 | 62.7 | 78.3 | 30 |
| | | | 72 | 68.3 | 23.1 | 5.0 | 5.3 | 33.9 | 105.3 | 20 |
| | | | 144 | 115.3 | 72.4 | 23.5 | 18.1 | 62.8 | 77.1 | 75.0 |
| | | | 384 | 317.7 | 179.0 | 134.8 | 104.9 | 56.3 | 77.8 | 60.0 |
| | | | 1152 | 978.2 | 601.9 | 182.6 | 193.9 | 61.5 | 106.2 | 81.0 |
| | | | 4608 | 3424.0 | 2147.6 | 286.1 | 253.1 | 62.7 | 88.5 | 85.0 |
| 0.5mM NaCl | NO MOPS | YES | 0 | 3.0 | | 2.2 | 1.2 | NA | NA | 5 |
| | | | 0+Na | 2.7 | 1.0 | 1.1 | 0.6 | NA | NA | 20 |
| | | | 30 | 23.8 | 15.0 | 1.2 | 2.2 | 63.0 | 187.3 | 10 |
| | | | 384 | 236.3 | 130.8 | 77.4 | 80.2 | 55.4 | 103.7 | 60 |
| | | | 4608 | 3417.8 | 1351.6 | 288.8 | 299.7 | 39.5 | 103.8 | 95 |
| 0.5mM NaCl | MOPS | NO | 0+Na+MOPS | 2.1 | 2.4 | 2.2 | 2.6 | NA | NA | 25 |
| | | | 30 | 25.2 | 17.5 | 3.0 | 2.6 | 69.3 | 86.5 | 20 |
| | | | 384 | 223.4 | 114.3 | 88.1 | 84.3 | 51.2 | 95.6 | 60 |
| | | | 4608 | 3055.1 | 995.8 | 42.5 | 31.5 | 32.6 | 74.2 | 100 |
| 1mM NaCl | MOPS | NO | 0+Na | 2.3 | 1.5 | 1.7 | 1.2 | NA | NA | 20.0 |
| | | | 0+Na+MOPS | 2.0 | 0.0 | 0.7 | 1.9 | NA | NA | 25.0 |
| | | | 30 | 20.0 | 11.9 | 3.0 | 2.3 | 59.2 | 77.2 | 0.0 |
| | | | 144 | 68.6 | 39.8 | 17.2 | 16.1 | 58.1 | 93.7 | 25 |
| | | | 384 | 194.8 | 93.0 | 60.3 | 55.4 | 47.8 | 91.9 | 40 |
| | | | 1152 | 857.8 | 307.3 | 103.4 | 81.6 | 35.8 | 78.9 | 40 |
| | | | 4608 | 3894.8 | 857.2 | 56.2 | 39.3 | 22.0 | 69.8 | 90 |
| 1mM NaCl | MOPS | YES | 0+Na | 3.3 | 2.2 | 3.2 | 2.3 | NA | NA | 15 |
| | | | 0+Na+MOPS | 3.3 | 2.8 | 2.4 | 2.1 | NA | NA | 5 |
| | | | 30 | 17.9 | 8.9 | 3.3 | 2.2 | 50.0 | 66.9 | 10 |
| | | | 144 | 103.7 | 65.9 | 30.0 | 26.4 | 63.5 | 88.0 | 35 |
| | | | 1230 | 879.5 | 632.9 | 98.9 | 79.4 | 72.0 | 80.2 | 55 |
| | | | 4608 | 3384.1 | 1823.8 | 66.9 | 44.0 | 53.9 | 65.8 | 55 |
| | | | 5760 | 4158.0 | 1966.7 | 64.9 | 27.1 | 47.3 | 41.7 | 90 |

## Table S1. Continued.

| Test type: | MOPS/NO MOPS | Valid Test | Nominal | Initial | | Final | | Initial | Final | % mortality |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | T | D | T | D | D/T% | D/T% | |
| 1mM NaCl | MOPS | NO | 0+Na | 3.3 | 1.9 | 1.8 | 1.9 | NA | NA | 15 |
| | | | 0+Na+MOPS | 3.5 | 2.2 | 1.6 | 0.9 | NA | NA | 25 |
| | | | 30 | 13.8 | 11.1 | 5.2 | 4.2 | 80.8 | 80.4 | 45 |
| | | | 144 | 99.6 | 90.7 | 34.6 | 30.6 | 91.1 | 88.5 | 85 |
| | | | 1230 | 768.8 | 738.9 | 169.6 | 183.6 | 96.1 | 108.3 | 65 |
| | | | 4608 | 1516.6 | 1205.9 | 103.7 | 106.1 | 79.5 | 102.3 | 80 |
| | | | 5760 | 2133.5 | 1681.8 | 123.9 | 117.8 | 78.8 | 95.1 | 100 |
| 2mM NaCl | MOPS | YES | 0+Na | 2.6 | 2.7 | 2.4 | 2.0 | NA | NA | 10 |
| | | | 0+Na+MOPS | 1.4 | 1.7 | 1.9 | 1.6 | NA | NA | 5 |
| | | | 30 | 12.4 | 6.9 | 5.2 | 4.9 | 55.6 | 95.5 | 15 |
| | | | 144 | 87.9 | 63.2 | 37.7 | 29.9 | 71.9 | 79.3 | 85 |
| | | | 1230 | 809.3 | 644.0 | 170.9 | 146.4 | 79.6 | 85.7 | 70 |
| | | | 4608 | 2988.4 | 1701.4 | 96.7 | 80.8 | 56.9 | 83.5 | 85 |
| | | | 9216 | 6636.3 | 2651.1 | 852.6 | 77.3 | 39.9 | 9.1 | 100 |
| 6mgC/L DOM | MOPS | YES | 0+DOM | 1.6 | 1.6 | - | - | NA | - | 10 |
| | | | 0+DOM+MOPS | 0.7 | 2.5 | - | - | NA | - | 15 |
| | | | 36 | 35.0 | 31.4 | - | - | 89.5 | - | 40 |
| | | | 144 | 130.9 | 127.7 | - | - | 97.5 | - | 25 |
| | | | 576 | 531.4 | 592.1 | - | - | 111.4 | - | 35 |
| | | | 2304 | 2260.4 | 1725.3 | - | - | 76.3 | - | 95 |
| | | | 9216 | 5550.7 | 2274.5 | - | - | 41.0 | - | 100 |
| 12mgC/L DOM | MOPS | YES | 0+DOM | 0.7 | 0.8 | 0.0 | 0.7 | NA | NA | 0 |
| | | | 0+DOM+MOPS | 2.2 | 1.7 | 1.0 | 0.5 | NA | NA | 5 |
| | | | 36 | 15.7 | 18.5 | 14.5 | 14.6 | 118.0 | 100.6 | 10 |
| | | | 144 | 141.7 | 127.3 | 109.3 | 105.5 | 89.8 | 96.5 | 5 |
| | | | 576 | 505.8 | 527.6 | 493.4 | 520.2 | 104.3 | 105.4 | 5 |
| | | | 2304 | 2549.1 | 2042.3 | 1899.5 | 1868.6 | 80.1 | 98.4 | 100 |
| | | | 9216 | 8587.8 | 2469.2 | 3070.0 | 2407.5 | 28.8 | 78.4 | 100 |

Table S2. Cerium speciation in *H. azteca* testing media (RSW) given as the % of total aqueous Ce concentration (from initial dissolved measurements) and predicted by WHAM (ver. 7.0.2). Cerium bound to colloidal Humic and Fulvic acids are represented as HA-Ce and FA-Ce, respectively. The units for nominal concentrations of Ca, Na and Mg trials are (mM) while DOM (mg C/L) and total Ce-$_D$ (µg/L).

| Test type: | Nom. Conc. | Total Ce | % Ce | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $Ce^{3+}$ | $CeCO_3^+$ | $CeHCO_3^{2+}$ | $CeOH^{2+}$ | $Ce(CO_3)_2^-$ | $CeCl^{2+}$ | $CeSO_4^+$ | $Ce(SO_4)_2^-$ | HA -Ce | FA -Ce |
| Ca | 0.1 | 16.7 | 1.2 | 0.5 | 0.0 | 0.0 | 0.00 | 0.0 | 0.1 | 0.0 | 10.0 | 88.1 |
| | 0.5 | 139 | 49.6 | 13.5 | 0.6 | 1.4 | 0.02 | 0.1 | 2.7 | 0.0 | 2.7 | 29.3 |
| | 1 | - | - | - | - | - | - | - | - | - | - | - |
| | 2 | 280 | 59.3 | 20.5 | 0.6 | 2.2 | 0.1 | 0.6 | 2.0 | 0.0 | 1.3 | 13.6 |
| Na | 0.101 | 16.7 | 1.2 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 10.0 | 88.1 |
| | 0.5 | 135 | 37.7 | 19.1 | 0.5 | 1.7 | 0.1 | 0.1 | 2.5 | 0.0 | 3.2 | 35.2 |
| | 1 | 345 | 47.4 | 29.0 | 0.6 | 2.6 | 0.1 | 0.2 | 2.8 | 0.0 | 1.4 | 15.8 |
| | 2 | 41.6 | 13.1 | 5.7 | 0.2 | 0.5 | 0.0 | 0.1 | 0.7 | 0.0 | 7.6 | 72.1 |
| Mg | 0.025 | 16.7 | 1.2 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 10.0 | 88.1 |
| | 0.1 | 180 | 38.0 | 19.7 | 0.5 | 1.7 | 0.1 | 0.0 | 10.1 | 0.0 | 2.5 | 27.3 |
| | 0.3 | 56.2 | 17.1 | 8.1 | 0.2 | 0.7 | 0.0 | 0.0 | 11.7 | 0.1 | 5.6 | 56.4 |
| DOM | 0.272 | 16.7 | 1.2 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 10.0 | 88.1 |
| | 6 | 852 | 0.7 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.9 | 90.0 |
| | 12 | 1026 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 9.8 | 90.1 |

# CHAPTER 3

*This chapter has been submitted to the Aquatic Toxicology journal - currently under review.*

# Modification of acute Dysprosium toxicity to *Hyalella azteca* and development of the biotic ligand approach.

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## Abstract

The toxicological understanding of rare earth elements (REEs) in the aquatic environment is very limited but of increasing concern. The objective of this research is to compare the toxicological effect of the REE dysprosium to the freshwater invertebrates *Daphnia pulex* and *Hyalella azteca* and in the more sensitive organism, understand the toxicity modifying influence Ca, Na, Mg, pH and dissolved organic matter (DOM). Standard methods (Environment Canada) were followed for testing and culture in media of intermediate hardness (60 mg $CaCO_3$ mg/L) at pH 7.8 with Ca at 0.5, Na 0.5, Mg 0.125 (mM) and 23ºC. Acute toxicity tests were done with <24 h old neonates for 48 h in the case of *D. pulex* and with 2-9 d old offspring for 96 h tests with *Hyalella*. The potential protective effect of cationic competition was tested with Ca (0.5 to 2.0 mM), Na (0.5 to 2.0 mM) and Mg (0.125 to 0.5 mM). The effect of pH (6.5 – 8.0) and Suwannee River DOM complexation (dissolved organic carbon (DOC) concentrations of 9 and 13 mg C/L). Dissolved Dy concentrations were lower than total (unfiltered) indicating precipitation, particularly at higher concentrations. Acute toxicity of Dy to *H. azteca* and *D. pulex* revealed *Hyalella* to be 1.4 times more sensitive than *Daphnia*. Additions of Ca and Na but not Mg provided significant protection against Dy toxicity to *Hyalella*. Similarly, low pH was associated with reduction in toxicity. Exposures which were pH buffered with and without MOPS were significantly different and indicated that MOPS enhanced Dy toxicity. DOM also mitigated Dy toxicity. Biotic ligand based parameters (Log K values) were calculated based on free ion relationships as determined by geochemical equilibrium modeling software (WHAM ver. 7.02). The log K value for $Dy^{3+}$ toxicity to *Hyalella* was 7.75 while the protective influence of Ca and Na were 3.95 and 4.10 respectively. This study contributes data towards the development of site specific water quality guidelines and criteria for Dy and possibly REEs in general and offers insight into the complex bio-geochemical nature of this element.

Keywords: Rare earth elements; invertebrates; toxicity modifying factors; biotic ligand model; water quality; risk assessment

## 3.0 Introduction

Rare earth elements (REEs) are a group of 17 similarly reactive metals that include 15 lanthanides, yttrium and scandium (Environment Canada, 2012; Migaszewski and Galuszka, 2015). These metals are divided into two groups: the light rare earth elements (LREEs), lanthanum to samarium and the heavy rare earth elements (HREEs) comprised of europium to lutetium as well as yttrium (Environment Canada, 2012; Migaszewski and Galuszka, 2015). Contrary to their name REEs are not rare; when compared to other metals, the soil REE concentrations are similar to iodine and cobalt and more abundant than those of mercury or silver (Environment Canada, 2012; Paul and Campbell, 2011). In general, the abundance of REEs decreases with increasing atomic number. Enriched deposits are limited and therefore there are a few areas where mining is economically viable particularly for less abundant HREEs (Paul and Campbell, 2011).

