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Do Toxicity Modifying Factors Influence Acute or
Chronic Toxicity of Thulium to *Hyalella azteca*?

by

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Honours BSc Biology, University of Guelph, 2012

THESIS

Submitted to the Department of Biology

Faculty of Science

in partial fulfillment of the requirements for

Master of Science in Integrative Biology

Wilfrid Laurier University

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Abstract

The industrial demand for rare earth elements (REEs) is growing and as a result, environmental exposure is a concern. Very little is understood about the toxicity of REEs in aquatic environments. The objective of this research is to evaluate the acute and chronic toxicity of Tm and to also understand the toxicity modifying influence of dissolved organic matter (DOM) and cationic competition (Ca^{2+} , Mg^{2+} and Na^{+}). Furthermore, the aim of this study was to determine linkages between Tm bioaccumulation, growth and survival during chronic exposures. Standard methods (Environment Canada) were followed for both 96h acute and 14d chronic tests, in media with a hardness of 60 mg CaCO_3/L , a pH of 7.6 at 23°C. *Hyaella azteca* neonates (2-9 d for acute and 0-3 d old for chronic) were used and mortality (acute and chronic) as well as dry weight and accumulation of Tm for survivors were the endpoints. For acute tests, the potential protective effect of cationic competition was tested with Ca (0.25-1.50 mM), Na (0.25-1.55 mM) and Mg (0.06-0.38 mM). The effect of Luther Marsh and Kouchibouguac DOM complexation (at dissolved organic carbon (DOC) concentrations of 2, 7 and 12 mg C/L) were also evaluated. For chronic tests, the potential protective effect of competition was tested with Ca (0.25 – 1.5 mM) and Luther Marsh DOM (7 mg C/L). Surviving *Hyaella* were dried, weighed, tissues dissolved and measured for Tm accumulation. Dissolved Tm concentrations were lower than total (unfiltered) Tm concentrations indicating that precipitation occurred and this was particularly the case at higher concentrations. No protective effect was seen for Na or Mg in acute tests, nor was a protective effect for Ca observed in both acute and chronic tests. However, dissolved organic matter was protective (both of the sources) at dissolved organic carbon (DOC) concentrations of 7 and 12 mg DOC/L for acute tests as well as at 7 mg DOC/L in the chronic test.

Bioaccumulation at 14 d of exposure was also shown to be reduced at higher concentrations of Ca even though survival was not. This study contributes data towards the understanding of Tm toxicity in aquatic environments and REEs in general.

Acknowledgements

I would like to thank my supervisor Jim McGeer for his essential guidance, teachings and encouragement over the last two years. Your influence will forever be impacting in both my career and in my life. Thank you so much Jim for everything. Thank you to Scott Smith for your patience in helping me to fill the large chemistry gaps in my knowledge that I so clearly had. My respect for you is enormous and you as well, have taught me many things besides chemistry! A huge thank you to my lab mates Che Lu, Oliver Vukov, Prachi Deshpande, Wes Troung, Holly Gray, Tamzin Blewett and Jon Ford for laboratory help, writing help, daily laughs and some long conversations about science and life in general. Thank you to Kevin Stevens for being on my committee and your contributions to my thesis. Thank you to Jeff Fehrenbach and all my wonderful friends at home who have been there on the late nights, during times of doubt and who have never doubted me. Lastly and foremost, thank you to my Mum, Kim Loveridge who was the inspiration, the motivation and the driving force behind everything I have done but most all, for teaching me that education is everything.

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Chapter 1: General Introduction

1.1 Introduction

1.1.1 Rare Earth Elements and Related Environmental Concerns

Rare earth elements (REEs) are a group of metals classified as lanthanides on the periodic table due to their ability form trivalent cations (Humphries, 2013). Unlike the name, REEs are not rare. In fact, some REEs are as abundant in the earth's crust as metals such as copper or lead (Castor and Hedrick, 2006). The REEs are divided into two groups: light rare earth elements (LREE) and heavy rare earth elements (HREE). LREEs are listed 57 through to 63 (Sc, La, Ce, Pr, Nd, Pm, Sm, Eu, and Gd) while HREEs are 64-71 (Y, Tb, Dy, Ho, Er, Tm, Yb, and Lu), 17 elements in total (Humphries 2013). These elements are typically found occurring together in deposits of mineral such as bastnaesite and monzonite (Humphries 2013). In fact, over 90% of the economically recoverable REEs are found in such primary mineral deposits (Humphries, 2013).

Global production and demand for REEs has increased dramatically with predictions of increased production nearly tripling over the next 25 years (Humphries 2013). This rapid growth in demand can be attributed to an increased use of these metals for the automotive industry, as sustainable resources for the use of wind energy, electronics and as well as use in the biomedical field (Alonso et al., 2012). While geographically REE deposits are sufficient for global demands, many countries may be restricted environmentally and economically from mining and processing (Humphries, 2013). Therefore, for many decades China has been the world's largest producer of REEs, accounting for about 97% of the global production (Humphries 2013). However, Canada is home to some of the largest HREE deposits worldwide, potentially making Canada one of the largest REEs producers in the world (Humphries 2013).

Thor Lake, Northwest Territories is home to Canada's largest HREE deposits and plans for extraction of these metals has been underway since 2010 (Humphries 2013). While point source exposure from mining can lead to leaching of these metals into aquatic systems, it is mainly the diffuse source of exposure through production and disposal of such REE containing products that causes the concern for potential environmental effects to Canada's aquatic systems (Lemy, 2002).

While metals such as copper, have been extensively studied through standard toxicological testing and research, little is known about potential risk and toxicity of REEs. Research is required to develop a better understanding of the mechanisms of REE toxicity in aquatic systems. Understanding the behavior of REEs under various geochemical changes and their subsequent toxicity will allow for improved toxicity prediction modeling for development of environmental guidelines (Environment Canada, 2013). Since each metal is unique in its mechanism of toxicity, they are tested to obtain data that can provide a dose that causes mortality to 50% of the tested species population. This is called an LC₅₀ and it is used to help develop thresholds for criteria and guidelines for water quality and environmental regulations. Canadian Water Quality Guidelines have been developed for many metals to provide maximum (acute and chronic) concentrations in aquatic systems (Canadian Council of Ministers of the Environment, 2003). However, no such guidelines currently exist for any of the REEs.

1.1.2 Thulium Industrial Uses and Toxicity

Thulium (Tm) is a HREE and the second rarest of the REEs (Emsley, 2011). While rare, Tm is used in the medical industry for its unique physical properties (Dai et al., 2013). Tm based complexes have gained interest for the use of magnetic resonance imaging (MRI) to measure temperature and pH in vivo and in vitro for biomedical research (Dai et al., 2013). The medical

industry has also begun to use Tm fiber based lasers for laser surgeries (Zeitels et al., 2006). Results found that the Tm laser in microlaryngeal surgery was even more effective than the frequently used CO² laser (Zeitels et al., 2006). Additionally, due to its radioactive properties, Tm has been used in portable x-ray devices in the dental industry (Krishnamurthy and Gupta 2005).

Little is understood about Tm toxicity in aquatic environments. However, in a previous study Tm was evaluated to be highly toxic, with a reported LC₅₀ value of 0.01 µg/L (Borgmann et al., 2005). This study is perhaps the only research ever conducted to understand Tm aquatic toxicity. The LC₅₀ reported by Borgmann et al., (2005), is very low, allowing for an assumption that Tm is highly toxic. For that reason, research is required to fully understand Tm toxicity and its mechanism of action in aquatic environments.

1.1.3 Toxicity Modifying Factors (TMFs)

For most metals, toxicity results from the uptake of free metal ions into the organism. For acute toxicity the mechanism of impact is the inhibition of ion transport functions within the organism (Niyogi and Wood, 2004). Freshwater organisms are osmoregulators and require a homeostatic balance of essential ions for many physiological functions to survive (Evans, 1980). Essential ions and metal ions, when sharing the same charge, can use the same transport channels to enter the organism. Therefore, when accumulation of a metal takes place at the transport sites on the cell (site of action) it prevents the uptake of these essential ions and disrupts the homeostasis of the organism, thus leading to mortality (Evans, 1980).

Water chemistry is a major influence on the toxicity of the free metal ion. Some of the factors that can alter the toxicity of a metal are pH, cations (such as Ca²⁺, Na⁺ and Mg²⁺), dissolved

organic matter (DOM) and inorganic ligands (such as Cl^- and carbonates). These factors can be grouped into two general categories; ones that complex the metal free ions and reduce bioavailability and ones that compete for uptake at the site of toxicity. Complexation and competition can dramatically alter toxicity. For example, Cu LC50 values can vary from approximately 100 $\mu\text{g Cu/L}$ to 1000 $\mu\text{g Cu/L}$ depending on the amount and quality of DOC present or the level of pH and water hardness (Santore et al., 2001). To develop a water quality guideline for Tm, it is important to gain an understanding on the toxicity of Tm, under varying conditions in the environment. To do this, how these metals interact with the different toxicity modifying factors (TMFs) such as competition and complexation will need to be assessed. This increased understanding of Tm toxicity will be useful in developing accurate guidelines and criteria for REEs and for assessing risk at a particular site while accounting for changes in toxicity brought about by water chemistry.