REEs are used and applied in many industries and this is reflective of their unique set of catalytic, magnetic and optical properties (Paul and Campbell, 2011). Dysprosium (Dy) is classified as a HREE and is used in lasers, hybrid electric vehicles, lighting, consumer electronics and permanent magnets (Navarro and Zhao, 2014; Stegen, 2015). For example Dy is a component of neodymium-iron-boron (Nd-Fe-B) based permanent magnets which greatly increase temperature resistance and this facilitates miniaturization of wind turbines and electric motors (Stegen, 2015). As well, Dy has numerous uses in the defense technologies such as military grade lasers, control and guidance systems, power generating devices and microwave communication (U.N.C.T.A.D., 2014). Its growing use makes Dy one of the five REEs (along with Nd, Eu, Tb and Y) identified by the United States Department of Energy as critical REEs for the development of future clean energy technologies (U.S.D.O.E., 2011). Recently, Elshkaki and Graedel (2014) estimated that the global demand for Dy will dramatically increase over the upcoming decades. While recycling of previously used Dy in obsolete equipment has been identified as an alternative to help offset the growing demand (Elshkaki and Graedel, 2014) and while novel approaches may contribute to more effective recovery of Dy in existing deposits (Horiike and Yamashita, 2015) new mining projects will be needed to meet future demand for REE. Several of these are under development in different countries around the world and in Canada the most advance HREE mining project is located in Thor Lake, Northwest Territories and owned by Avalon Rare Metals Inc.

In freshwaters REEs occur at relatively low dissolved concentrations (usually ng/L to µg/L, Leybourne and Johannesson, 2008; Verplanck et al., 2001) and dissolved LREEs are generally more abundant than HREEs (Migaszewski and Galuszka, 2015). As one of the critical REEs, Dy is of particular concern in terms of the potential for impacts, particularly in the context of the northern Canadian environments where enriched deposits occur and mining may develop. There are few studies on natural occurrences of Dy in freshwaters. Dy concentrations were relatively low (1.3 µg/L) in an uncontaminated naturally acidic lake located in Nunavut (Johannesson and Lyons, 1995). Concentrations of Dy at reference sites for a REE mine were below 22 µg/L (Verplanck et al., 2001) but at some mining projects Dy concentration has been recorded as high as 595 µg/L in the surrounding groundwater (Miekeley et al., 1992). Whether these Dy concentrations have the potential to induce deleterious effects in aquatic biota is poorly understood. There are no water quality guidelines in Canada and this is also the case for water quality criteria in the United States. The lack of guidelines and criteria is reflective of the limited number of aquatic toxicity studies with Dy. Even less is known about the influence of toxicity modifying factors (TMFs) on Dy toxicity.

The influence of water chemistry TMFs on metal toxicity can be categorized as anionic factors that influence the bioavailability of metal via complexation of free metal ions and cationic factors that antagonistically compete with the free-metal ions for uptake at the site of toxic action. Some factors, such as pH can influence toxicity via both categories (i.e. changes in $H^+$ as well as carbonate complexation for example). The free-metal ion form of a metal is generally considered to be the most bioavailable species (Campbell, 1995) but it is unclear whether the REEs, which occur as trivalent ions, (e.g. $Dy^{3+}$, Migaszewski and Galuszka, 2015) follow this model. Out of the few published studies on the toxicity of REEs, the most notable were conducted with algae (EL-Akl et al., 2015; Tai et al., 2010; Hao et al., 1997; Yang et al., 2014; Zhao and Wilkinson, 2015), sea urchin (Oral et al., 2010), a microcosm of algae, protozoa, and bacteria (Fuma et al., 2005), *Daphnia* (Barry and Meehan, 2000) and *Hyalella* (Borgmann et al., 2005). These studies although performed with different REEs under various conditions, illustrate that toxicity is influenced by water chemistry. For instance, Barry and Meehan (2010) conducted acute La exposures to *Daphnia carinata* and showed that increased water hardness decreased La toxicity. They argued that the physiochemical similarity of $La^{3+}$ to $Ca^{2+}$ have resulted in toxicity through the inhibition of many Ca dependent biological systems and that elevated Ca concentrations were protective of La toxicity via cationic competition. Fuma et al. (2005) worked with Dy exposures in a microbial microcosm and identified pH as a major factor in controlling toxicity and attributed the increase in EC50 at elevated pH because of increased complexation with inorganic ligands. A recently published study by EL-Akl et al. (2015) examining Ce uptake in algae demonstrated a decrease in uptake with increasing dissolved organic matter (DOM). The study of Borgmann et al. (2005) included acute Dy toxicity tests with *Hyalella azteca* and showed that the LC50 in soft water was lower than in intermediate hardness water but differences were not significant and for the latter test measured concentrations were not reported. These studies indicate that water chemistry may influence Dy toxicity and stress the need for further investigations of Dy effects in aquatic settings.

Currently, the most advanced method to comprehensively account for the influence of water chemistry on metal toxicity is the biotic ligand model (BLM). The BLM predicts toxicity based on the interaction of the free-metal ions with the site of toxic action (the biotic ligand). It accounts for the influence of TMFs such as competing cations, and complexation with inorganic and organic ligands (Di Toro et al., 2001; Niyogi and Wood, 2004; Paquin et al., 2000; Paquin et al., 2002). The BLM simultaneously accounts for the influence of TMFs within a geochemical equilibrium context to predict metal speciation and subsequently toxicity to specific organisms. Mortality is predicted when the concentration of the metal bound to the biotic ligand surpasses the threshold accumulation for that organism. This threshold is defined as the accumulation (at the biotic ligand) that is associated with 50% effect, for the lethality end point the LA50. The practical application of the BLM is to predict metal toxicity on a site-specific basis, as a function of local water chemistry conditions (Paquin et al., 2000).

The objective of this study was to develop data on the acute toxicity of Dy to sensitive aquatic invertebrates, including an understanding of the influence of water chemistry. A BLM approach was applied through a systematic investigation of potential TMFs, including $Ca^{2+}$, $Na^{+}$, $Mg^{2+}$, $H^{+}$ and DOM. The initial step in this study was to compare the sensitivities to Dy between *Daphnia pulex* and *Hyalella azteca*.

## 3.1 Materials and Methods

### *3.1.1 Invertebrate culturing*

#### *3.1.1.1 Hyalella azteca*

*Hyalella* were collected from Hannah Lake near Sudbury Ontario and had been cultured at Wilfrid Laurier University for over two years. The collected organisms were identified morphologically (Pennak, 1978) and genetically as *Hyalella azteca* belonging to the inner Sudbury clade **(**Babin-Fenske et al., 2012). Culturing followed Environment Canada standardized Biological Test Method EPS 1/RM/33 2nd edition (Environment Canada, 2013). A reconstituted aquatic medium (RAM) was used, based on a 50% dilution of the standardized aquatic medium for *Hyalella* growth and reproduction described by Borgmann (1996). RAM was made with analytical grade salts (Sigma Aldrich, Mississauga, ON) of $CaCl_2$, $NaHCO_3$, $MgSO_4$, KCl and NaBr which when diluted with deionized water (18 Mohm, Milli-Q A30, Millipore Corporation, Fisher Scientific Nepean, ON) were at nominal concentrations of 500, 500, 125, 25 and 5 µM, respectively. Hardness was 60 mg $CaCO_3$/L and the solution pH was 7.6 ± 0.2 (measured using Radiometer PHM240 meter equipped with pHC2701 electrode (ATI Scientific, Mississauga, ON)).

Cultures were maintained in 2 L polypropylene beakers (VITLAB® Griffin, VWR International, Mississauga, ON) with 30-80 adult organisms in 1.6 L of RAM. Beakers were covered with a glass lid and a water change was done weekly using 650 and 275 µm mesh to separate adults from neonates. After each water change a new piece cotton gauze of approximately 5 by 5 cm was placed in solution to act as substrate (Borgmann et al., 1989). *Hyalella* cultures were fed 5 mg ground fish flakes (TetraMin, Tetra Holding (US) Inc., Blacksburg, VA) three times weekly, on non-consecutive days. Neonates used for toxicity testing were collected into during water changes and were 2 to 9 d of age at the beginning of tests. Cultures were kept in a controlled environment chamber (CMP6010 Conviron, Winnipeg, MB), where temperature was 23 ± 1$^{o}$C, light intensity was 500-1000 lux and photoperiod was 16 h light: 8 h dark.

#### *3.1.1.2 Daphnia pulex*

*Daphnia* was purchased from Aquatic Research Organisms Inc. (Hampton, NH) and cultured in 1 L glass beaker. Procedures for culturing *Daphnia pulex* followed standard methods (Environment Canada, 1996) with RAM. Water changes were carried out daily in order to separate neonates from adults. *Daphnia* feeding followed each water change and consisted of 10 mL of an algae mixture (30% *Chlorella vulgaris* and 70% *Pseudokirchneriella subcapitata*) and 5 mL of a yeast, cerophyl and trout chow mix (YCT, Aquatic Research Inc., Hampton, NH). The temperature was 21 ± 1$^{o}$C with lighting between 400-1000 lux and a photoperiod of 16 h light and 8 h dark cycle. Toxicity tests were carried out without food using neonates less than 24 h of age.

### *3.1.2 Acute toxicity testing*

Acute toxicity tests for *Hyalella* (96 h) and *Daphnia* (48 h) were carried out following standard methods (Environment Canada, 2013 and 1996, respectively). In brief, Dy exposure solutions were prepared in duplicate by adding the appropriate amount of Dy stock solution generated from a 1 g/L atomic absorption standard solution (Inorganic Ventures, Christiansburg, VA) to culture medium, adjusting to pH 7.3 and then allowing for 24 h of equilibration prior to test start. Mortality patterns from Dy exposures revealed *Daphnia* as the more resistant organism and therefore, further TMF tests were performed with the more sensitive *Hyalella*.

#### ***3.1.2.1 Testing with TMFs***

A series of tests were done for each TMF, beginning with the culture medium and then in a progressive manner systematically modifying concentrations in order to understand how Ca, Mg, Na, pH and DOM influence acute Dy toxicity to *Hyalella*. In each test series one chemical parameter was varied while keeping others constant. Test were done in duplicate in 400 mL polyethylene beakers with 240 mL of exposure solution. Test solutions were equilibrated for 24 h before the test started. During equilibration of test solutions gauze pieces (2 cm x 2 cm) were also equilibrated but in a separate plastic beaker. Exposures were initiated by adding the gauze and then 10 *Hyalella* 2-9 d of age to each beaker. Beakers were covered and held in a controlled environment chamber (described above) for 96 h.

Each test included a control (media with the modified parameter) and five exposure concentrations ranging from 1.2 - 79 µM. The exact range was dependent on degree of toxicity modification anticipated by the TMF. Culture medium was altered by sequentially changing the concentration of the selected TMF while keeping others constant. Ca was added as $CaCl_2$, Mg as $MgSO_4$ and Na as NaCl. The effect of pH was evaluated by using 1 mM 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (De Schamphelaere et al., 2004) to stabilize pH at either 6.5, 6.8 or 8.0 by additions of either KOH or $HNO_3$. Additionally, the potential influence of MOPS alone was evaluated in side-by-side tests with 1 mM MOPS and without added MOPS (unmodified culture medium at pH 7.6). The effect of DOM on Dy toxicity was assessed by adding Suwannee River organic matter (SR-DOM, 1R101N, International Humic Substances Society, St. Paul, MN) to the nominal exposure solutions at either 8 or 15 mg C/L. Measured exposure water chemistry is summarized in Table 2.

### *3.1.2.2 Exposure sampling and characterization*

Temperature and pH were characterized as described above and were measured prior to the start and end of the test. Water samples were collected shortly after solutions were made and when exposures ended (96 h) in order to characterize water chemistry and Dy content. For each sampling time and each exposure concentration, a pair of 10 mL samples were collected, one unfiltered and the other filtered (pre-rinsed 0.45 µm, Acrodisc HT tuffryn membrane, Pall Corporation, MI). Samples were stored in 15 mL tubes (Celltreat, Mandel Scientific, Guelph, ON) and preserved with 2% v/v 16 N $HNO_3$ (trace metals grade, Fisher Scientific, Nepean, ON) before being analyzed. Tests containing DOM were 0.45 µm filtered for dissolved organic carbon (DOC) and 50 mL of sample was collected between each exposure replicate for the time period when the solutions were made and when the test ended. DOC samples were stored at 4$^o$C and not acidified before being measured with the total organic carbon analyzer (TOC-LCPH, Shimadzu, Mandel Scientific, Guelph, ON). RAM DOC concentration was also measured, at 0.4 mg C/L.

Dy concentrations were determined for filtered (dissolved Dy: Dy-$_D$) and unfiltered (total Dy: Dy-$_T$) samples using inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 8000, PerkinElmer Inc., Woodbridge, ON) and solution cations (Ca, Na and Mg) were measured using an atomic absorption spectrophotometer in flame mode (AAS, SpectAA-880, Varian Inc., Palo Alto, CA). Analysis parameters and wavelengths followed manufacturer recommendations. Quality assurance procedures for Dy measurements involved analysis of reference standards (Standard 26, Inorganic Ventures Inc., Christiansburg, VA) and reagent blanks (2% $HNO_3$) throughout each run. The average measured concentrations for Dy in controls

(RAM medium) for Dy-$_T$ was 3.9 µg/L (stdev= 5.7, n=23) and for Dy-$_D$ was 1.8 µg/L (stdev =3.6, n=23).

### *3.1.2.3 Calculations and statistics*

Dy concentration resulting in 50% mortality (LC50) were calculated based on Dy-$_T$ and Dy-$_D$ measured exposure concentrations at 96 h with the Comprehensive Environmental Toxicity Information System software (CETIS, Tidepool Software, 2005) using the trimmed Spearman Karber method (Hamilton et al., 1977). Significant differences in LC50s were determined using the Litchfield and Wilcoxon method (1949, Environment Canada, 2005).