DOM is a heterogeneous mixture of organic compounds and exists in all systems (Al-Reasi et al., 2013). As plants and dead organic matter slowly break down and decay in the water, DOM is formed (Al-Reasi et al., 2013). The composition and concentrations of DOM is variable between locations is dependent on differences between ecosystems and the types of plant biomass present. It is usually made up of humic substances (humic acid is made up of carboxyl and phenolate groups and fulvic acid) as well as carbohydrates, proteins, and amino acids (Al-Reasi et al., 2013). DOM is typically reported as dissolved organic carbon in mg C /L . The presence of DOM in freshwater ecosystems has previously been shown to provide a protective effect from metal toxicity (Di Toro et al., 2001). Ample research has shown how DOM may be able to do this; the free metal ions in the water can complex with the large DOM molecules, making the ion unavailable to bind to the site of toxic action on the organism (Al-Reasi et al.,

2011). For instance, the site of binding to the free metal ion appears to be at the gill for a fish (DiToro et al., 2001). This can vary among species however, so we will treat the binding site of action on the organism as the biotic ligand. Since DOM is variable between locations, its protective effect may also vary between locations. For example, as pH changes, so does the binding capacity of the metal to the DOM molecule. As the pH decreases (becomes more acidic) ions tend to separate from the DOM molecule and remain in their free metal ion state (Wood et al., 2011).

Differences in the pH of a solution can effect toxicity in other ways as well. Generally, as pH increases, what occurs is an increased competition between cations and decreased complexation with organic and inorganic molecules (Meador 1991). For instance, as pH increases (becomes more basic) the metal binds with OH^- creating metal hydroxide complexes and in so doing, makes the metal less bioavailable and thereby reducing the toxicity (DiToro et al., 2001). This will also increase the hydrogen cations available in solution increasing cationic competition with the free metal ion at the site of binding on the organism. Overall, toxicity of a metal may differ between aquatic systems since pH will vary between these environments. Thus, pH is an important factor to be taken into consideration when assessing the toxicity of REEs.

Water hardness increases when calcium and magnesium concentrations increase and this also varies between aquatic systems. These elements in their cationic form, modify toxicity by competition (Paquin et al., 2002). Freshwater species are hyperosmotic regulators, meaning that they must regulate their blood osmotic pressures (Hill et al., 2008). Being hyperosmotic means that the animal tends to gain water by osmosis and dilutes their body fluids (Hill et al., 2008). Since the animal must maintain certain ion concentrations in the blood plasma to survive, they have mechanisms in place to actively transport ions back into their blood (Hill et al., 2008). This

is accomplished against a gradient through ion transport channels (i.e. in the gills of a fish) and by excreting highly dilutes urine (Hill et al, 2008). Therefore, an increase in these essential cations modifies toxicity by increasing cationic competition at the ion transport binding sites (Niyogi and Wood 2004). Competition prevents free metal ions from binding to those sites and reduces toxicity to the species (Niyogi and Wood 2004). Another essential cation is Na⁺ and also influences toxicity through competition. Na is required for many physiological processes essential to the organism's survival. Competition between Na and the metal disrupts these balances within the organism and much like the previous cations mentioned, when present in larger amounts it causes increased competition.

1.1.4 Models for Uptake and Bioavailability

The importance of competition, particularly with hardness ions, is illustrated by the Free Ion Activity Model (FIAM). This model focuses on cationic metal binding to critical sites on aquatic organisms. It emphasizes the importance of free metal ion activities in determining uptake and toxicity (Brown and Markich, 2000). However, there are some limitations to the FIAM. The focus of the FIAM is that metal ions are the primary cause of toxicity, when in a natural environment toxicity to the organism at the site of action should encompass not just the free metal ion, but other possible metal complexes that are also able to react directly with the site of action (Brown and Markich, 2000). New models have since been developed to integrate the concepts of the FIAM with geochemical speciation and bioaccumulation at the site of action at the biotic ligand.

The BLM is a tool used for site specific assessment of toxicity that incorporates the concepts of cationic competition, geochemical speciation and bioaccumulation at the biotic ligand (Di

Toro *et al.*, 2001; Niyogi and Wood, 2004; Paquin *et al.*, 2002). Since the BLM is designed to account for differences in water geochemistry among ecological sites, it can be used at a site specific basis (Niyogi and Wood, 2004). It is for these reasons that the BLM has gained a reputation as a reliable tool for environmental risk assessment. Figure 2 is a schematic of the BLM and illustrates how the BLM approach considers interactions between TMFs to predict the toxicity and bioavailability of the metal (Di Toro *et al.*, 2001; Niyogi and Wood, 2004; Paquin *et al.*, 2002). Thus, by estimating the bioavailability of the metal, toxicity to the organism can be predicted.

The foundation of the BLM is prediction of toxicity based on a bioaccumulation at the biotic ligand (Di Toro *et al.*, 2001; Niyogi and Wood, 2004; Paquin *et al.*, 2002). Bioaccumulation will cause mortality to the organism when the free metal ion accumulates at the biotic ligand beyond a certain threshold for that specific species. This is called the (LA₅₀) which is the lethal accumulation at 50% mortality of the organism. An accumulation threshold at the biotic ligand is characterized using the metal bioavailability predictions and Log K values of the cation metal (M^{Z+}) and the biotic ligand (L⁻) (Brown and Markich, 2000). Thus, accumulation at the biotic ligand can be estimated given a certain exposure.

The BLM predicts toxicity by incorporating such interactions. Environmental risk assessors at a given site can use the BLM as a tool to make site specific predictions and objectives. The BLM can be applied to many well studied metals and has so far been developed for Cu, Ag, Zn and Ni (Niyogi and Wood, 2004). However, due to a lack of knowledge or testing of REE toxicity and exposure in aquatic systems, no such predictive tools exist for their toxicity in these environments. With improved understanding of the interactions between Tm and water chemistry

as well as its toxicity on aquatic invertebrates we hope to be able to contribute data to develop REE specific toxicity prediction models.

1.2 Goals and Objectives

The overall goal of this research is to gain an understanding of the potential impacts of REEs to aquatic biota. The specific objectives are:

- A) Develop acute toxicity data on Tm to a sensitive aquatic invertebrate and gain an understanding of the influence of TMFs
 - Assess if Tm toxicity is influenced by cationic competition with Ca^{2+} , Mg^{2+} and Na^+
 - Assess if Tm complexes with DOM, decreasing its bioavailability

- B) Develop chronic toxicity data on Tm to a sensitive aquatic invertebrate and the influence of selective TMFs

- C) To determine if bioaccumulation can be used to estimate Tm toxicity
 - Determine if Tm acute toxicity is linked with Tm bioaccumulation
 - Determine the long term effect of chronic Tm exposures and if bioaccumulation is a factor.

The hypotheses for these objectives are:

- 1) Tm has adverse acute and chronic effects on *Hyalella azteca*
 - Cationic competition does influence the toxicity of Tm

- The presence of DOM will decrease Tm bioavailability thus, reducing its toxicity
- 2) Bioaccumulation will occur in *Hyalella azteca* in both acute and chronic Tm exposures
 - TMFs will reduce toxicity by reducing accumulation of Tm

1.3 Significance of Research

Understanding the toxicity of Tm has both small scale and large scale implication in the field of toxicology and for the environment as a whole. This research will gather data on a metal that is so far has not been studied. As well, it is not understood in terms of its aquatic geochemistry. As production and demand increases for REEs, exposure in the environment becomes a concern. The data obtained in this research can be contributed to a better understanding of Tm toxicity in Canadian aquatic environments. Long term, this could lead to government policies and regulations that can protect and sustain Canadian ecosystems.

This research is integrative because it utilizes many fields in science including chemistry, biology, toxicology as well as elements of ecology to fully understand the toxicological impacts of Tm on aquatic ecosystems. It integrates knowledge of chemical interactions in terms of the aquatic geochemistry of Tm and links those interactions to effects on an invertebrate species. Furthermore, this research is integrated with the principles of government policy and regulation.