$Dy^{3+}$ concentrations were estimated for LC50 values (Dy-$_D$ basis) using the Windermere Humic-Aqueous Model (WHAM ver. 7.02., Tipping et al., 2011) and measured water chemistry (Table 2). DOC entered into WHAM inputs for DOM assumed it to be in colloidal phase and at 90% Fulvic and 10% Humic acid (Santore et al., 2001). Speciation data from WHAM was used to develop stability constants for competitive interaction of cations (including $Ca^{2+}$ and $Na^{+}$) on $Dy^{3+}$ toxicity. These were calculated according to the method developed by De Schamphelaere and Janssen (2002). In short, linear regression analysis of free cationic activities of Ca on $Dy^{3+}$ LC50 were performed in the presence of constant $Na^{+}$ to derive the slope and intercept values from which a system of linear equations was solved and an estimate of Log K Ca-$_{BL}$ developed (De Schamphelaere and Janssen, 2002). Likewise, regression variables for the toxicity mitigating effect of $Na^{+}$ activity on $Dy^{3+}$ activity at the LC50 concentration and in the presence of constant $Ca^{2+}$ were used to estimate the Log K for the binding of Na to the BL. The conditional equilibrium constant used to quantify $Dy^{3+}$ binding to the biotic ligand (Log K Dy-$_{BL}$) was derived as the negative log of the average of the intercepts from the three individual cation regression relationships: $Dy^{3+}$ on $Ca^{2+}$, $Na^{+}$ and $Mg^{2+}$ (De Schamphelaere and Janssen, 2002).

## 3.2 Results

### *3.2.1 Exposure characterization*

Both Dy-$_{T}$ and Dy-$_{D}$ were measured and the overall results indicate increased precipitation at higher concentrations as well as during the course of the exposure except in tests with low pH and with added DOM (Figure 1, Supplementary data Table S1). Dy-$_{T}$ concentrations measured immediately after solution preparation were very close to nominal concentrations. These solutions were mixed before sampling and therefore could have contained precipitated Dy that was brought back into solution upon sample acidification. Dissolved concentrations in these initial samples of exposure solutions were <51% of total at the lowest exposure concentration and 34% at the highest (Figure 1). Measured Dy-$_{T}$ at the end of the test (collected without disturbing the solutions) were 1.6, 1.7, 1.9 and 3.4 fold less than initial Dy-$_{T}$ concentration in nominal 200, 1600, 6400 and 12800 µg/L, respectively (Figure 1). After 96 h of exposure, the dissolved concentrations were generally close to initial Dy-$_{D}$ being slightly higher up to nominal 800 µg Dy/L and then lower above this concentration (Figure 1). In low pH tests the concentrations of Dy-$_{D}$ were much closer to Dy-$_{T}$ (Supplementary data Table S1). Similarly, when DOM was added in the test medium there was a tendency for much less precipitation (Supplementary data Table S1). Measurement of additional samples, collected from test beakers that had been completely acidified to 2% with concentrated $HNO_3$ showed that when precipitated Dy was brought back into solution the measured concentrations matched nominal ones (data not shown). Given the variation in Dy concentrations we decided to base estimates of toxicity (i.e. calculation of LC50 values) on concentrations measured at the end of the tests. Because some jurisdictions use total metal and others dissolved we have reported both Dy-$_{T}$ and Dy-$_{D}$ based endpoints. Dy-$_{\mathrm{D}}$ LC50 values were also converted to $Dy^{3+}$ in order to develop modelling parameters (see below).

### *3.2.2 Species sensitivity to Dy*

Acute 96 h Dy exposure to *Daphnia pulex* and *Hyalella azteca* was performed under the same water chemistry conditions (unmodified RAM) and showed the latter organism to be more sensitive (Figure 2). Therefore, testing to quantify the potential influence of toxicity modifying factors was done using the more sensitive *Hyalella*.

### *3.2.3 Effect of cationic competition and pH on Dy toxicity to Hyalella azteca*

Increases in Ca concentration significantly decreased acute Dy toxicity (Table 1, Figure 3A). Based on Dy-$_{\mathrm{T}}$ measurements at 96 h the LC50 increased 1.8 times over the range of Ca added (from 0.5 to 2 mM). A less distinct, exposure-effect pattern was evident for Dy-$_{\mathrm{D}}$ and it is notable that for the test at 2 mM Ca it was only possible to estimate the LC50 value when intermediate exposure concentration results (nominal 800 and 1600 µg/L) were omitted (Supplementary data Table S1). Increased Na also significantly decreased Dy toxicity, by a factor of 1.4 times based on measured Dy-$_{\mathrm{T}}$, with trends being somewhat less clear based on Dy-$_{\mathrm{D}}$ but clearer (2.6 fold change) for $Dy^{3+}$ over the range of added Na (Table 1, Figure 3B). Tests with added Mg generally indicated no consistent protective effect although there was a significant difference in LC50 values for Dy-$_{\mathrm{T}}$ as well as $Dy^{3+}$ (Table 1, Figure 3C) although the calculation of the latter was likely influenced by the low pH of that particular test (7.4 vs 7.7, Supplemental data Table S1). The linear regression for the relationships of $Dy^{3+}$ LC50 showed clear positive correlations for $Ca^{2+}$ (Figure 3A) and $Na^{+}$ (Figure 3B).

Tests at different pH values demonstrated that toxicity decreased as pH decreased (Table 1) and there was a strong linear relationship between Dy toxicity and $H^+$ across the pH range of 6.5 – 8.0 (Table 1, Figure 3D). LC50 values decreased 8.2 and 8.9-fold for Dy-$_T$ and Dy-$_D$, respectively (Table 1) while those based on $Dy^{3+}$ revealed a pronounced 482-fold decrease in toxicity (pH 8.0 vs 6.5, Table 1, Figure 3D). Comparison of tests with and without addition of MOPS at pH 7.8 showed that MOPS increased the toxicity of Dy by 33% (Table 1).

### *3.2.4 Effect of dissolved organic matter complexation on Dy toxicity*

Addition of NOM from Suwannee River to test solutions significantly decreased toxicity (Table 1, Figure 4). The 96 h LC50s for Dy-$_T$ and Dy-$_D$ increased 3.6 and 3.3-fold respectively as DOC concentrations were increased 0.4 to 13.0 mg C/L (Table 1). There was a positive correlation in the linear relationship between DOC (mg C/L) and LC50 for Dy-$_D$ (Table 1, Figure 4A). Estimates of $Dy^{3+}$ LC50 values were negatively correlated with DOC concentration (Figure 4B). $Dy^{3+}$ LC50 decreased 125-fold over the tested DOC concentration range (Table 1, Figure 4B).

### *3.2.5 Estimating Log K values*

Estimates of conditional equilibrium constants for the interaction of free ion activities of Ca, Na and Dy on the biotic ligand (see Table 2) were derived from geochemical speciation modeling using WHAM and based on measured dissolved water chemistry (Table 1). The derived Log K value for Dy-$_{BL\text{-}Ca}$ was 3.95 while for Dy-$_{BL\text{-}Na}$ was 4.10 and for the interaction of $Dy^{3+}$ with the biotic ligand (Dy-$_{BL}$) it was 7.75 (Table 2). In the case of the reduction in toxicity due to pH (Table 1), a Log K value for the competitive effect of $H^+$ was not calculated because of the potential for other factors influencing toxicity (e.g. carbonate complexes and MOPS, see discussion). It was not possible to determine the effect of $H^+$ competition alone.

### 3.3 Discussion

In this study we were able to determine acute Dy toxicity to two sensitive freshwater invertebrate species and show that water chemistry can have a significant influence on toxic responses. Dy precipitated during the 96 h exposures (Figure 1) and because dissolved concentrations tended to be lower at the end of the tests (Figure 1) we based our LC50s on the end concentrations. In the intermediate hardness RAM medium, the solubility limit of Dy appeared to be around 600 µg/L (Figure 1) just above the LC50 for $\text{Dy-}_\text{D}$. In tests with added DOM or low pH, the solubility of Dy was higher (Supplementary data Table S1, Figure 1). Acute toxicity of Dy to *H. azteca* and *D. pulex* was compared and in moderately soft water the LC50, based on $\text{Dy-}_\text{D}$, showed *H. azteca* to be 1.4 times more sensitive than *D. pulex* (Figure 2). This may have been associated with the longer duration of the standard test for *Hyalella* tests (96 h vs. 48 h for *Daphnia* spp.) in addition to inherent differences in sensitivity. Given the greater sensitivity of *H. azteca*, it was used for subsequent testing with toxicity modifying factors. Additions of Ca and Na but not Mg (Table 1, Figure 3) provided significant protection against Dy toxicity. Similarly, low pH was associated with reduction in toxicity (Table 1). DOM also mitigated Dy toxicity and significant differences were determined when compared to the control for both DOC additions (Table 1).

### *3.3.1 Precipitation of Dy in solution*

Characterizing exposure proved challenging because of significant loss of Dy in all test solutions except for low pH trials (Table 1, Figure 1). Dy-$_{D}$ was much lower than Dy-$_{T}$ for all exposures and at higher concentrations this was more pronounced. Whether Dy adhered to the exposure container walls or precipitated was not explored in this study but Dy equilibration in solution appears to be rapid as the initial Dy-$_{D}$ concentrations did not change substantially over the course of exposures. Dy-$_{T}$ concentrations decreased dramatically between initial and final samples (Figure 1) and the difference increased with increasing concentrations. The difference in Dy-$_{T}$ can be explained by the fact that the initial solutions were stirred vigorously before sampling while final solutions were not. Therefore, initial Dy-$_{T}$ measurements undoubtedly contained precipitated forms of Dy. The exception to this was tests at low pH where initial and final Dy-$_{T}$ and Dy-$_{D}$ concentrations were similar and both were close to nominal concentrations (Supplemental data Table S1, Figure 1). Overall the most significant observation on Dy behavior in solution was the very low concentrations of Dy-$_{D}$, which appear to plateau at approximately 600 µg/L in the RAM media (Figure 1). While this apparent solubility limit in the culture and testing medium was above the LC50 for Dy-$_{D}$ it often resulted in dramatic increases in mortality over a very narrow range of final Dy-$_{D}$ concentrations (Supplemental data Table S1). Mortality-exposure patterns based on final Dy-$_{T}$ were more pronounced and this feature of our study is worth further investigation.

Precipitation in aquatic toxicity tests has been demonstrated previously for REEs (e.g. Borgmann et al., 2005; Barry and Meehan, 2000). Few studies on REEs provide measured concentrations and those that do (e.g. Bowmer et al., 1993; Borgmann et al., 2005) generally agree with our observations that using nominal Dy concentrations to calculate endpoints underestimates toxicity (Gonzalez et al., 2014). In their study with La exposures to *Daphnia carinata*, Barry and Meehan (2000) noted precipitation and decided to use the mean of the nominal and measured concentrations at the end of the test in order to derive their EC50 values. We based our LC50 calculations on Dy-$_{D}$ concentrations measured after 96 h of exposure as these values would represent the most conservative estimates of toxicity. We also calculated LC50s based on final Dy-$_{T}$ concentrations in order to provide information for the Canadian regulatory context. Given the differences between nominal and Dy-$_{D}$, the use of nominal concentrations in LC50 determination would dramatically underestimate toxicity.

The loss of Dy in test solutions increased with increased concentrations and this could have been due to adsorption or precipitation (or both). Johannesson and Hendry (2000) indicate that the particular charge of the REE species is one of the factors controlling adsorption. For instance, the authors argue that enrichment of HREEs can result from adsorption to oppositely charged particulate or solid phases (Johannesson and Hendry, 2000). Thus adsorption to surfaces in our tests, such as the polyethylene beakers (Benes and Paulenova, 1973), could have enhanced loss of Dy-$_{D}$ from solution. The low concentrations in the study may also be due to precipitation of Dy. As reviewed by Gonzalez et al. (2014) and demonstrated by Gonzalez et al. (2015), precipitation is a feature of most REE toxicity studies using artificial media and this is because of phosphate and/or carbonate complexes which have low solubility (Jiang and Ji, 2012). It seems likely that some of these processes contributed to the loss of Dy-$_{D}$ in solution. These results highlight the importance of exposure characterization of test solutions (Gonzalez et al., 2014) and stress the need for further understanding of how total and dissolved Dy effect toxicity.

### *3.3.2 Sensitivity difference to Dy toxicity between H. azteca and D. pulex*

Acute Dy exposures to *Daphnia pulex* (48 h) and *Hyalella azteca* (96 h) under the same water chemistry conditions demonstrated greater sensitivity in the latter (Figure 2). There are very few published studies on the toxicity of Dy (Gonzalez et al., 2014). One notable study is that of Borgmann et al. (2005) where the 7 d LC50 for Dy-$_D$ (samples collected at the end of the test) was 162 µg/L (95% CI of 34-769 µg/L) and this compares well to our results 340 µg/L (308-390). The Borgmann et al. (2005) study was for 7 d exposures (vs 4 d in this study) and in softer water than our test medium (hardness of 18 mg $CaCO_3$/L vs 60 for our RAM) and this may explain the lower toxicity value. Borgmann et al. (2005) also conducted tests in hard water (120 mg $CaCO_3$/L) and the reported 7 d LC50 value was 897 µg/L. This value was based on nominal loadings and our study indicates that nominal concentrations may be misleading, however it did demonstrate that toxicity in hard water was reduced.