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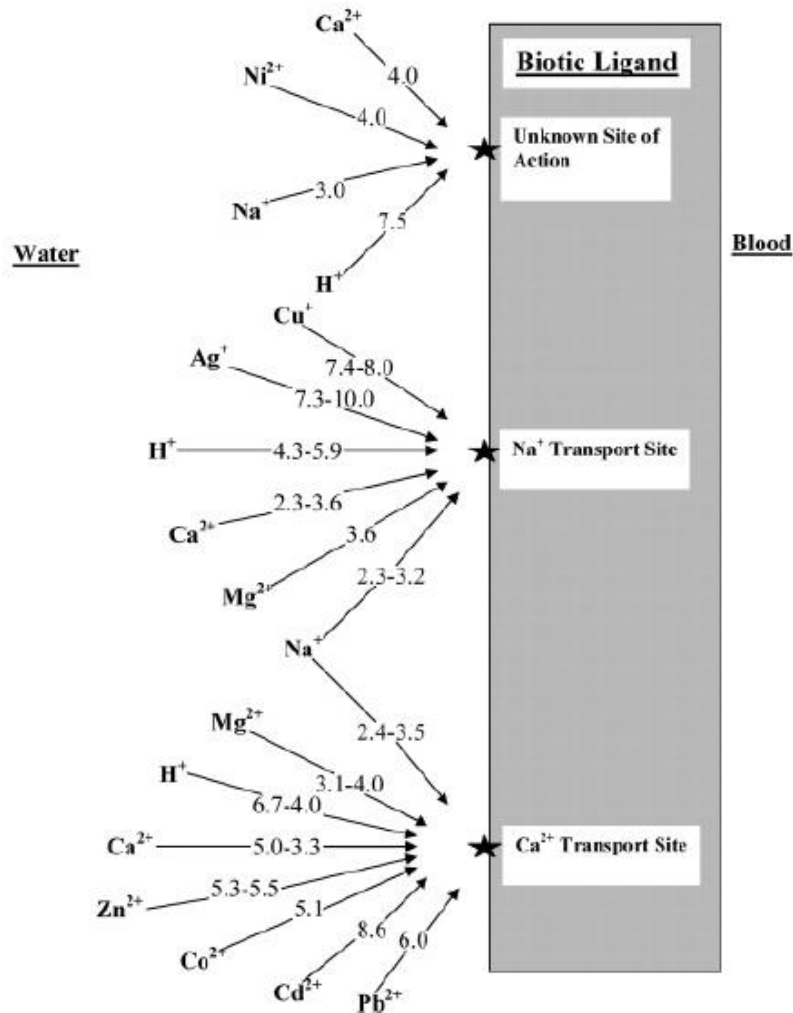


Figure 1.1: Schematic of Ion transport sites in aquatic organisms are used for passive diffusion against a gradient to regulate blood plasma ion concentrations within the organism. Binding affinities (Log K where K is the affinity constant) reflect the cationic competition between ions within an aquatic system. Ions vital for the survival of the organism include Ca^{2+} , Mg^{2+} , Na^+ . However, free ion metal contaminants, interacting at these transport sites can block uptake of these vital ions, causing a toxic action. From

BIOTIC LIGAND MODEL

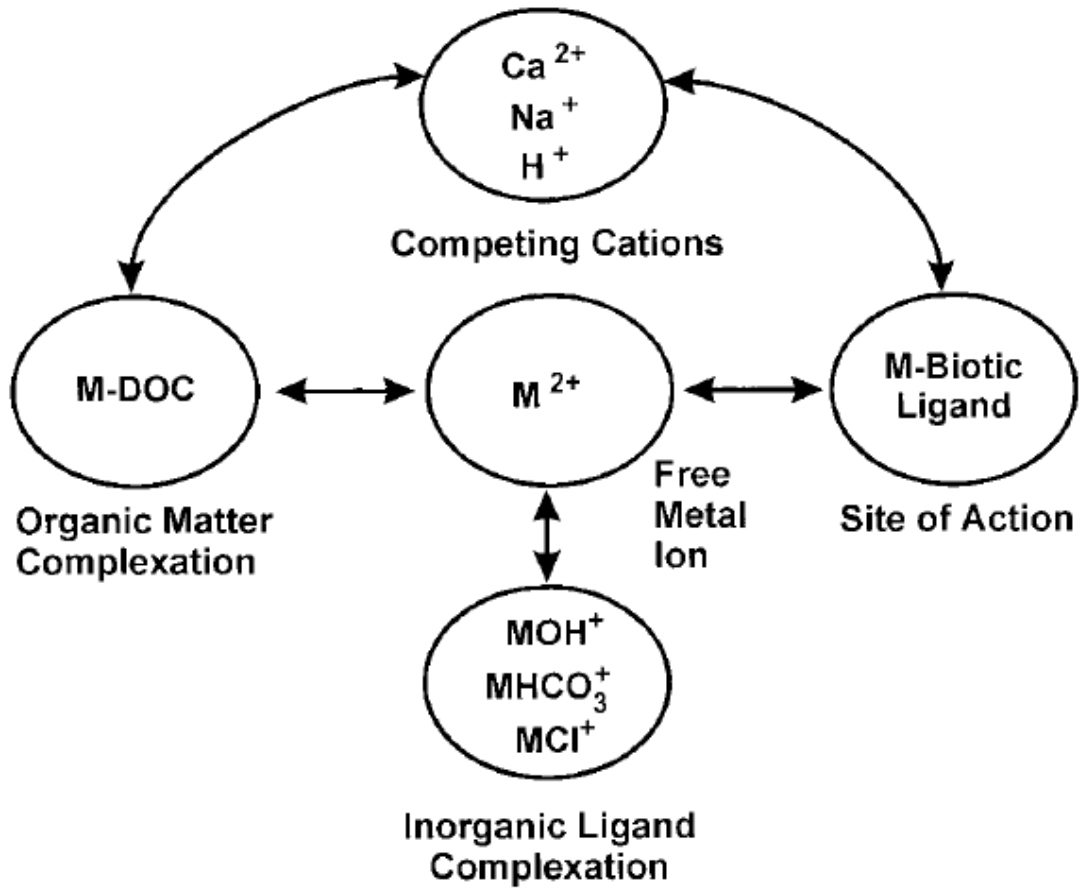


Figure 1.2: Schematic representation of a Biotic Ligand model illustrating complexation with organic matter, inorganic matter, free metal ions and cationic competition, and the interaction of the metal (M^{2+}) with the biotic ligand from Figure 1 of the Biotic Ligand Model by Di Toro et al., 2001.

Chapter 2: Acute Tm Toxicity

2.1.0 Introduction to Acute Tm Toxicity

2.11 Global Demand of Rare Earths and Potential Environmental Concerns

Rare earth elements (REEs) are a group of 17 metals with similar chemical and physical properties that include 15 lanthanides as well as yttrium and scandium (Environment Canada, 2013). REEs are not rare and crustal abundance is similar to other metals such as copper or lead (Castor and Hedrick, 2006). However, enriched REE deposits are uncommon (Humphries 2013; Paul and Campbell, 2011). Global demand for REEs has increased dramatically with predictions of increased production nearly tripling over the next 25 years (Humphries 2013). Increases in demand are attributed to a growing number of uses related to their optical, magnetic and catalytic properties (Alonso et al., 2012). Canada is home to some of the largest REE deposits, particularly heavy REEs (elements 64 to 71; Y, Tb, Dy, Ho, Er, Tm, Yb and Lu) potentially making it a leading global producer (Humphries 2013).

There is limited information about potential environmental impacts of REEs in aquatic systems. This is particularly the case for Thulium (Tm). To my knowledge however, there is only one study where Tm toxicity was evaluated. Borgmann et al. (2005) found Tm to be highly toxic to *Hyalella azteca* with a reported measured dissolved LC₅₀ value of 0.01 µg/L or 5.9 nmol/L (reported nominal LC₅₀ value of 721 µg/L). This result was from exposures in very soft waters (12.4 mg CaCO₃/L) Borgmann et al. (2005). Borgmann et al. (2005) also conducted tests in water with an elevated hardness (Lake Ontario, hardness of 124 mg CaCO₃/L) and reported a LC₅₀ of 739 µg/L. However, this was a nominal value and measured were not reported. The difference in nominal and measured LC₅₀ values for the soft water test in that study indicates that precipitation was occurring within Tm exposure solutions. With both nominal results being similar and no measured result reported for Tm exposures in the hard water medium, it is hard to

interpret if water hardness had any mitigating effect for Tm toxicity. Since there is only limited information available a greater understanding of Tm toxicity is needed.

2.1.2 Toxicity Modifying Factors (TMFs)

For many well studied metals, acute toxicity results from the uptake of free metal ions into the organism and the resulting disruption of essential ion balance (Niyogi and Wood, 2004). Water chemistry is a major influence on the toxicity of metals. Cations (eg. H^+ , Ca^{2+} , Na^+ and Mg^{2+}), dissolved organic matter (DOM) and inorganic ligands (e.g. Cl^- and carbonates) have been known to modulate responses (El-Akl et al., 2015). These factors can be grouped into two general categories: complexation where a negatively charged ligand complexes the metal free ion and reduces bioavailability; competition where cations compete for uptake at the site of toxicity (Santore et al., 2001). Complexation and competition can dramatically alter toxicity. For example, in a study by Vukov et al. (2016), the addition of Ca^{2+} in solution with Dy exposure to *H. azteca* found to decrease toxicity significantly. In that same study, increased concentrations of DOM positively correlated with decreased toxicity. To develop data for water quality criteria and guidelines for Tm, not only is it important to gain an understanding of its toxicity but also how TMFs might influence responses. An improved understanding of the site specific toxicity of Tm will be useful for application in risk assessment.

DOM is ubiquitous in aquatic systems and plays an important role in mitigating metal toxicity (Wood et al., 2011). It arises from both autochthonous and terrigenous inputs and there are significant differences in composition among sources (Al-Reasi et al., 2011). This variability in composition can result in significant differences in the protective capacity of DOM for metals (Al-Reasi et al., 2013; Wood et al., 2011). DOM is a large, heterogeneous, complex molecule and metal will bind to functional groups such as carboxylates and phenols (Al-Reasi et al., 2013).

With growing amounts of research on DOM, it has become well understood of its importance in metal toxicity mitigation and therefore, has become a key variable in predicting site specific metal toxicity (Wood et al., 2011).

Toxicity reduction through cationic competition occurs because the free metal ion form of the dissolved metal is generally considered to be the most toxic form (Di Toro et al., 2001). This is particularly the case for monovalent and divalent metals such as Cu^{2+} , Zn^{2+} , Pb^{2+} , Ag^+ and Co^{2+} (Niyogi and Wood, 2004). However, it is not known if this is the case for REEs, which occur as trivalent ions. In previous research, La toxicity to *D. carinata* was shown to decrease with increased hardness (Barry and Mehan, 2000). A positive correlation between Ca^{2+} and LC_{50} was also seen in Dy toxicity to *H. azteca* (Vukov et al., 2016). Borgmann et al. (2005) also saw decreases in some REE toxicity with an increase in water hardness although in that study water chemistry was altered by dilution and therefore changes in hardness co-occurred with changes in other TMFs including DOM. These acute toxicity studies illustrate that cations such as Ca and Mg may compete antagonistically with REE^{3+} cations for uptake and/or binding to the site of toxic action.

2.1.3 The Biotic Ligand Model Approach

The Biotic Ligand Model (BLM) is a tool that has become widely accepted for the site specific assessment of toxicity (Niyogi and Wood, 2004). This tool predicts toxicity of a metal based on the bioavailability of the free metal ion and incorporates the influences of TMFs such as cationic competition and complexation with inorganic and organic ligands (Paquin et al., 2000).

Essentially the BLM defines toxicity through accumulation thresholds of the free metal ion at the site of action (the biotic ligand). Since very little is known about the toxicity of REEs, we have

applied a BLM approach to understand how TMFs influence Tm toxicity and if indeed the BLM is a tool that can be used to predict the bioavailability of REEs.

2.1.4 Hyalella azteca: A Sensitive Invertebrate

In this study *H. azteca* was the species used for Tm toxicity tests and it was chosen so for a number of reasons. *H. azteca* are a species of amphipods that are commonly found in fresh water lakes, streams and marshes across North America (Environment Canada, 2013). *H. azteca* reproduction in lab is continuous and therefore harvest of neonates for testing is predictable (Environment Canada, 2013). They have been used widely for toxicity tests for many decades due to their sensitivity to contaminants. Environment Canada has a standard biological tests method for culturing and testing of this species for both water only toxicity and sediment toxicity tests (Environment Canada, 2013). Within this standard method, based on the study of the ion requirements of *H. azteca* (Borgmann, 1996) a standard artificial medium is provided. This standard artificial medium allows consistency and comparability between *H. azteca* toxicity tests.

2.2 Objectives

The objective of this study is to develop acute toxicity data on Tm to a sensitive aquatic invertebrate, *H. azteca* and to also gain an understanding of the influence of TMFs. The main goals are to assess if Tm acute toxicity is influenced by cationic competition with Ca^{2+} , Mg^{2+} , and Na^+ as well as to assess if Tm complexes with DOM, therefore decreases its bioavailability.

We hypothesize that increased Ca and Mg will decrease Tm toxicity but that Na will not. We also hypothesize that the presence of DOM will decrease Tm bioavailability and thus, reduce its toxicity. Furthermore, there will be a difference in protective effect between DOM sources.

2.3 Material and Methods

2.3.1 *H. azteca* culture

Culture and test procedures followed the Environment Canada method for *Hyaella azteca* (Environment Canada, 2013) and organisms were originally collected from the shore of Eabamet Lake, ON and maintained in the lab for 2 y. Culture and testing was in a reconstituted medium (RM) as described by Vukov et al (2016) and made with analytical grade CaCl₂, NaHCO₃, MgSO₄, KCL and NaBr (Sigma-Aldrich, Mississauga, ON) at 500, 500, 125, 25 and 5µM respectively to give a hardness of 60 (mg CaCO₃/L) and pH of 7.6 ± 0.2. Cultures of 20-30 adults were kept in 2L beakers with 1600 ml of RM and held at 23°C ± 2 in an incubator (LTCB-19 BioChamber, BioChambers Inc., Winnipeg MN) with full spectrum lighting at 400 to 1,000 lux and a 16:8hr light: dark photoperiod. *H. azteca* were fed on Mon., Wed. and Fri. with 5 mg of finely ground tropical fish food (TetraMin, Tetra, Blacksburg, VA). Neonates between 0 and 7 d of age were separated from cultures at the weekly media renewal using 650 and 275 µm mesh polyethylene mesh. At RM renewal, a fresh piece of cotton gauze (approx. 10 x 5cm) was added to the beakers (Borgmann et al., 1989).

2.3.2 Acute *Tm* Toxicity Tests

Testing procedures followed the Environment Canada standard aquatic test method for *Hyaella azteca* published in 2013 (EPS1/RM/33) (Environment Canada, 2013) with mortality as the endpoint. Acute tests were conducted using 2-9 day old neonates and consisted of up to 7 exposure concentrations (including control). Thulium exposure solutions were made using a neutralized (pH 7.3 ± 0.05, Environment Canada, 2013) stock solution (30 mg/L) created from an analytical standard (Inorganic Ventures Inc., Christiansburg, VA) that was 5% HNO₃. Exposures were done in duplicate in 400 mL polyethylene beakers (Fischer Scientific) with

240mL of solution made by appropriate dilution of the stock solution. Test solutions were equilibrated for 24h prior to test start (0h) after which pH was measured prior to starting the test. A 10cm X 5cm piece of cheese cloth was placed in a 40mL plastic cup with 10mL of the exposure solution, for equilibration. After equilibration, the cheesecloth was added to the exposure beaker along with 10 neonates that were 2-9 days old. At the beginning of the test, two 15mL water samples were taken from each beaker, one was not filtered and the other was filtered with a 45 μ m, (HT Tuffryn membrane, Pall, Sigma Aldrich, Mississauga, ON). For the duration of the 96 h test, beakers were kept at 23°C \pm 2, a 16:8 light:dark photoperiod with lighting between 400 and 1000 lx and no feeding. At 96h, dead and surviving neonates were counted and recorded and filtered (dissolved Tm: Tm_{-D}) and unfiltered (total Tm: Tm_{-T}), were collected (as described above). All water samples were acidified to 2% with v/v 16N HNO₃ (trace metals grade, Fischer Scientific, Nepean, ON). All samples were stored in 15mL tubes (Celltreat, Mandel Scientific, Guelph, ON).

To understand the influence of TMFs, tests were conducted (as described above) beginning with culture medium, then followed by modified concentrations of Ca, Mg, Na and finally additions of DOM. Only one parameter was altered at a time while keeping the others consistent. To adjust Ca concentrations, either CaCl was added or removed when making RM. The range of concentrations tested were 0.3mM to 1.5mM. Mg concentrations ranged between 0.06mM to 0.38mM and to alter these concentrations MgSO₄ was either reduced or added to RM. NaCl was added or reduced to test for the influence of Na on Tm toxicity and the range in Na concentration was 0.3mM to 1.5mM. The effect of DOM on Tm toxicity was tested by the addition of two different sources of DOM (Table A2). One was DOM collected from Kouchibouguac, NB, Canada. The concentration of dissolved organic carbon (DOC) in this source was 394mgC/L and

was made to a nominal concentration of 7mgC/L by dilution. The second source of DOM was collected from Luther Marsh (Luther Marsh, Grand Valley, ON). DOC concentration from this source was 697mgC/L and was made to nominal concentrations of 2mgC/L to 12 mgC/L by appropriate dilution. For tests with DOC, 50mL of 0.45 μ m filtered sample were taken at test start and at test end for each replicate. DOC samples were not acidified.

2.3.3 Water Chemistry and Characterization Tests

To understand how Tm acts in solution, a bench test was conducted with five replicates of five Tm concentrations (2.2, 4.4, 8.9, 17.8, 35.5 mM). Solutions were made up using the RM and put into tri-corner polyethylene beakers. Samples were taken at 0hrs, 24hrs and 96hrs. and as described above. All samples were acidified to 2% with TraceMetal™ Grade HNO₃. To account for possibility of human error in solution making, one replicate of the five was acidified completely to 2% at 0hrs and one more replicate at 96 hrs.