### *3.3.3 Toxicity modifying effects of water chemistry to the toxicity of Dy to H. azteca*

The protective effect of waterborne Ca on Dy toxicity to *H. azteca* was demonstrated for Dy-$_T$ and Dy-$_D$ (Table 1, Figure 3A) although it is noteworthy that it was only possible to calculate the LC50 value for Dy-$_D$ in the 2 mM Ca test with an edited exposure-response relationship where the nominal concentrations of 800 and 1600 µg/L were not included (see supplemental data Table S1). While mortality increased with increased nominal and also Dy-$_T$ concentrations, the final Dy-$_D$ concentrations were all similar and at the apparent solubility limit for Dy in RAM (Figure 1). Precipitation and issues in calculating endpoints was noted by Barry and Meehan (2000) in their study on La exposures to *Daphnia carinata*.

Our results showing the protective effect of waterborne Ca are generally in agreement with the studies of Borgmann et al. (2005) and Barry and Meehan (2000) which showed that REE toxicity was reduced as water hardness increased. For example, in the study of Borgmann et al. (2005) acute tests were done in very soft (18 mg $CaCO_3$/L) and intermediate hardness (124 mg $CaCO_3$/L) water and Dy toxicity increased 1.8 times for the latter (nominal concentration basis). This change in toxicity cannot be attributed exclusively to Ca as Borgmann et al. (2005) altered test solution chemistry by dilution of the hard water (Burlington tap water) with deionized water. The protective effect of Ca on the uptake of REEs into algae has been previously shown. A 10,000 fold increase in Ca concentration resulted in a 30% reduction of La in the algal species *Scenedesmus pannonicus* (subsp. Berlin) but no effect on uptake in fungus (*Aureobasidium pullulans*; Demon et al., 1989). More recently, EL-Akl et al. (2015) working with the freshwater algae (*Chlamydomonas reinhardtii*) showed a threefold decrease in Ce uptake as $Ca^{2+}$ increased from 0.01 to 1 mM. Although there are a limited number of studies, our results with Dy support the hypothesis of a competitive interaction between Dy and Ca.

In this study, we use WHAM for speciation modeling of measured $Dy\text{-}_D$ concentrations to estimate $Dy^{3+}$ content in solution. If $Dy^{3+}$ and $Ca^{2+}$ compete for uptake at the biotic ligand then the LC50 concentrations for $Dy^{3+}$ would have been positively correlated with $Ca^{2+}$ concentration and this was observed (Figure 3A). It was not surprising to observe the protective effect of Ca on Dy toxicity to *Hyalella* since lanthanides and Ca share similar physiochemical properties. For instance, lanthanides have comparable ionic radii to $Ca^{2+}$ (Hirano and Suzuki, 1996) and as a result are effective Ca channel blockers (Sandvig and Olsnes, 1982; Lansman, 1990). Also, lanthanides have been shown to replace Ca in nutrient deficient tests with alga indicating a shared biological function (Goecke et al., 2015). In this study we demonstrate that

Dy toxicity decreases with increasing waterborne Ca concentration and the mechanism underlying this protective effect is worthy of further study.

Increased waterborne Na was also protective of Dy toxicity to *Hyalella azteca* (Table 1, Figure 3B) suggesting the possibility of competition between $Dy^{3+}$ and $Na^{+}$ for Na uptake was not anticipated. Studies with Mg additions show no clear protective effect, interestingly LC50s based on $Dy\text{-}_{T}$ showed a significant reduction in toxicity with increasing Mg but based on $Dy\text{-}_{D}$ there was no change (Table 1). Studies on the effects of metals such as Cd, Zn, Cu, Co, Pb and others show that acute adverse effects are caused by inhibition of one of the major cation transporters. For instance $Cd^{2+}$, $Zn^{2+}$, $Co^{2+}$ and $Pb^{2+}$ act on the Ca transporter and block $Ca^{2+}$ uptake while $Cu^{2+}$ and $Ag^{+}$ inhibit the Na transporter and interfere with the uptake of $Na^{+}$ and $Cl^{-}$ across the fish gill (Niyogi and Wood, 2004). It is not known whether Dy interferes with ion uptake processes on the respiratory surface but this study provides indirect evidence that link it to Ca and Na.

The potential mechanisms of acute adverse effect of Dy and other REEs on aquatic organisms have not been studied and is not known. It could be that trivalent free ion forms disrupt essential ion regulation as with mono and divalent metals (Paquin et al., 2002). Alternatively, other mechanisms may be implicated. Studies using gadolinium (Gd) exposure to erythrocyte cells have revealed that $Gd^{3+}$ alters membrane permeability through pore formation (Cheng et al., 1999a), whose size is concentration dependent (Cheng et al., 1999b) and because of cellular entrance (Cheng et al., 1999a, 1999b) likely induces mitochondrial apoptosis (Liu et al., 2003). If waterborne $Dy^{3+}$ induces pore formation in apical surfaces then this may lead to a generalized disruption in ion regulation and might explain, at least partially, the protection by both Ca and Na however this theory is highly speculative. Recently, Senatore et al. (2014) have identified unique T type Ca channels in *Lymnaea stagnalis* that are permeable to Na as well as Ca instead of being highly selective for Ca as observed in vertebrate organisms. If these types of channels are present and have a role in Ca and Na ion regulation in *Hyalella* then an increases in either of the ions would explain the decrease in Dy toxicity through cationic competition. While our study was not designed to elucidate the mechanisms of Dy toxicity it offers some insights and indicates that a focus on Ca and Na balance may yield interesting results.

Solution pH, tested from 6.5 to 8.0, resulted in dramatic differences in LC50 values with low pH values yielding much lower toxicities. This influence on Dy toxicity resulted in a strong linear relationship between the $H^+$ and $Dy^{3+}$ LC50 values (Table 2). For LC50 values calculated on the basis of final $Dy\text{-}_D$ measurements an 8.9 fold change in toxicity was associated with the 32 fold change in $H^+$ concentration in exposure solutions from pH 8 to 6.5. On the basis of $Dy^{3+}$, the same 32 fold change in $H^+$ resulted in a 482 fold change in LC50 values, overall yielding a slope of 9.1 (i.e. 1 nM change in $H^+$ resulted in a 9.1 nM change in $Dy^{3+}$ toxicity). The overwhelming effect of $H^+$ appears to suggest that other dissolved species, in addition to $Dy^{3+}$, are involved in toxic responses as pH increases. The most likely candidates for this would be carbonate complexes. This is supported by the analysis of Gonzalez et al. (2014). Decreased toxicity at lower pH has been reported for several metals (Campbell and Stokes, 1985) and the effect of pH has been incorporated into BLMs as both the $H^+$ cation reducing toxicity via competition as well as by accounting for the contributions of complexes (e.g. CuOH species) to toxicity (De Schamphelaere and Janssen 2002; Di Toro et al., 2001). Recently, the EL-Akl et al. (2015) work with algae and Ce show protective effect of $H^+$ at low pH values. The authors speculated that cationic competition between $H^+$ and $Ce^{3+}$ was taking place at the biotic ligand since reduced biouptake was observed with increasing proton concentration (EL-Akl et al., 2015).

Cationic competition between the $H^+$ and $Dy^{3+}$ at low pH is undoubtedly part of the explanation for the strong decreased in Dy toxicity and it is also possible that Dy-carbonate complexes may be bioavailable and induce toxic response. However, there are additional uncertainties to consider. The pH series of tests were all conducted with the addition of MOPS buffer in order to stabilize the pH. MOPS was chosen as it does not interact/bind with free metal ions (Good and Izawa, 1972) and therefore would not alter free ion concentrations (De Schamphelaere et al., 2004). MOPS has been previously used in metal toxicity studies (e.g. De Schamphelaere and Janssen, 2002; De Schamphelaere et al., 2004; Kozlova et al., 2009; Clifford and McGeer, 2009; Clifford and McGeer, 2010). However, the study of Esbaugh et al. (2013) suggested that MOPS has a physiological effect that enhances toxic response of Pb to fathead minnow *Pimephales promelas*. As part of our studies we compared the effect of MOPS in side-by-side tests at pH 7.8 and found that exposures with MOPS increased the toxicity of Dy. The results from our investigation are not as pronounced as those of Esbaugh et al. (2013) but indicate that Dy tests that use MOPS should be interpreted cautiously. The mechanism underlying this effect of MOPS is not known but Esbaugh et al. (2013) attributed it to physiological stress on the organism due to indirect inhibition of Na uptake and $NH_3$ excretion. It may also be that MOPS binds $Ca^{2+}$ and this is supported by Altura et al. (1980) who examined several biological buffers and proposed that the decrease in prostaglandin mediated smooth muscle contraction resulted from MOPS interaction with Ca ions. These possibilities await further investigation.

Tests with Suwanee River DOM at nominal concentrations of 8 and 15 mg DOC/L demonstrated an exposure dependent decrease in toxicity to *Hylaella* (Table 1, Figure 4). The chosen concentrations for our experiments are generally representative of Canadian Shield waters (David et al., 1997). The protective effects of DOM are well established for metals (Wood et al., 2011) and while little is known about how it influences REE toxicity in natural waters, at circumneutral pH the REEs are known to bind to organic molecules (Tang and Johannesson, 2003; Johannesson et al., 2004). Our hypothesis was that while LC50 values for $Dy_{-D}$ increase with added DOC, when toxicity was expressed on a $Dy^{3+}$ basis the values would be similar across DOC trials. Instead, dramatic reductions in $Dy^{3+}$ LC50 values are predicted by WHAM software as DOC increases (Table 1, Figure 4B). Modeling of exposure chemistry for the lowest DOC addition (9.3 mg C/L) indicated that 99.9% of $Dy_{-D}$ in solution was bound to humic and fulvic complexes suggesting that very small amounts are bioavailable (Supplemental data Table S2). The negative relationship between DOC concentrations and the LC50 for $Dy^{3+}$ can be explained by overestimation of DOM-$Dy^{3+}$ complexation and/or that DOM-Dy complexes are bioavailable and contribute to toxicity. Fuma et al. (2005) suggests that the decrease in Dy toxicity to microbial microcosm results from reduction in $Dy^{3+}$ through complexation with autochthonous organic matter. EL-Akl et al. (2015) showed decreased Ce bioavailability to algae under increasing presence of Suwannee River DOM but also concluded that some REE-organic ligands complexes (e.g. malic and citric acids) may be bioavailable and toxic (Yang et al., 2014; Zhao and Wilkinson, 2015). The mitigating effect of NOM to Dy toxicity in our study is generally consistent with other observations on REEs and indicates that Dy bioavailability is strongly influenced by DOM complexation. Further studies measuring the bioaccumulation of

Dy under different exposure conditions could contribute to an improved understanding of bioavailability.

### *3.3.4 BLM development*

This study shows that Ca, Na, pH and DOM can all influence the toxicity of Dy and therefore we suggest that the BLM approach to estimating Dy toxicity on a site specific (water chemistry) basis could be useful. Previous investigation has concluded that some REEs, such as Lu, also follow BLM principles (Weltje et al., 2004). Recently, conditional equilibrium constants for REEs have been developed for algae (Yang et al., 2014; El-Akl et al., 2015) but no Log K values exist to quantify Dy interaction with *Hyalella*. Our Dy-$_{\mathrm{BL}}$ (Log K = 7.75, Table 2) closely resembles the values obtained from other REEs such as Ce-$_{\mathrm{BL}}$ (7.3), Sm-$_{\mathrm{BL}}$ (7.0), Eu-$_{\mathrm{BL}}$ (7.0) and Tm-$_{\mathrm{BL}}$ (7.1, Yang et al., 2014; El-Akl et al., 2015) and our calculation for Ca binding to the BL (3.95, Table 2) correlates well to the value of 4.3 obtained by El-Akl et al. (2015) in their work with Ce. This is the first study where equilibrium binding constants for Dy and *Hyalella azeca* have been calculated and the data derived in this investigation are applicable for BLM development in freshwaters of moderate hardness. Remaining uncertainties highlighted in our study include the effect of pH on Dy toxicity and the complexation of $Dy^{3+}$ by DOM.

## 3.4 Conclusions

This study demonstrates that water chemistry can have a significant influence on the acute toxicity of Dy to *Hyalella azteca*. Increased Ca, Na and DOM decreased toxicity while decreased pH decreased toxicity. Additions of Mg had no clear effect on toxicity. These results are consistent with other metals where free ion forms are correlated to toxicity and TMFs mitigate via complexation or cationic competition. However, our studies are preliminary in this regard and further mechanistic study is required in order to fully understand if it is appropriate to apply a BLM framework for site specific toxicity predictions. Towards this end we have determined Log K values for the interaction of $Dy^{3+}$ on the biotic ligand as well as for the protective effect of Ca and Na. The influence of pH on Dy toxicity was dramatic and it may be that Dy-carbonate complexes in solution contribute to toxicity.

### 3.5 Acknowledgements

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## 3.6 Tables and Figures

Table 1. Measured water chemistry from acute toxicity tests after 96 h of exposures and the corresponding Dy toxicity to *H. azteca* as either total (Dy-$_{T}$), dissolved (Dy-$_{D}$) or $Dy^{3+}$ (calculated using WHAM ver. 7.02 from Dy-$_{D}$ values). All concentrations are µM except for pH, DOC (mg C/L) and $Dy^{3+}$ (nM). The $^{m}$ superscript represents tests where MOPS was added and * highlights the 2 mM Ca test where the LC50 calculation was based on the modified dataset (see text).

| | Ca | Na | Mg | pH | DOC | LC50 Dy-$_{T}$ | 95% | | LC50 Dy-$_{D}$ | 95% | | LC50 $Dy^{3+}$ | 95% | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | LCL | UCL | | LCL | UCL | | LCL | UCL |
| Ca | **590** | 597 | 182 | 7.8 | 0.4 | $2.2^{a}$ | 2.0 | 2.5 | $2.1^{a}$ | 1.9 | 2.4 | $18.8^{a}$ | 16.1 | 21.8 |
| | **1130** | 587 | 180 | 7.7 | 0.4 | $3.0^{b}$ | 2.6 | 3.5 | $2.1^{a,b}$ | 1.6 | 2.9 | $26.6^{a,b}$ | 17.8 | 38.7 |
| | **2177** | 639 | 188 | 7.7 | 0.4 | $4.1^{b}$ | 3.0 | 5.5 | $2.8^{b}$* | 2.5 | 3.1 | $34.4^{b}$* | 30.1 | 39.3 |
| Na | 590 | **597** | 182 | 7.8 | 0.4 | $2.2^{a}$ | 2.0 | 2.5 | $2.1^{a}$ | 1.9 | 2.4 | $18.8^{a}$ | 16.1 | 21.8 |
| | 594 | **1112** | 183 | 7.5 | 0.4 | $2.4^{a,b}$ | 1.8 | 3.3 | $1.6^{b}$ | 1.4 | 2.0 | $30.2^{b}$ | 22.8 | 39.3 |
| | 543 | **2166** | 156 | 7.6 | 0.4 | $3.2^{b}$ | 3.0 | 3.4 | $2.9^{c}$ | 2.8 | 3.0 | $48.7^{c}$ | 46.7 | 50.8 |
| Mg | 590 | 597 | **182** | 7.8 | 0.4 | $2.2^{a}$ | 2.0 | 2.5 | $2.1^{a}$ | 1.9 | 2.4 | $18.8^{a}$ | 16.1 | 21.8 |
| | 519 | 606 | **341** | 7.4 | 0.4 | $2.2^{a}$ | 1.7 | 2.8 | $1.9^{a}$ | 1.5 | 2.4 | $47^{b}$ | 33.7 | 64.2 |
| | 570 | 577 | **653** | 7.7 | 0.4 | $3.6^{b}$ | 2.7 | 4.8 | $1.8^{a}$ | 1.5 | 2.1 | $20.4^{a}$ | 16.4 | 25.2 |
| pH | 542 | 584 | 179 | **$6.5^{m}$** | 0.4 | $12.4^{a}$ | 8.8 | 17.5 | $12.5^{a}$ | 8.8 | 17.5 | $2845^{a}$ | 1970 | 4085 |
| | 629 | 620 | 187 | **$6.8^{m}$** | 0.4 | $7.9^{a}$ | 5.4 | 11.5 | $7.9^{a}$ | 5.3 | 11.6 | $976^{b}$ | 635 | 1483 |
| | 590 | 597 | 182 | **7.8** | 0.4 | $2.2^{b}$ | 2.0 | 2.5 | $2.1^{b}$ | 1.9 | 2.4 | $18.8^{c}$ | 16.1 | 21.8 |
| | 652 | 594 | 186 | **$7.8^{m}$** | 0.4 | $1.5^{c}$ | 1.3 | 1.8 | $1.4^{c}$ | 1.2 | 1.6 | $10.7^{d}$ | 8.5 | 13.3 |
| | 598 | 589 | 180 | **$8.0^{m}$** | 0.4 | $1.6^{c}$ | 1.3 | 2.0 | $1.4^{c}$ | 1.1 | 1.7 | $5.9^{e}$ | 4.3 | 7.9 |
| DOC | 590 | 597 | 182 | 7.8 | **0.4** | $2.2^{a}$ | 2.0 | 2.5 | $2.1^{a}$ | 1.9 | 2.4 | $18.8^{a}$ | 16.1 | 21.8 |
| | 554 | 575 | 157 | 7.5 | **9.3** | $8.8^{b}$ | 7.7 | 10.1 | $4.2^{b}$ | 4.0 | 4.3 | $0.09^{b}$ | 0.1 | 0.1 |
| | 552 | 577 | 174 | 7.5 | **13.0** | $8.1^{b}$ | 7.1 | 9.3 | $6.9^{c}$ | 6.2 | 7.7 | $0.15^{c}$ | 0.1 | 0.2 |

Table 2. Linear regression coefficients calculated for the effect of $Ca^{2+}$, $Na^{+}$, $Mg^{2+}$ and $H^{+}$ on acute $Dy^{3+}$ toxicity. The units of slope and intercept are on a molar basis.

| Relationship | Slope | Intercept | r | Derived Log K |
|---|---|---|---|---|
| $Dy^{3+}$ to $Ca^{2+}$ | 0.01454 | 1.3 x$10^{-8}$ | 0.998 | 3.95 |
| $Dy^{3+}$ to $Na^{+}$ | 0.02037 | 8.0 x$10^{-9}$ | 0.998 | 4.10 |
| $Dy^{3+}$ to $Mg^{2+}$ | -0.01064 | 3.2 x$10^{-8}$ | 0.110 | NA |
| $Dy^{3+}$ to $H^{+}$ | 9.07 | -1.7 x$10^{-7}$ | 0.990 | NA |
| $Dy^{3+}$ to BL | - | - | - | 7.75* |

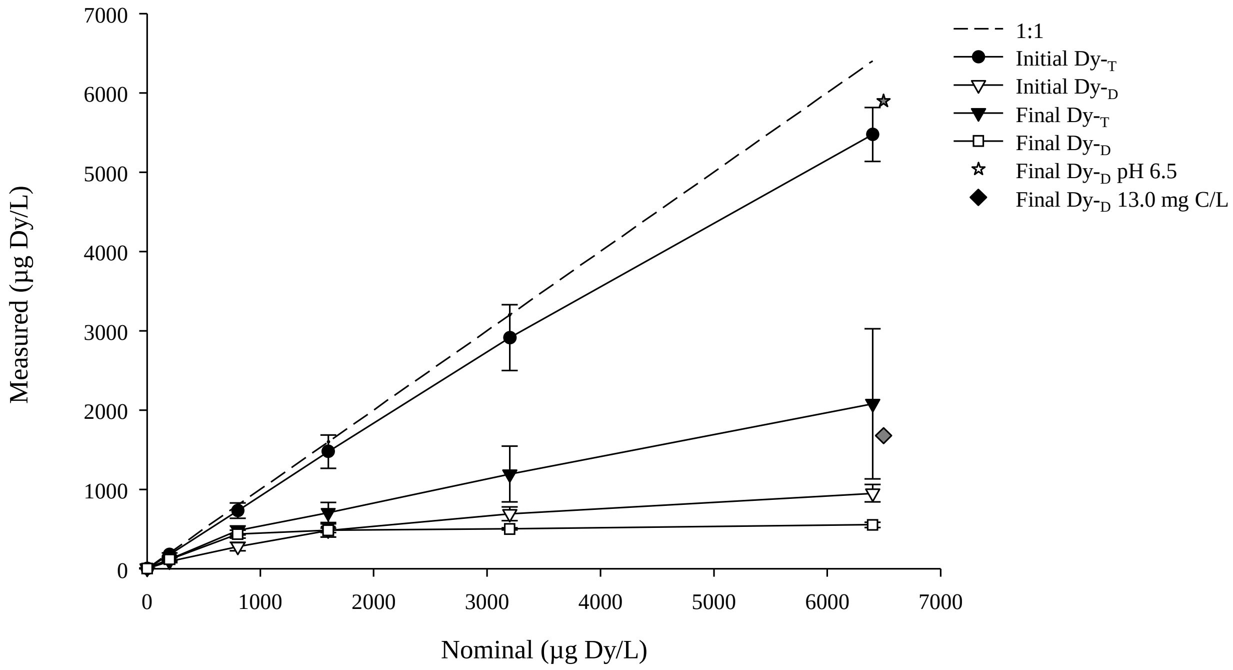

Figure 1. Comparison of nominal and measured Dy measurements for total (Dy-$_T$) and dissolved (Dy-$_D$) concentrations from the initial and final time periods. Tests involving cationic competition are summarized and exclude data with pH and DOM alterations except for 6400 µg/L data at low pH (grey star) and high DOM (grey diamond) which have been offset to the right. Error bars are represented as ±SEM. The number of observations in this subset of tests varies n= 7 to 10.

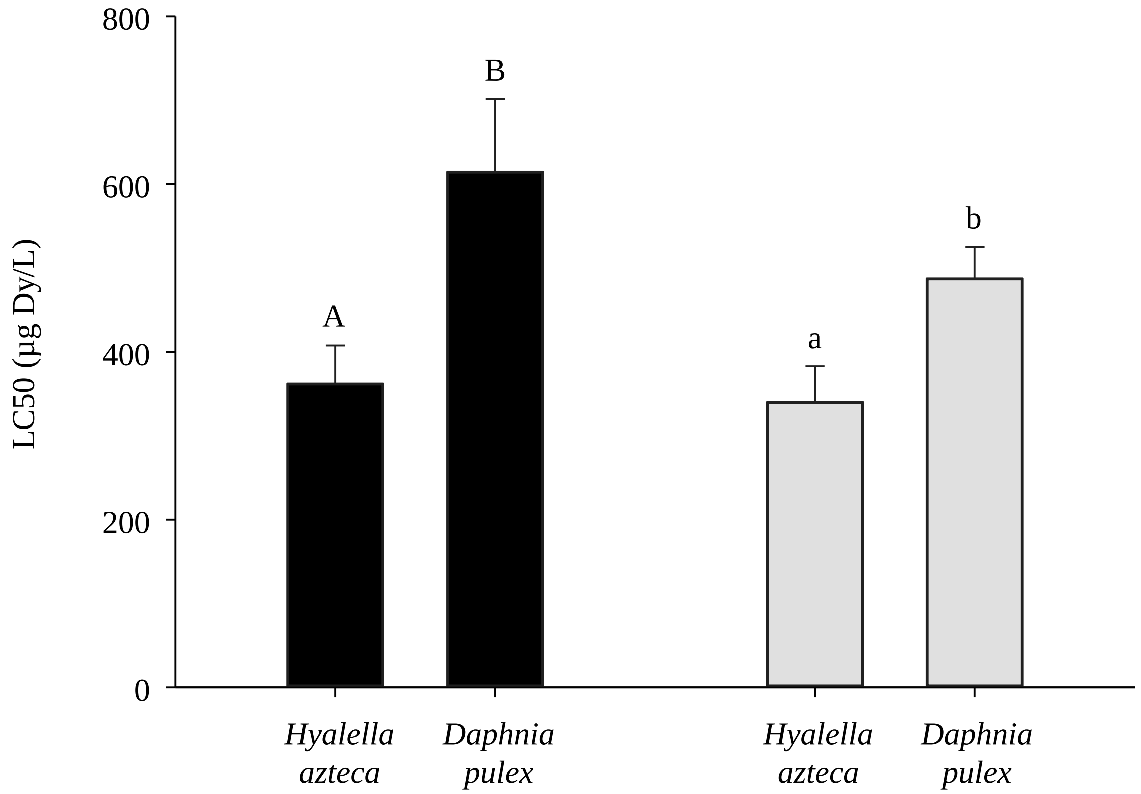

Figure 2. LC50 values (with upper 95% confidence interval) for *Hyalella azteca* and *Daphnia pulex* exposure to Dy. The LC50 values are based on 48 h (*Daphnia*) and 96 h (*Hyalella*) tests and measured total (Dy-$_T$ black bars) and dissolved (Dy-$_D$ gray bars) concentrations. Different letters indicate LC50 values that are statistically different for either Dy-$_T$ or Dy-$_D$.