2.3.4 Sample Measurements and Calculation and Statistics

Measured concentrations were determined for both Tm-T and Tm-D using the inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 8000, Perkin-Elmer Inc., Woodbridge, ON) as well as all solution cations (Ca, Na, Mg). Analysis parameters and wavelengths were selected using manufacturer guidelines and recommendations. Reference standards were made using analytical standards (Inorganic Ventures Inc., Christiansburg, VA) and were acidified to 2% with v/v 16N HNO₃ (trace metals grade, Fisher Scientific, Nepean, ON) and were frequently referred to throughout the run for quality assurance. For measurement of DOC concentrations, the total organic carbon analyzer was used (TOC-LCPH, Shimadzu Corporation, Mandel Scientific, Guelph, ON). This machine acidified the samples during readings.

Lethal concentration at 50% (LC₅₀) was calculated for both Tm_{-T} and Tm_{-D} measured concentrations at 96h. Calculations were done using SPSS probit analysis where 95% confidence intervals were also calculated. Significant differences between LC₅₀ values were established according to Litchfield and Wilcoxon (1949, cited in Environment Canada, 2005).

2.4 Results

2.4.1 Tm Water Chemistry and Characterization

In the experiments for Tm water chemistry only (*H. azteca* not exposed), the amount of Tm recovered was not the same as the nominal concentration added (Table 1 and 2, Fig. 1). As nominal concentrations increased, the measured Tm_{-T} decreased. By 120h, this recovered Tm_{-T} ranged between 12% and 89% and there was an inverse correlation between nominal concentration and percent recovery of Tm_{-T} (Table 1 and 2, Fig. 1). In the first 24 h of solution production, there was a dramatic reduction in Tm_{-T}. For example, at a nominal concentration of 35 µM the percent Tm_{-T} recovered was 38% immediately after preparation (0 h) and then 14% after 24 h. Equilibration was evident by 24 h with little difference in recovered Tm_{-T} between 24 h and 120 h (Table 1 and 2, Graph 1). By 120h, Tm_{-D} was 99% of the Tm_{-T} (Table 2).

2.4.2 Tm Toxicity and Cationic Competition

H. azteca mortality increased with increasing concentrations of Tm and a typical exposure response curve is shown in (Fig. 2). The results for effects of Ca for Tm_{-T} showed significantly lower LC₅₀ at the 0.5 mM Ca treatment (unaltered RM medium) than LC₅₀ values for Tm_{-T} at 0.3mM and 1.5mM Ca treatments (Fig. 3). However, for Tm_{-D} LC₅₀ at the 0.5mM Ca treatment was only significantly higher than the Tm_{-D} LC₅₀ at the 1.5 mM Ca treatment (Fig. 3). No other significant differences were seen between Ca treatments. There was some variability between LC₅₀ values within the different Na treatments but there were so significant differences found

(Fig. 4). The results for effects of Mg for Tm-T showed significantly lower LC₅₀ at the 0.1 mM Mg treatment (unaltered RM medium) than the Tm-T LC₅₀ value at 0.06 mM Mg (Fig. 5).

However, no other significant differences were seen between Mg treatments for both Tm-T and Tm-D LC₅₀s.

2.4.3 The Protective Effect of DOC

Results for Luther Marsh DOC treatments showed significant differences in both Tm-T and Tm-D LC₅₀s at 7 mg C/L and 12 mg C/L when compared to 0 mg C/L (Fig. 6). There were increases in both Tm-T and Tm-D LC₅₀ values at 2 mg C/L. However, these differences were not significantly different than 0 mg C/L DOC LC₅₀ values (Fig. 6). Results for the Kouchibouguac DOC experiment showed a significant increase in both Tm-T and Tm-D LC₅₀ values in the 7 mg C/L treatment (Fig. 7).

2.5 Discussion

In this study, we obtained results that contribute to a better understanding of the toxicity of Tm to a sensitive freshwater invertebrate, *H. azteca*, as well as the unique influence that water chemistry has on REEs. A correlation between Tm exposure concentration and acute toxicity was observed in the 96 hr acute tests (Figure 2). As concentrations of Tm increase, mortality of *H. azteca* also increases, indicating that high concentrations of Tm are toxic. Results indicate that Na and Mg do not provide a protective effect while those with Ca are less clear. However, DOC did prove to have a protective effect. One of the main goals of this research was to understand more about Tm toxicity. This was considered important because in the Borgmann et al. (2005) study a very low Tm-D LC₅₀ of 0.01ug/L was reported for a soft water medium. , That study also reported a LC₅₀ value for tests done in relatively hard water but only nominal concentration was

given. The difference between nominal and Tm-D LC₅₀ values in the soft water medium in Borgmann et al (2005) indicate precipitation was occurring. Borgmann and co-workers reported nominal LC₅₀ values for soft and hard water mediums that were very similar indicating that hardness may have no influence on Tm toxicity. However the Borgmann et al. (2005) study results with Tm are somewhat hard to interpret because of a lack of measured concentrations.

In our study, we used the same species as Borgmann et al. (2005) however they were not the same source. We also used a very similar general water composition. In the Borgmann et al. (2005) study the soft water tests used a medium created by dilution of the original medium (dechlorinated Burlington tap water) with deionized water, thus changing the entire chemistry of the medium. In our tests, we specifically changed concentrations of each of our ions (Ca, Na and Mg) keeping the rest of the water chemistry consistent. Methodology in the Borgmann et al. (2005) study was also different than standard toxicity tests since he was evaluating a large quantity of metals at once. In our study, we used standard Environment Canada toxicity methods for *H. azteca* (Environment Canada, 2013) for the purpose of focusing on the toxicity of just Tm. These may be some of the reasons that explain why our results show a toxicity for Tm that is approximate 300 fold less than that reported by Borgmann et al. (2005).

2.5.1 Thulium Precipitation and the Importance of Water Chemistry Characterization

Based on previous REE studies where precipitation was observed during toxicity tests (Barry and Meehan, 2000; Borgmann et al., 2005; Vukov et al., 2016) we conducted tests to focus on Tm water chemistry. We found that water chemistry characterization was of upmost importance in this research, as a loss of Tm due to precipitation, resulted in LC₅₀ values that were much lower than that of nominal. In the case of other metals where precipitation does not generally occur in simple aquatic media the nominal and measured LC₅₀ values can be similar.

However, based on our results from the bench tests conducted, we saw that recovered concentrations of Tm were much lower than nominal concentrations and therefore LC₅₀ values determined using nominal concentrations would greatly underestimate the actual toxicity of Tm (Table 1, Figure 2).

Precipitation has occurred in previous REE toxicity tests as well. As described above, Borgmann et al. (2005) reported a nominal LC₅₀ of 721ug/L with a measured LC₅₀ of 0.01ug/L. The differences in LC₅₀ values indicates a loss of Tm in solution. Similar results for other REEs have also been seen. Lanthanum aquatic toxicity tests using *D. carinata* demonstrated that La readily precipitated out of solution and that measured values were always less than 30% of the nominal concentrations (Barry and Meehan, 2000). Additionally, Vukov et al. (2016) found that increased precipitation of Dysprosium (Dy) correlated with increasing exposure concentration. It was reported that at high exposure concentrations of Dy, dissolved concentrations were less than 34% of total concentrations (Vukov et al., 2016). High levels of precipitation could be accounted for by the formation of insoluble salts with carbonates in solution (Jiang and Ji, 2012). The RM used in the tests contained NaHCO₃. The carbonates in solution may have contributed to an increase in pH that was observed throughout the 96 hr test. Both an increase in pH, as well as the low solubility carbonates present in solution may be the contributing factors to the reduced solubility of Tm (Janssen and Verweij, 2003). Furthermore, several studies have indicated that dominant REE species in solution are sulphates, carbonates and chloride species (Janssen and Verweij, 2003; Jiang and Ji, 2012). Based on the water chemistry of the RM used in our tests, it is possible these Tm species were present and contributing to precipitation.

An additional observation was that while precipitation increased with higher exposure concentrations, tests done with DOC present in solution caused the amount of precipitation to

decrease and the difference between Tm_{-D} and nominal concentrations was reduced. In RM, Tm_{-T} and Tm_{-D} would range between 89%-12% of the nominal concentration (Table 1 and 2) with the recovery decreasing as there was increasing exposure concentrations. When 12mgC/L of DOC was present in solution, the range was between 99%-46% of the nominal concentrations. This may indicate that DOC complexation with Tm kept more of the Tm dissolved in solution. While free metal ions are considered the most bioavailable form, we did not calculate LC_{50} for Tm^{3+} but we do report values for Tm_{-D} to allow for subsequent calculations of free ions. Tm_{-D} is also used in some jurisdictions and generally provides a more conservative estimate of toxicity compared to Tm_{-T} . However, Canadian Water Quality Guidelines require that total measured amounts of a metal be used and therefore, we also calculated LC_{50} values for Tm_{-T} (Canadian Council of Ministers of the Environment, 2003).