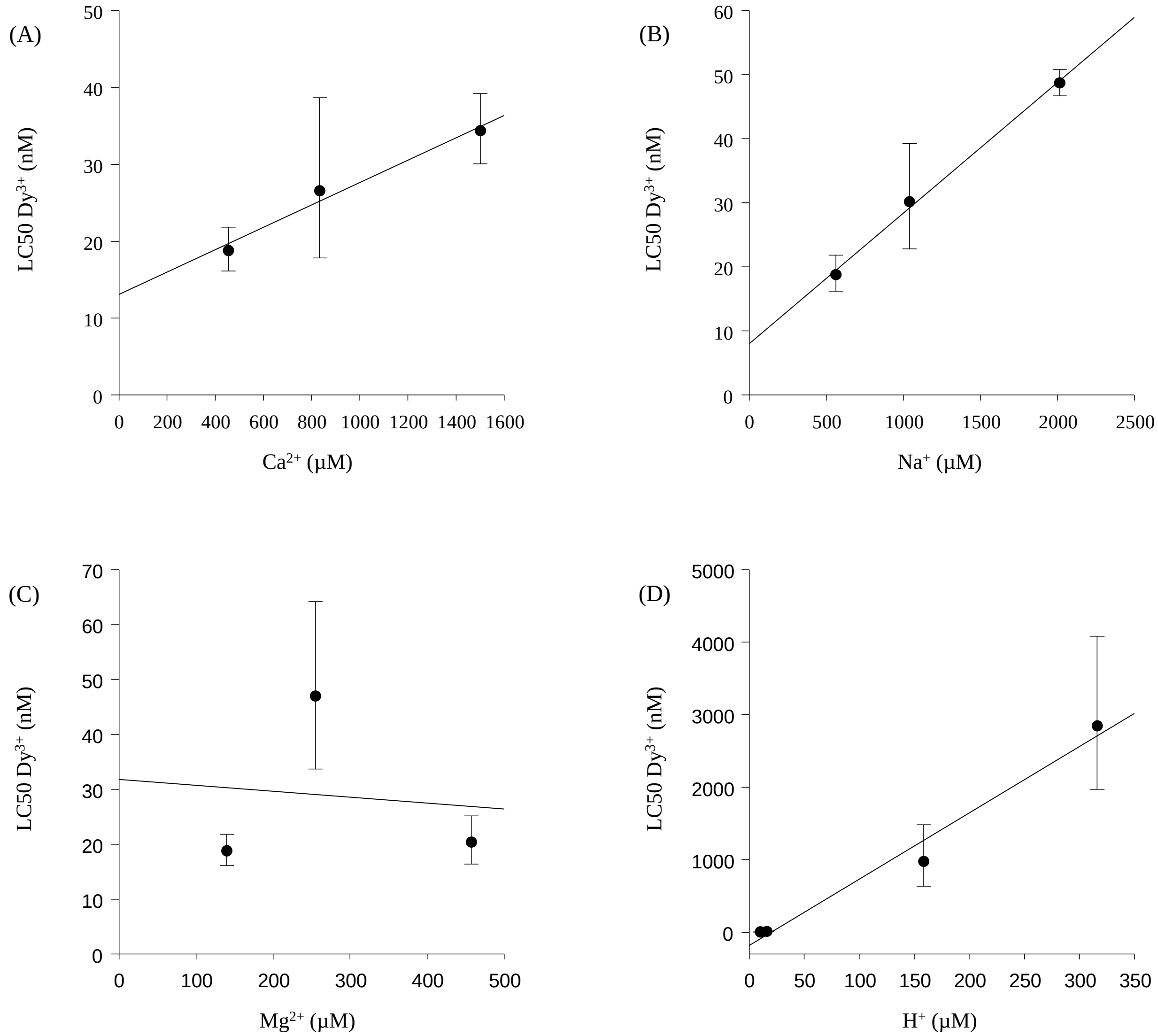

Figure 3. LC50 values (with 95% confidence interval) and the competitive effect of $Ca^{2+}$ (A), $Na^{+}$ (B), $Mg^{2+}$ (C) and $H^{+}$ (D) on $Dy^{3+}$ to *Hyalella azteca*. Linear regression line is shown and ion activities were calculated from measured dissolved 96 h concentrations and modelled using WHAM 7 (ver. 7.02). See Table 3 for linear regression coefficients and associated Log K values.

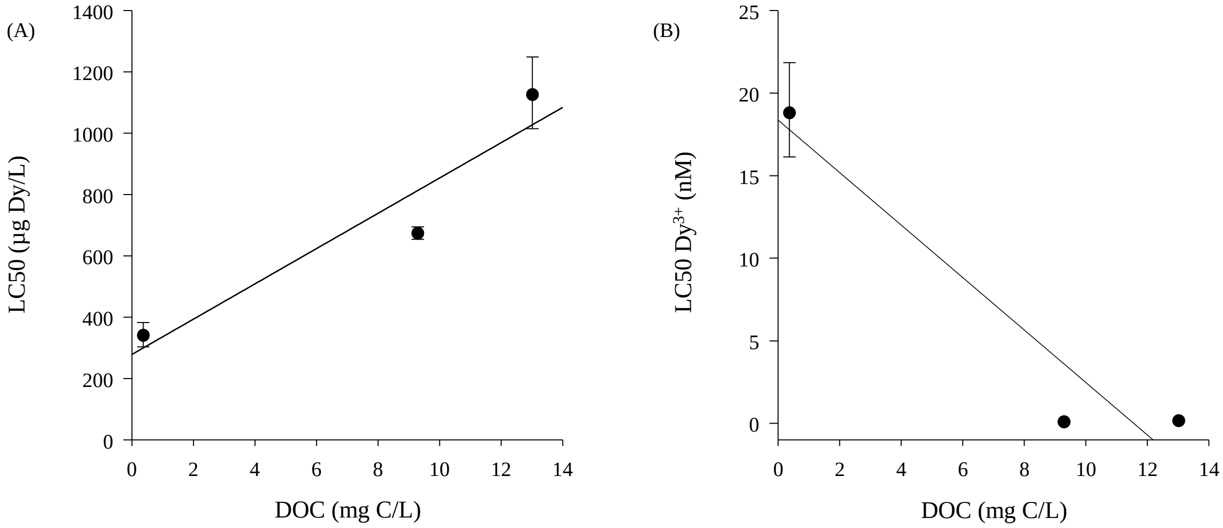

Figure 4. LC50 values (with 95% confidence intervals) and the protective effect of natural organic matter on Dy toxicity to *H. azteca*. Both panels are from 96 h measured dissolved and (A) shows regression as a function of Dy toxicity while (B) as a function of $Dy^{3+}$ activity. Single source of NOM was used from Suwannee River (SR-NOM). Regression panel (A) LC50 $Dy\text{-}_D$ = 57.58 (SR-DOM) + 278 (r = 0.949) and panel (B) LC50 $Dy^{3+}$ = -1.589 (SR-DOM) + 18.36 (r = 0.957).

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## 3.8 Supplementary data

Table S1. Detailed exposure chemistry with mortalities for successful *H. azteca* tests. Measured values at the end of each exposure are given (all in µM except for pH, DOC (mg C/L), and nominal, Dy-$_T$ (T) and Dy-$_D$ (D) which are in µg Dy/L) and pH and mortality are averages of two replicates per concentration.

| Test Type: | Final | | | | | Initial | | | Final | | Initial | Final | Final/Initial | % Mortality |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ca | Mg | Na | pH | DOC | Nominal | T | D | T | D | D/T % | D/T % | D % | |
| RAM trial 1 | 558 | 156 | 543 | 7.8 | 0.4 | 0 | 4.7 | 10.4 | 14.1 | 0.2 | NA | NA | NA | 5 |
| | 599 | 210 | 777 | 7.7 | 0.4 | 200 | 94.4 | 35.7 | 51.4 | 42.5 | 37.8 | 82.7 | 118.9 | 20 |
| | 593 | 184 | 599 | 7.8 | 0.4 | 800 | 246 | 111 | 152 | 149 | 45.2 | 98.0 | 134.1 | 5 |
| | 607 | 190 | 596 | 7.7 | 0.4 | 1600 | 418 | 158 | 286 | 289 | 37.8 | 101 | 183.3 | 65 |
| | 551 | 183 | 572 | 7.7 | 0.4 | 3200 | 756 | 278 | 486 | 450 | 36.7 | 92.7 | 162.1 | 100 |
| | 548 | 178 | 573 | 7.8 | 0.4 | 6400 | 3517 | 498 | 509 | 453 | 14.2 | 88.9 | 90.9 | 100 |
| RAM trial 2 | 566 | 153 | 540 | 8.1 | 0.4 | 0 | 1.0 | 3.4 | 7.5 | 0.0 | NA | NA | NA | 5 |
| | 571 | 176 | 564 | 7.9 | 0.4 | 200 | 303 | 155 | 229 | 225 | 51.1 | 98.2 | 145.4 | 15 |
| | 629 | 188 | 599 | 7.8 | 0.4 | 800 | 1257 | 236 | 533 | 497 | 18.8 | 93.2 | 210.4 | 48 |
| | 633 | 190 | 590 | 7.8 | 0.4 | 1600 | 2626 | 356 | 513 | 468 | 13.6 | 91.2 | 131.3 | 67 |
| | 632 | 195 | 613 | 7.8 | 0.4 | 3200 | 5112 | 500 | 679 | 474 | 9.8 | 69.9 | 94.9 | 95 |
| 1mM CaCl$_2$ | 1201 | 171 | 610 | 7.7 | 0.4 | 0+Ca | 0.7 | 5.8 | 16.0 | 0.8 | NA | NA | NA | 15 |
| | 1184 | 184 | 612 | 7.7 | 0.4 | 200 | 152 | 177 | 135 | 132 | 116 | 98.2 | 74.6 | 5 |
| | 1106 | 183 | 576 | 7.7 | 0.4 | 800 | 645 | 602 | 489 | 509 | 93.2 | 104 | 84.6 | 33 |
| | 1114 | 180 | 572 | 7.7 | 0.4 | 1600 | 1307 | 955 | 544 | 513 | 73.1 | 94.3 | 53.7 | 60 |
| | 1114 | 185 | 580 | 7.7 | 0.4 | 3200 | 2627 | 1140 | 720 | 479 | 43.4 | 66.5 | 42.0 | 90 |
| | 1061 | 175 | 574 | 7.7 | 0.4 | 6400 | 5318 | 1215 | 611 | 482 | 22.9 | 79.0 | 39.7 | 100 |
| 2mM CaCl$_2$ | 2133 | 173 | 616 | 7.8 | 0.4 | 0+Ca | 5.7 | 0.5 | 0.5 | 0.0 | NA | NA | NA | 0 |
| | 2126 | 181 | 607 | 7.7 | 0.4 | 200 | 190 | 84 | 168 | 166 | 44.2 | 98.9 | 197.9 | 0 |
| | 2313 | 210 | 685 | 7.7 | 0.4 | 800 | 751 | 253 | 625 | 610 | 33.7 | 97.5 | 240.9 | 14 |
| | 2260 | 203 | 674 | 7.8 | 0.4 | 1600 | 1531 | 484 | 689 | 567 | 31.6 | 82.3 | 117.3 | 25 |
| | 2150 | 186 | 629 | 7.7 | 0.4 | 3200 | 3093 | 798 | 796 | 516 | 25.8 | 64.8 | 64.6 | 60 |
| | 2082 | 172 | 623 | 7.7 | 0.4 | 6400 | 6057 | 1089 | 571 | 529 | 18.0 | 92.6 | 48.5 | 100 |
| 0.25mM MgSO$_4$ | 497 | 150 | 591 | 7.3 | 0.4 | 0 | 0.4 | 0.8 | 0.3 | 0.4 | NA | NA | NA | 15 |
| | 513 | 317 | 590 | 7.4 | 0.4 | 0+Mg | 0.9 | 0.4 | 0.3 | 0.3 | NA | NA | NA | 20 |
| | 519 | 333 | 596 | 7.4 | 0.4 | 200 | 166 | 40.9 | 66.7 | 58.8 | 24.6 | 88.1 | 143.8 | 10 |
| | 573 | 372 | 628 | 7.4 | 0.4 | 800 | 739 | 198 | 461 | 421 | 26.8 | 91.2 | 212.3 | 50 |
| | 542 | 352 | 608 | 7.4 | 0.4 | 1600 | 1450 | 361 | 602 | 515 | 24.9 | 85.5 | 142.6 | 80 |
| | 513 | 346 | 612 | 7.4 | 0.4 | 3200 | 2898 | 684 | 688 | 547 | 23.6 | 79.5 | 80.0 | 90 |
| | 457 | 326 | 602 | 7.3 | 0.4 | 6400 | 5595 | 1355 | 798 | 686 | 24.2 | 86.0 | 50.6 | 100 |
| 0.5mM MgSO$_4$ | 567 | 612 | 544 | 7.8 | 0.4 | 0+Mg | 1.2 | 1.1 | 16.1 | 14.1 | NA | NA | NA | 15 |
| | 575 | 642 | 574 | 7.7 | 0.4 | 200 | 178 | 72.5 | 128 | 130 | 40.7 | 102 | 179.3 | 10 |
| | 624 | 702 | 600 | 7.7 | 0.4 | 800 | 739 | 264 | 613 | 534 | 35.7 | 87.1 | 202.2 | 55 |
| | 595 | 675 | 583 | 7.7 | 0.4 | 1600 | 1556 | 412 | 1299 | 514 | 26.5 | 39.6 | 124.8 | 85 |
| | 548 | 660 | 582 | 7.7 | 0.4 | 3200 | 2934 | 617 | 2836 | 482 | 21.0 | 17.0 | 78.1 | 100 |
| | 514 | 624 | 579 | 7.7 | 0.4 | 6400 | 5798 | 773 | 5840 | 524 | 13.3 | 9.0 | 67.8 | 100 |
| 1mM NaCl | 608 | 166 | 1006 | 7.7 | 0.4 | 0+Na | 0.1 | 1.1 | 0.6 | 0.5 | NA | NA | NA | 10 |
| | 624 | 190 | 1239 | 7.6 | 0.4 | 200 | 171 | 75.7 | 118 | 116 | 44.2 | 98.8 | 153.6 | 15 |
| | 622 | 192 | 1282 | 7.6 | 0.4 | 800 | 737 | 187 | 586 | 371 | 25.4 | 63.3 | 198.5 | 70 |
| | 589 | 185 | 1066 | 7.6 | 0.4 | 1600 | 1494 | 499 | 1222 | 535 | 33.4 | 43.7 | 107.2 | 90 |
| | 578 | 183 | 1053 | 7.4 | 0.4 | 3200 | 3018 | 766 | 2761 | 557 | 25.4 | 20.2 | 72.8 | 100 |
| | 541 | 180 | 1024 | 7.4 | 0.4 | 6400 | 6042 | 834 | 5655 | 665 | 13.8 | 11.8 | 79.7 | 100 |
| 2mM NaCl | 517 | 138 | 602 | 7.5 | 0.4 | 0 | 3.5 | 6.9 | 6.8 | 2.7 | NA | NA | NA | 5 |
| | 566 | 141 | 2135 | 7.6 | 0.4 | 0+Na | 4.4 | 7.0 | 5.2 | 2.0 | NA | NA | NA | 5 |
| | 577 | 159 | 2180 | 7.6 | 0.4 | 200 | 171 | 97.4 | 74.8 | 67.2 | 57.0 | 89.7 | 68.9 | 10 |
| | 577 | 165 | 2177 | 7.6 | 0.4 | 800 | 733 | 376 | 401 | 392 | 51.3 | 98.0 | 104.4 | 10 |
| | 553 | 163 | 2161 | 7.6 | 0.4 | 1600 | 1430 | 632 | 508 | 468 | 44.2 | 92.2 | 74.1 | 40 |
| | 511 | 156 | 2168 | 7.6 | 0.4 | 3200 | 2862 | 760 | 572 | 503 | 26.6 | 87.9 | 66.1 | 65 |
| | 473 | 150 | 2177 | 7.6 | 0.4 | 6400 | 5988 | 891 | 566 | 529 | 14.9 | 93.5 | 59.4 | 100 |

Table S1. Continued.

| Test Type: | Final |  |  |  |  | Initial |  |  | Final |  | Initial | Final | Final/Initial | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | Ca | Mg | Na | pH | DOC | Nominal | T | D | T | D | D/T % | D/T % | D % | Mortality |
| 8mg C/L DOM | 553 | 142 | 577 | 7.5 | 10.5 | 0+DOM | 0.0 | 4.2 | 0.6 | 2.5 | NA | NA | NA | 0 |
|  | 566 | 161 | 572 | 7.6 | 9.8 | 800 | 728 | 529 | 515 | 511 | 72.7 | 99.2 | 96.5 | 5 |
|  | 604 | 162 | 577 | 7.6 | 9.4 | 1600 | 1516 | 696 | 951 | 616 | 45.9 | 64.8 | 88.5 | 5 |
|  | 572 | 163 | 576 | 7.5 | 9.6 | 3200 | 3042 | 1070 | 1601 | 690 | 35.2 | 43.1 | 64.5 | 70 |
|  | 522 | 158 | 587 | 7.5 | 8.4 | 6400 | 6005 | 1354 | 3164 | 806 | 22.5 | 25.5 | 59.5 | 100 |
|  | 506 | 155 | 562 | 7.4 | 8.1 | 12800 | 12058 | 882 | 4610 | 803 | 7.3 | 17.4 | 91.0 | 100 |
| 15mg C/L DOM | 547 | 156 | 566 | 7.5 | 15.4 | 0+DOM | 0.1 | 1.4 | 1.4 | 0.5 | NA | NA | NA | 0 |
|  | 576 | 181 | 586 | 7.5 | 14.9 | 800 | 758 | 641 | 680 | 672 | 84.6 | 98.8 | 104.9 | 10 |
|  | 593 | 184 | 584 | 7.5 | 14.0 | 1600 | 1543 | 1242 | 1093 | 1032 | 80.5 | 94.4 | 83.1 | 30 |
|  | 579 | 181 | 576 | 7.6 | 13.0 | 3200 | 3033 | 2262 | 1594 | 1183 | 74.6 | 74.2 | 52.3 | 55 |
|  | 516 | 171 | 575 | 7.5 | 11.0 | 6400 | 6145 | 4676 | 1829 | 1678 | 76.1 | 91.7 | 35.9 | 100 |
|  | 501 | 173 | 576 | 7.4 | 9.7 | 12800 | 12389 | 7476 | 2498 | 2145 | 60.3 | 85.9 | 28.7 | 100 |
| pH 6.5 MOPS | 505 | 154 | 572 | 7.3 | 0.4 | 0 | 0.1 | 0.3 | 0.5 | 0.7 | NA | NA | NA | 5 |
|  | 488 | 159 | 572 | 6.5 | 0.4 | 0+MOPS | 0.0 | 0.0 | 13.9 | 11.3 | NA | NA | NA | 9 |
|  | 486 | 172 | 590 | 6.5 | 0.4 | 200 | 179 | 181 | 86.1 | 85.2 | 101 | 98.9 | 47.1 | 15 |
|  | 505 | 182 | 582 | 6.5 | 0.4 | 800 | 764 | 753 | 581 | 576 | 98.6 | 99.2 | 76.5 | 20 |
|  | 584 | 187 | 589 | 6.5 | 0.4 | 1600 | 1482 | 1525 | 1374 | 1402 | 103 | 102 | 92.0 | 45 |
|  | 611 | 188 | 596 | 6.5 | 0.4 | 3200 | 2959 | 2997 | 2913 | 2923 | 101 | 100 | 97.5 | 60 |
|  | 579 | 185 | 574 | 6.5 | 0.4 | 6400 | 6036 | 6024 | 6012 | 5897 | 99.8 | 98.1 | 97.9 | 90 |
| pH 6.8 MOPS | 586 | 155 | 606 | 7.0 | 0.4 | 0 | 0.3 | 1.7 | 1.6 | 1.5 | NA | NA | NA | 10 |
|  | 562 | 164 | 609 | 6.8 | 0.4 | 0+MOPS | 0.0 | 0.4 | 0.6 | 0.4 | NA | NA | NA | 0 |
|  | 556 | 170 | 618 | 6.8 | 0.4 | 200 | 172 | 168 | 73.4 | 68.2 | 97.4 | 92.9 | 40.7 | 0 |
|  | 604 | 198 | 632 | 6.8 | 0.4 | 800 | 754 | 728 | 406 | 401 | 96.6 | 98.9 | 55.1 | 25 |
|  | 675 | 210 | 603 | 6.8 | 0.4 | 1600 | 1484 | 1531 | 1095 | 1069 | 103 | 97.6 | 69.9 | 33 |
|  | 697 | 191 | 620 | 6.7 | 0.4 | 3200 | 2979 | 2909 | 2910 | 2986 | 97.6 | 103 | 102.6 | 62 |
|  | 680 | 193 | 639 | 6.7 | 0.4 | 6400 | 6142 | 6109 | 6230 | 6402 | 99.5 | 103 | 104.8 | 100 |
| pH 7.8 MOPS | 570 | 152 | 578 | 7.6 | 0.4 | 0 | 1.1 | 1.2 | 0.5 | 0.1 | NA | NA | NA | 40 |
|  | 569 | 155 | 569 | 7.8 | 0.4 | 0+MOPS | 2.3 | 0.5 | 0.2 | 0.0 | NA | NA | NA | 5 |
|  | 607 | 180 | 595 | 7.8 | 0.4 | 200 | 195 | 76.4 | 107 | 99.4 | 39.1 | 93.0 | 130.1 | 0 |
|  | 675 | 195 | 606 | 7.8 | 0.4 | 800 | 785 | 189 | 320 | 302 | 24.1 | 94.3 | 159.5 | 70 |
|  | 716 | 194 | 598 | 7.8 | 0.4 | 1600 | 1556 | 315 | 501 | 458 | 20.2 | 91.3 | 145.3 | 85 |
|  | 695 | 201 | 599 | 7.8 | 0.4 | 3200 | 3090 | 445 | 508 | 437 | 14.4 | 86.1 | 98.2 | 100 |
|  | 652 | 192 | 597 | 7.8 | 0.4 | 6400 | 6872 | 588 | 606 | 526 | 8.6 | 86.8 | 89.4 | 100 |
| pH 8.0 MOPS | 518 | 147 | 564 | 7.6 | 0.4 | 0 | 0.8 | 0.7 | 0.4 | 0.6 | NA | NA | NA | 5 |
|  | 549 | 166 | 600 | 8.0 | 0.4 | 0+MOPS | 0.7 | 0.2 | 0.2 | 0.5 | NA | NA | NA | 5 |
|  | 562 | 177 | 588 | 8.0 | 0.4 | 200 | 185 | 41.4 | 73.5 | 66.2 | 22.4 | 90.0 | 159.7 | 10 |
|  | 613 | 186 | 588 | 8.0 | 0.4 | 800 | 762 | 121 | 306 | 278 | 15.9 | 90.6 | 229.0 | 45 |
|  | 638 | 187 | 584 | 8.0 | 0.4 | 1600 | 1498 | 187 | 404 | 322 | 12.5 | 79.7 | 172.4 | 80 |
|  | 628 | 183 | 590 | 8.0 | 0.4 | 3200 | 3036 | 249 | 493 | 328 | 8.2 | 66.5 | 131.6 | 100 |
|  | 598 | 183 | 584 | 8.0 | 0.4 | 6400 | 6110 | 224 | 509 | 361 | 3.7 | 71.0 | 161.0 | 100 |

Table S2. Dysprosium speciation in *H. azteca* testing media given as the % of total aqueous Dy concentration (from 96 h dissolved measurements) and predicted by WHAM (ver. 7.0.2). Dysprosium bound to colloidal Humic and Fulvic acids are represented as HA-Dy and FA-Dy, respectively. The $^{m}$ subscript indicates tests which used MOPS as pH buffer.

| Test Type: | Conc. | pH | % Dy | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $Dy^{3+}$ | $DyCO_3^{+}$ | $Dy(CO_3)_2^{-}$ | $DyHCO_3^{2+}$ | $DySO_4^{+}$ | $DyOH^{2+}$ | $Dy(SO_4)_2^{-}$ | HA - Dy | FA - Dy |
| Ca | 0.5mM | 7.8 | 1.5 | 52.1 | 17.5 | 0.2 | 0.5 | 1.6 | 0.001 | 2.1 | 24.5 |
| | 1.0mM | 7.7 | 2.3 | 55.7 | 14.3 | 0.3 | 0.6 | 1.8 | 0.001 | 2.0 | 23.0 |
| | 2.0mM | 7.7 | 2.6 | 59.7 | 16.2 | 0.3 | 0.6 | 2.0 | 0.001 | 1.5 | 17.2 |
| Na | 0.5mM | 7.8 | 1.5 | 52.1 | 17.5 | 0.2 | 0.5 | 1.6 | 0.001 | 2.1 | 24.5 |
| | 1.0mM | 7.5 | 3.1 | 52.4 | 8.6 | 0.4 | 1.0 | 1.7 | 0.002 | 2.6 | 30.2 |
| | 2.0mM | 7.6 | 3.0 | 60.7 | 12.6 | 0.3 | 0.8 | 1.9 | 0.001 | 1.6 | 19.0 |
| Mg | 0.125mM | 7.8 | 1.5 | 52.1 | 17.5 | 0.2 | 0.5 | 1.6 | 0.001 | 2.1 | 24.5 |
| | 0.25mM | 7.4 | 4.1 | 54.9 | 7.1 | 0.5 | 2.4 | 1.7 | 0.009 | 2.3 | 26.8 |
| | 0.5mM | 7.7 | 2.1 | 51.0 | 12.9 | 0.2 | 2.1 | 1.7 | 0.013 | 2.4 | 27.6 |
| DOC | 0.4mg /L | 7.8 | 1.5 | 52.1 | 17.5 | 0.2 | 0.5 | 1.6 | 0.001 | 2.1 | 24.5 |
| | 9.3mg /L | 7.5 | 0.003 | 0.1 | 0.009 | 0.000 | 0.001 | 0.002 | 0.000 | 7.2 | 92.7 |
| | 13.0mg /L | 7.5 | 0.004 | 0.1 | 0.010 | 0.000 | 0.001 | 0.002 | 0.000 | 7.6 | 92.3 |
| pH | pH 7.8 | 7.8 | 1.5 | 52.1 | 17.5 | 0.2 | 0.5 | 1.6 | 0.001 | 2.1 | 24.5 |
| | pH 7.8$^{M}$ | 7.8 | 1.3 | 45.0 | 15.1 | 0.2 | 0.4 | 1.4 | 0.001 | 2.9 | 33.7 |
| | pH 6.5$^{M}$ | 6.5 | 36.9 | 39.8 | 0.4 | 2.8 | 12.2 | 2.0 | 0.023 | 0.5 | 5.4 |
| | pH 6.8$^{M}$ | 6.8 | 20.6 | 57.8 | 1.6 | 2.0 | 6.9 | 2.2 | 0.014 | 0.7 | 8.2 |
| | pH 8.0$^{M}$ | 8.0 | 0.7 | 39.8 | 21.2 | 0.1 | 0.2 | 1.2 | 0.000 | 2.9 | 33.8 |

# CHAPTER 4

# 4.0 General discussion

## *4.1 Summary of major results*

The overall objective of this research was to contribute data towards the development of water quality guidelines for rare earth elements (REEs). Since REEs are divided into two groups, the light (LREEs) and heavy (HREEs) rare earths, cerium (Ce, a LREE) and dysprosium (Dy, a HREE) were chosen as representative of these metals. Acute Ce and Dy exposures were performed on *Hyalella azteca* and *Daphnia pulex* in media typical of Canadian Shield waters. Based upon these tests, the more sensitive organism was used to evaluate the toxicity modifying effect of water chemistry. The central findings of this research were as follows:

1) Both Ce and Dy precipitated in solution. Dissolved concentrations sampled at the end were always significantly lower than initial except for tests with added dissolved organic matter (DOM) and low pH (in the case of Dy, see Chapter 2 and 3, Figure 1)
2) Acute Ce and Dy exposures to *Daphnia* (24 h) and *Hyalella* (96 h) revealed the latter as the more sensitive organism in similar water chemistry conditions. Therefore, the effects of toxicity modifying factors (TMFs) on Ce and Dy toxicity were performed using *Hyalella* (see Chapter 2 and 3, Figure 2).
3) The effects of cationic competition are as follows: an increase in waterborne Ca was protective of Ce and Dy toxicity to *Hyalella* (see Chapter 2 and 3, Figure 3A). Similarly, an increase in Na was protective of Dy toxicity to *Hyalella* but no ameliorative effect was observed with Ce exposures (see Chapter 2 and 3, Figure 3B). Increasing the concentration of Mg had no effect on REE toxicity (see Chapter 2 and 3, Figure 3C). Lastly, decreasing the pH was strongly protective of Dy toxicity to *Hyalella* (see Chapter 3, Figure 3D).