Based on our Tm bench tests, recoverable Tm_{-T} reached 4 $\mu\text{mol/L}$ (Figure.1) before a precipitation threshold was evident. In our study however, we used a highly sensitive species and calculated LC_{50} values were always below this threshold. However, since beyond this threshold, no more Tm can be dissolved in solution a less sensitive species may not show toxicity if the effective concentration is never reached due to precipitation. Research on Tm toxicity to different aquatic species would need to be done to confirm this hypothesis.

2.5.2 Cationic Competition

We hypothesized that increases in Ca would have a protective effect to Tm toxicity but this did not occur. We saw significant increases in LC_{50} values at the decreased Ca treatment of 0.3mM. There was no consistent trend across the range of Ca tested (Fig. 3). This was unexpected because other studies show that Ca provides significant protection against REE toxicity. Vukov et al. (2016) showed a 1.8-fold decrease in toxicity to *H. azteca* over a 3-fold

increase in Ca concentration. In this study, *H. azteca* was the tested species, however it was from another source (Hannah Lake, Sudbury, ON) and Dy was the tested metal. However, a similar reconstituted medium of the Borgmann (1996) aquatic medium was used and similar methods to test the influence of waterborne Ca on Dy toxicity (Vukov, et al. 2016). Since our methods in this study were very similar to that of Vukov et al. (2016), it could be that Dy has a competitive interaction with Ca and Tm does not. Our study was also not in agreement with other studies such as Barry and Meehan (2000) which showed that REE toxicity was reduced as water hardness increased. However, in this study, all three water medium tested had varying water chemistries therefore the change in toxicity with hardness can not be exclusively attributed to Ca. In the case of the Borgmann et al. (2005) study, changes in hardness did not show a change in toxicity when comparing nominal LC₅₀s for Tm. As mentioned previously however, due to precipitation it is hard to interpret nominal results. Borgmann et al. (2005) did however report measured Dy LC₅₀ values showing that increased hardness did decrease Dy toxicity. This comparison of Tm and Dy in the Borgmann et al. (2005) study are in agreement with the hypothesis that Dy may have a competitive interaction with Ca and Tm does not. However, more research is required to compare the toxicities of different REEs.

There was no protective effect with increasing Na concentrations. Again, this result is different to the Vukov et al., (2016) study where increased Na significantly decreased Dy toxicity by a factor of 1.4 times. However, these results were based on total Dy concentrations and the study states that LC₅₀s for dissolved Dy concentrations were much less clear (Vukov et al., 2016). In our results we did not see a protective effect with Mg either. This result did agree with the Vukov et al. (2016) study where Mg additions did not show a clear protective effect.

Previous toxicity studies on divalent cations such as Cd, Zn, Cu and Pb have caused toxicity by blocking major divalent cation transporters such as a Ca transporter (Niyogi and Wood, 2004). Therefore, a competitive interaction between a trivalent REE and the tested cations (Ca, Na, and Mg) would be very unexpected. However, there are previous studies that have discussed possible mechanisms of how a REE would competitive interact with a divalent cation. Evans (1983) describes that Ln^{3+} , a lanthanide, behaves similarly to Ca^{2+} regardless of differences in charge. Reasons for this include that both Ca^{2+} and Ln^{3+} have a similar ionic radius, they both bind ionically and prefer atoms in which donate oxygen (Evans 1983). Evans (1983) also describes the ability of Ln^{3+} to replace Ca^{2+} at binding sites on proteins. Studies with human erythrocytes and gadolinium (Gd) showed that Gd increased permeability of the cell membrane by pore formation (Cheng, et al., 1999.) While these studies provide possible uptake mechanisms for REEs, our study focused on the toxicity of Tm. Nonetheless research into the physiological mechanisms of Tm uptake in *H. azteca* is an interesting direction for future study.

2.5.3 The Protective Effect of DOC

In this study we were able to look at two different sources of DOC (Table A2). Results demonstrated a strong positive correlation between DOC concentration and LC_{50} (Fig. 6, Fig. 7). The concentrations of DOC chosen for testing were based on DOC measurements from 23 lakes surrounding Yellowknife, NWT (Pientitz and Smol, 1993). It is already well understood that DOC has a strong ability to complex with metals reducing bioavailability and therefore, mitigating toxicity (Al-Reasi et al., 2013; Di Toro et al., 2001; Wood et al., 2011). In the study by Vukov et al., (2016) similar results were found in that as DOC concentrations increased, LC_{50} values for dissolved Dy increased. Opposing results were seen in a study by Zhao and Wilkinson

(2015) where in the presence of organic ligands, increased bioavailability and therefore toxicity to a species of algae *C. reinhardtii*. Our results are generally consistent however, with studies that show the strong mitigating effects of DOC to metal toxicity. It is important to highlight that the protective effect of DOC occurred in solutions with increased concentrations of Tm (i.e increased Tm_T and Tm_D) due to reduced precipitation.

Our results for Kouchibouguac DOC was tested at 7 mg C/L and illustrated a strong protective effect increasing the LC₅₀ value by almost 3-fold. An almost 4-fold increase was seen however, when 7 mg C/L of Luther Marsh DOC was in solution indicating that DOC collected from Luther Marsh may have stronger protective effect than Kouchibouguac. Both DOC sources are optically dark which is known to indicate that its composition consists mainly of allochthonous DOC (or land-based sources) and therefore is more protective (Wood et al., 2011). However, given the highly diverse composition of natural DOC molecules, studies are showing that each source of DOC may have a distinctive protective effect and this could be due to a number of factors such as the number of phenolic rings and humic acid content (Al-Reasi et al., 2013; Pempkowiak et al., 1999). Further study is required however, to fully understand the mechanisms that cause DOC to mitigate toxicity.

2.6 Conclusions

In this study, we were able to contribute data on Tm toxicity to *H. azteca* and found Tm to be much less toxic than what was reported in Borgmann et al. (2005) results. Results indicate that Mg and Na did not have a protective effect on Tm toxicity and that there is a less clear interaction between Ca and Tm. These results were not consistent with previous REE toxicity research where increased hardness, Ca and Na had protective effects (Barry and Meehan, 2000; Vukov et al., 2016). However, we did see a significant protective effect with the addition of

DOC and also found that there were differences in the protective effect between sources of DOC, which is consistent with previous literature. This study is an introductory examination of Tm toxicity however, and much more research is required to fully understand the influence of water chemistry on Tm toxicity. Also, further study is required to understand if the BLM is a model that can be applied to REE aquatic toxicity.

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Table 2.1: Nominal, Total Acidified beaker (TA), Measured total (T_{m-T}) and dissolved (T_{m-D})

T_m concentrations at 0 h, 24 h and 120 h reported in µg/L. T_m solutions were made in RM.

Results indicate that T_m is precipitating out within the first 24hrs and as concentrations increase.

Nominal µg/L	0 h			24 h		120 h		
	T _{m-T}	T _{m-D}	TA	T _{m-T}	T _{m-D}	T _{m-T}	T _{m-D}	TA
375	357	307	365	328	320	332	326	330
750	537	410	604	519	517	529	527	558
1500	800	572	930	689	689	670	669	865
3000	1473	801	1600	819	810	710	703	1736
6000	2304	991	4060	865	839	720	696	5643

Table 2.2: Tm Nominal, measured total (Tm-T) and dissolved (Tm-D) Tm concentrations and the percentage differences at for dissolved measurements, between total and dissolved measurements and between nominal and dissolved and nominal and total measurements. Tm solutions were made in RM. Results indicate that Tm is precipitating out within the first 24hrs and as concentrations increase. By 120 h, Tm-D concentrations are 99% of Tm-T.