4) Dissolved organic matter increase was protective of Ce and Dy toxicity to *Hyalella*. Expressing Dy toxicity on the basis of the free metal ion did not follow BLM principles (see Chapter 2 and 3, Figure 4).
5) Comparing Dy exposures to *Hyalella* with and without 3-(N-morpholino)propanesulfonic acid (MOPS) indicated that the buffer increases metal toxicity (see Chapter 3).
6) Both Ce and Dy follow BLM principles. The Log K values quantifying the binding of Ce to the biotic ligand (BL, Ce-$_{BL}$) and similarly Dy (Dy-$_{BL}$) were determined to be 7.3 and 7.75, respectively. The binding constants for Ca binding to the BL are 3.9 and 3.95 for Ce and Dy, respectively. The Log K for Na binding to the biotic ligand was determined in Dy exposures with the value of 4.10 (see Chapter 2 and 3, Table 2).

### *4.2 Discussion of major results*

The corresponding discussion relates to previously outlined summary of major results and includes:

1) In this study, both REEs precipitated during acute exposures and concentrations sampled at the end were much lower in comparison to initial or nominal. Precipitation in REEs tests has been observed previously (Borgmann et al., 2005; Barry and Meehan, 2000) and testing based on nominal values has been shown to underestimate toxicity (Gonzalez et al., 2014). This study demonstrates that LC50s based on end dissolved concentrations provide the most conservative estimates of toxicity and recommends future LC50s to be based on final concentrations.

   Tests with low pH and high DOM demonstrate increased dissolved Ce and Dy concentrations. Results from low pH exposures were not surprising since most of the metal would be present as the free metal ion and because of higher solubility of this species the resulting metal concentrations are elevated. The observed increase in dissolved Ce and Dy concentration was surprising for tests with added DOM. One previous study with Ce nanoparticles (Ce-NP) speculated that DOM constituents (such as humic and fulvic acids) stabilize Ce-NP in natural waters through colloidal associations (Quik et al., 2012) and this could explain the elevated Ce and Dy concentrations observed in this study.

2) Acute toxicity tests with *Hyalella* (96 h) and *Daphnia* (48 h) were carried out following standard methods (Environment Canada, 2013 and 1996, respectively). *Hyalella* was determined to be more sensitive to Ce and Dy exposures and as a result further tests with TMFs were performed using this organism. *H. azteca* heightened sensitivity may have been associated with the longer duration of the standard test (96 h for *Hyalella* vs. 48 h for *Daphnia* spp.) in addition to inherent sensitivity difference. This study demonstrates sensitivity differences exist between commonly used invertebrates and recommends future species comparisons to have equivalent exposure length.
3) The effect of cationic competition was evident but varied with the type of REE exposed to *Hyalella*. Increase in waterborne Ca had a protective effect on *Hyalella* survival for both REEs. Geochemical modeling of the same data suggests cationic competition between $Ca^{2+}$ and the REE free metal ion. The observed protective effect of Ca on Ce or Dy toxicity to *Hyalella* was expected since lanthanides and Ca share similar physiochemical properties. For instance, lanthanides have comparable ionic radii to $Ca^{2+}$ (Hirano and Suzuki, 1996) and as a result are effective Ca channel blockers (Sandvig and Olsnes, 1982; Lansman, 1990). Recently, lanthanides have been shown to replace Ca in

nutrient deficient tests with alga indicating a shared biological function (Goecke et al., 2015). The protective effects observed in this study are supported with previous REE toxicity investigations (Barry and Meehan, 2000; EL-Akl et al., 2015) which highlight Ca involvement in decreasing toxicity.

The protective effect of Na on Ce or Dy toxicity to *Hyalella* varied with the type of REE. For instance, increasing Na was not protective in exposures with Ce but Dy tests demonstrated clear ameliorating effects. Both of these metals were exposed to the same source of *Hyalella azteca,* but testing with these REEs was performed at two different water chemistries. For instance, exposures with Ce were performed in low hardness while Dy in high hardness conditions and this might have contributed to the observed difference in Na protective effect. Recently, Senatore et al. (2014) have identified unique T-type Ca channels in *Lymnaea stagnalis* that are permeable to Na as well as Ca instead of being highly selective for Ca as observed in vertebrate organisms. If these types of channels are present and have a role in Ca and Na ion regulation in *Hyalella* then increases in either of the ions would explain the decrease in Dy toxicity through cationic competition. It is possible that more of these T-type Ca channels are available at higher hardness (such as Dy exposures) and less available at lower hardness (such as Ce tests) conditions. This notion is highly speculative but future identification of this transporter might offer some explanation to the difference in toxicity between these REEs. Additionally, performing future REE exposures to *Hyalella* using similar water chemistry would explain if Na protective effects were exclusive to Dy exposures and offer insight into whether or not REEs have the same mode of toxicity to this organism.

In both REE exposures to *Hyalella* increasing Mg had no protective effect. Previous studies with metals such as Cd, Zn, Cu, Co and Pb have identified that adverse effects from acute metal exposures result from inhibition of major ionoregulatory transporters. For instance $Cd^{2+}$, $Co^{2+}$, $Pb^{2+}$ and $Zn^{2+}$ affect the Ca transporter and block $Ca^{2+}$ uptake while $Cu^{2+}$ and $Ag^{+}$ inhibit the Na transporter and interfere with the uptake of $Na^{+}$ and $Cl^{-}$ across the fish gill (Niyogi and Wood, 2004). The most probable explanation to the lack of Mg effect on Ce and Dy toxicity is that these REEs do not act on the Mg transporter.

The effect of pH change on REE toxicity was evaluated with Dy exposures to *Hyalella*. Decreases in pH dramatically lowered Dy toxicity to *H.azteca*. These results were unexpected since lower pH would increase the most bioavailable metal form (e.g. $Dy^{3+}$) and thus increase toxicity (Campbell, 1995). The linear relationship between $H^{+}$ and $Dy^{3+}$ LC50 suggests strong competitive effect between the proton and the free metal ion as pH decreases. Decreased toxicity at lower pH has been reported previously with other metals (Campbell and Stokes, 1985) and recent work with Ce exposures to algae (EL-Akl et al., 2015) support the competitive effect of $H^{+}$ observed in this study. At higher pH, toxicity might be related to the presence of carbonate bound complexes. Future investigations with different REEs should evaluate pH influence on toxicity.

4) Increased DOM concentration was protective of Ce and Dy toxicity to *Hyalella*. Dissolved organic matter has been identified as one of the primary factors ameliorating metal toxicity (Wood et al., 2011) and since REEs are known to bind organic molecules (Tang and Johannesson, 2003; Johannesson et al., 2004) the decrease in Ce and Dy toxicity was not surprising. Previous investigations with Ce (EL-Akl et al., 2015) and Dy

(Fuma et al., 2005) support the ameliorative effects observed in this study with organic matter.

Interestingly, DOM trials with Dy did not follow BLM principles. In this study, the hypothesis was that the LC50 values (based on dissolved Dy) when expressed as $Dy^{3+}$ would be similar across the DOM trials (Santore et al., 2001). Instead, dramatic reductions in $Dy^{3+}$ LC50 values were predicted by WHAM software as DOM increased. The negative relationship between DOM concentrations and the LC50 for $Dy^{3+}$ can be explained by overestimation of DOM-$Dy^{3+}$ complexation and/or that DOM-Dy complexes are bioavailable and contribute to toxicity. The latter idea probably does not apply since increased DOM decreased Dy LC50s (see Chapter 3, Figure 4A) and thus the stability constants for Dy binding to DOM are likely defined poorly in WHAM and require refinement. This pattern was only observed for Dy data and the results with Ce were not affected.

5) The effect of MOPS buffer on REE toxicity was evaluated in Dy exposures. Comparison of side-by-side tests with and without MOPS showed that the buffer increased Dy toxicity to *Hyalella*. Previous investigation with Pb exposure to fathead minnow *Pimephales promelas* suggested that the buffer has a physiological effect and that it enhanced Pb toxicity (Esbaugh et al., 2013). Results in this study agree with Esbaugh et al. (2013) and suggest caution when interpreting results from tests that utilized this buffer. Moreover, MOPS should not be used as a pH buffer in future toxicity studies with REEs and *Hyalella*.
6) This study demonstrates that increased Ca and DOM concentration decrease acute toxicity to both REEs and that increase Na and $H^+$ are protective of Dy toxicity to

*Hyalella*. Previous investigation has concluded that some REEs, such as Lu, also follow BLM principles (Weltje et al., 2004) and this is also the case with Ce and Dy in this study. The equilibrium binding constant for Ce-$_{BL}$ (Log K = 7.3) and Dy-$_{BL}$ (Log K = 7.75) closely resembles the values obtained from other REEs such as Ce-$_{BL}$ (7.3), Sm-$_{BL}$ (7.0), Eu-$_{BL}$ (7.0) and Tm-$_{BL}$ (7.1, Yang et al., 2014; El-Akl et al., 2015) and our calculation for Ca binding to the BL (Ce tests = 3.9 and Dy tests = 3.95) correlates well to the value of 4.3 obtained by El-Akl et al. (2015) in their work with Ce. This is the first study where equilibrium binding constants for Ce and Dy to *Hyalella azteca* have been calculated and the data derived in this investigation can be applied for future development of the freshwater BLM for these metals.

### *4.3 Study limitations*

The main limitations of this study were: 1) the uncertainty in predicting REE speciation using WHAM7 geochemical software and 2) the lack of investigating how precipitated phases effect REE toxicity estimates.

1) The use of WHAM7 was limiting since Dy binding to DOM seemed to be poorly defined (as discussed above) and speciation software did not take into account the effect of redox chemistry of some REEs (e.g. Ce). The predicted Dy-DOM interactions were overestimated and this could be due to the notion that they were based on one published paper (Tipping et al., 2011) using antiquated thermodynamic values. Future investigations should look into refining REE-DOM binding parameters and the data generated in this study should be updated accordingly. Moreover, WHAM7 modeling software did not take into account the complex redox chemistry

of some REEs, such as Ce, nor did it take into account the formation of precipitated forms of both REEs. This was an oversight in the speciation program since precipitation was clearly demonstrated in Ce and Dy exposures and for the former the redox chemistry most likely amplified the precipitation process. Future modeling software needs to incorporate precipitation of these metals in order to predict the bioavailable REE species with greater certainty.

2) The development of BLM binding parameters (Log K values) in this study were based on dissolved metal concentrations. However, the use of total (unfiltered) concentrations should also be investigated, since precipitated REE forms might also contribute to toxicity. Recent development of water quality guidelines for aluminum (Al) in coastal marine waters of Australia indicated that toxicity is influenced by both dissolved and precipitated phases (Golding et al., 2015). Since $Al^{3+}$ and $REE^{3+}$ share similar charge and both metals precipitate, it is possible that the precipitated forms of REEs have comparable effects on toxicity. Future development of BLM binding parameters for REEs should investigate if measurements from total (unfiltered) concentrations generate improved predictions of toxicity.

### *4.4 Conclusion and significance*

Rare earth elements are a group of metals that are used in a variety of industries and thus are of vital importance to modern society. The demand for these elements is expected to increase and as a result new mining projects are being developed. In Canada, the most advanced REE mining project is located in the Northwest Territories and is owned by Avalon Rare Metals Inc. With the increase in REE mining development, there is a need to set safe discharge limits that are protective of aquatic organisms. Currently, there are no Canadian Council of Ministers of the

Environment water quality guidelines and this is also true in the United States where there are no water quality criteria set by the Environmental Protection Agency. This study evaluates the effect of water chemistry on the acute toxicity of Ce (a light REE) and Dy (a heavy REE) to *Hyalella azteca*. For both metals, Ca and DOM had a protective effect while Mg was not protective. Increase in Na was not protective of Ce toxicity but in the case of Dy was strongly protective. This might be related to the difference in the mode of metal toxicity between the light and heavy rare earths or the difference in exposure chemistries (ie *Hyalella* was acclimated in harder water for Dy tests). Tests involving pH change and Dy exposures have revealed that $H^+$ is strongly protective particularly at low pH. Although, change in pH has been shown to be protective in Dy exposures, caution should be taken with the interpretation of pH tests since the buffer used (e.g. MOPS) has been demonstrated to enhance Dy toxicity in this study. Further tests with different REEs need to be performed in conditions of changing pH to adequately understand if the protective effects observed in this study are characteristic of this metal group. Ultimately, Ce and Dy exposures to *Hyalella* are likely to cause concern in waters characteristic of having low Ca and DOC content.

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