Nominal µg/L	Tm-D / Tm-T (%)			Tm-T / Tm-Nominal (%)			Tm-D / Tm-Nominal (%)		
	0 h	24 h	120 h	0 h	24 h	120 h	0 h	24 h	120 h
375	86	98	98	95	88	89	82	85	87
750	76	100	100	72	69	71	55	69	70
1500	72	100	100	53	46	45	38	46	45
3000	54	99	99	49	27	24	27	37	23
6000	43	97	97	38	14	12	17	14	12

Measured Total vs. Nominal Concentrations

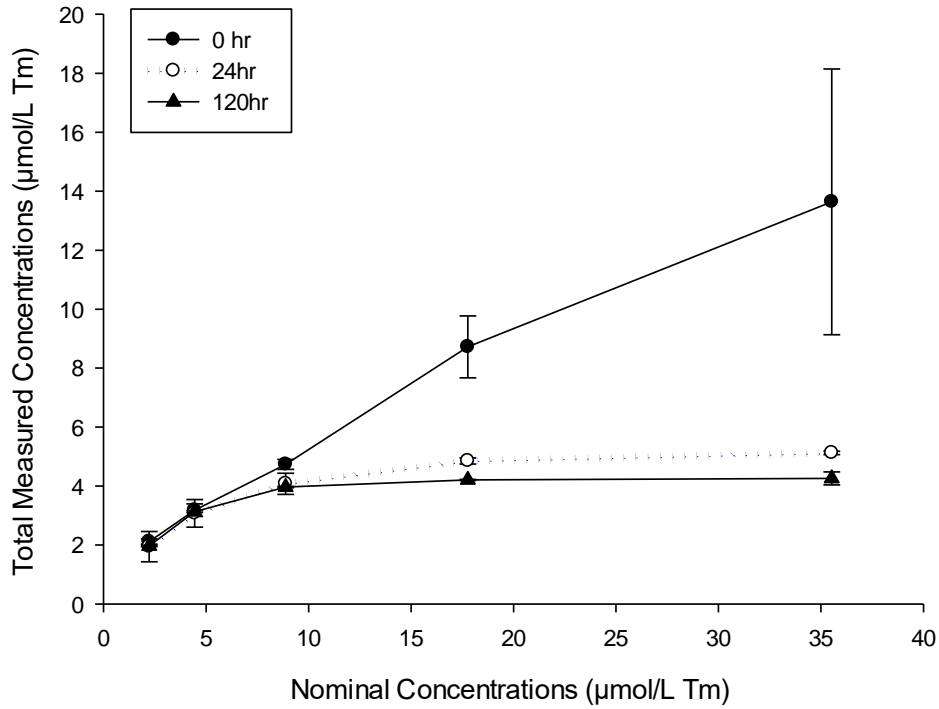


Figure 2.1: Comparison of nominal and measured Tm measurements for total (Tm-T). concentrations from the time periods of 0h, 24h and 120h for water chemistry test. Means of Tm-T concentrations are shown with error bars represented as \pm SEM. The number of observations of this test is n=5.

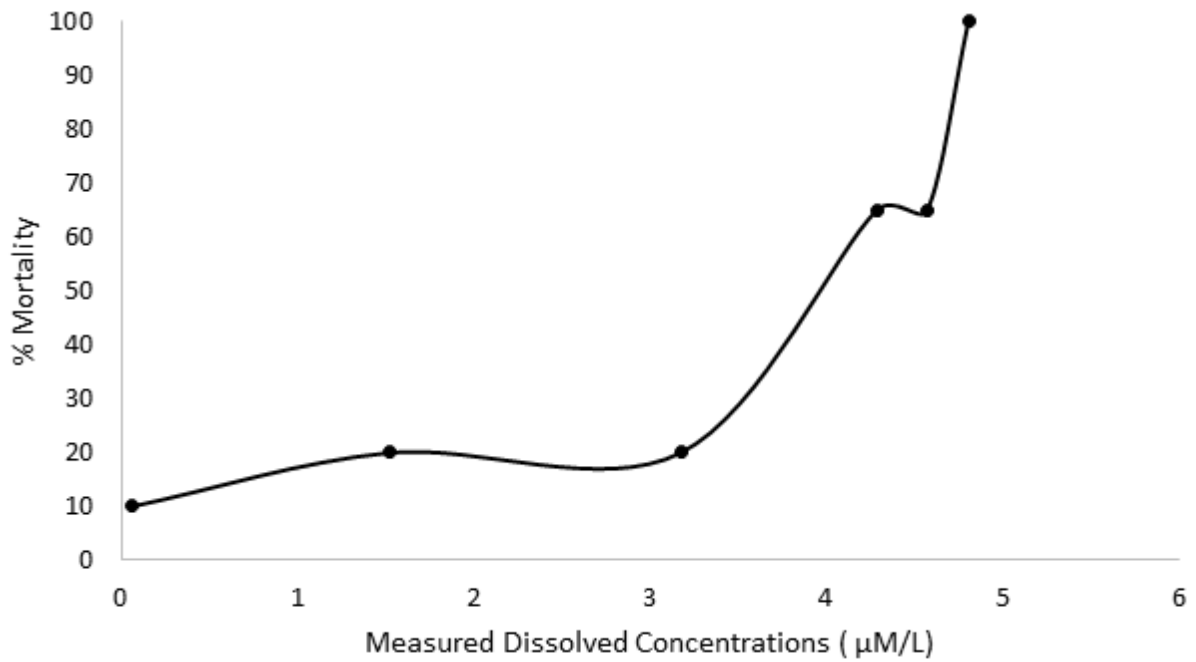


Figure 2.2: Typical exposure response curve showing average mortality (%) for *H. azteca* in an acute 96h Tm toxicity test done using RM. The tests consisted of n=2 replicates at each concentration with n=10 organisms per concentration.

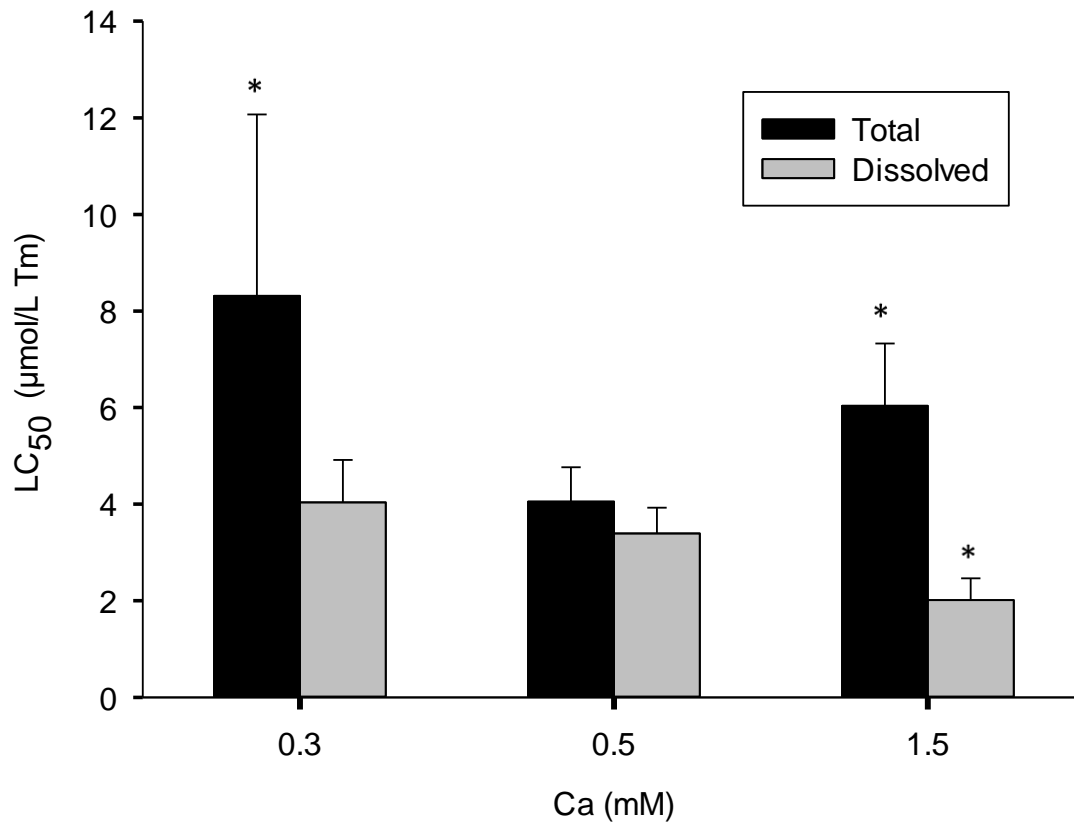


Figure 2.3: LC₅₀ values (with upper 95% confidence intervals) for *Hyalella azteca* exposure to Tm at different Ca concentrations. The LC₅₀ values are based on 96h tests and measured total (Tm-T black bars) and measured dissolved (Tm-D grey bars) concentrations. The stars indicate LC₅₀ values significantly different for either Tm-T or Tm-D from acute test in RM at 0.5 mM Ca.

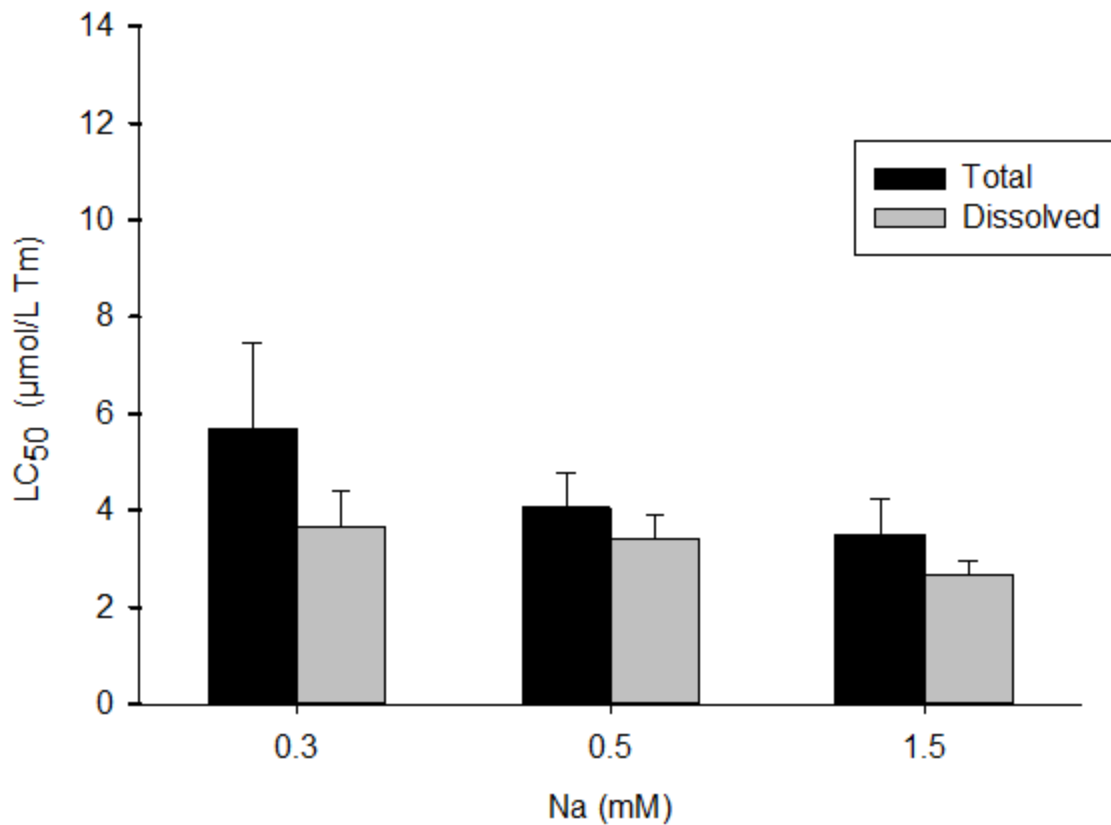


Figure 2.4: LC₅₀ values (with upper 95% confidence intervals) for *Hyalella azteca* exposure to Tm at different Na concentrations. The LC₅₀ values are based on 96h tests and measured total (Tm-T black bars) and measured dissolved (Tm-D grey bars) concentrations. There were no significant differences between LC₅₀ values for Tm-T or for Tm-D when compared to the acute test in RM at 0.5 mM Na.

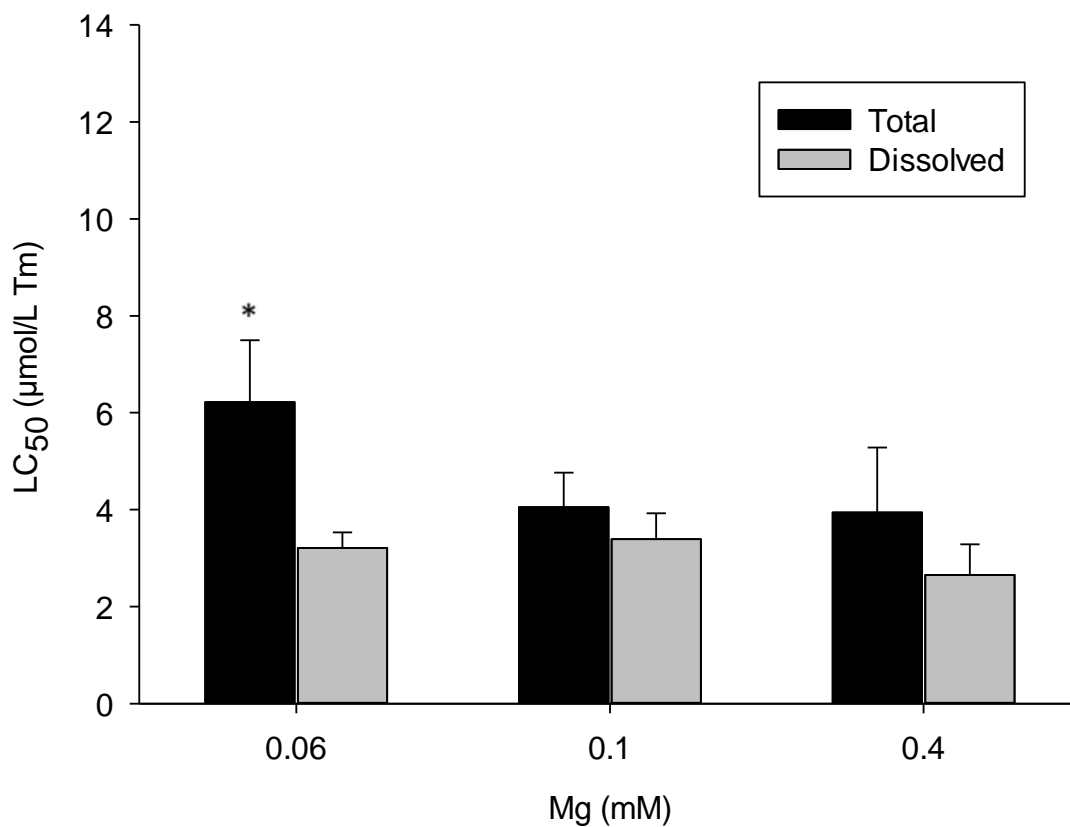


Figure 2.5: LC₅₀ values (with upper 95% confidence intervals) for *Hyalella azteca* exposure to Tm at different Mg concentrations. The LC₅₀ values are based on 96h tests and measured total (Tm-T black bars) and measured dissolved (Tm-D grey bars) concentrations. The stars indicate LC₅₀ values significantly different for either Tm-T or Tm-D from acute test in RM at 0.1 mM Mg.

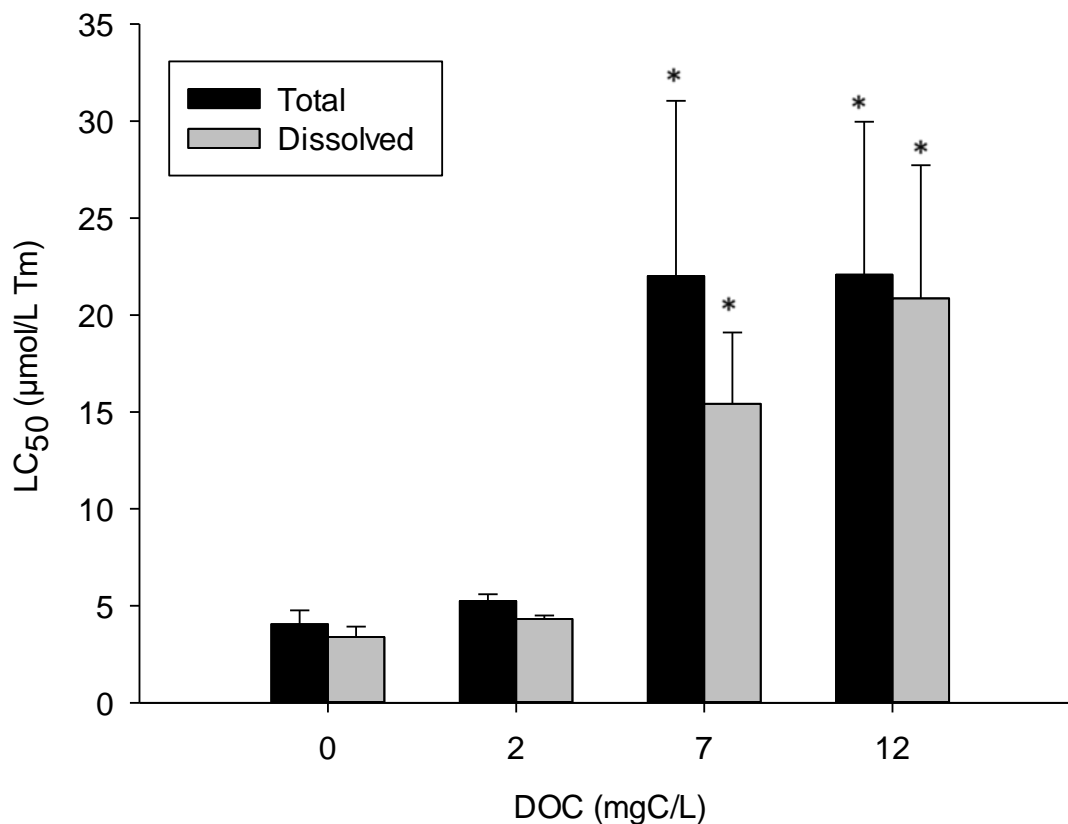


Figure 2.6: LC₅₀ values (with upper 95% confidence intervals) for *Hyalella azteca* exposure to Tm at different Luther Marsh DOC concentrations. The LC₅₀ values are based on 96h tests and measured total (Tm-T black bars) and measured dissolved (Tm-D grey bars) concentrations. The stars indicate LC₅₀ values significantly different for either Tm-T or Tm-D from acute test in RM at 0 mg C/L DOC.

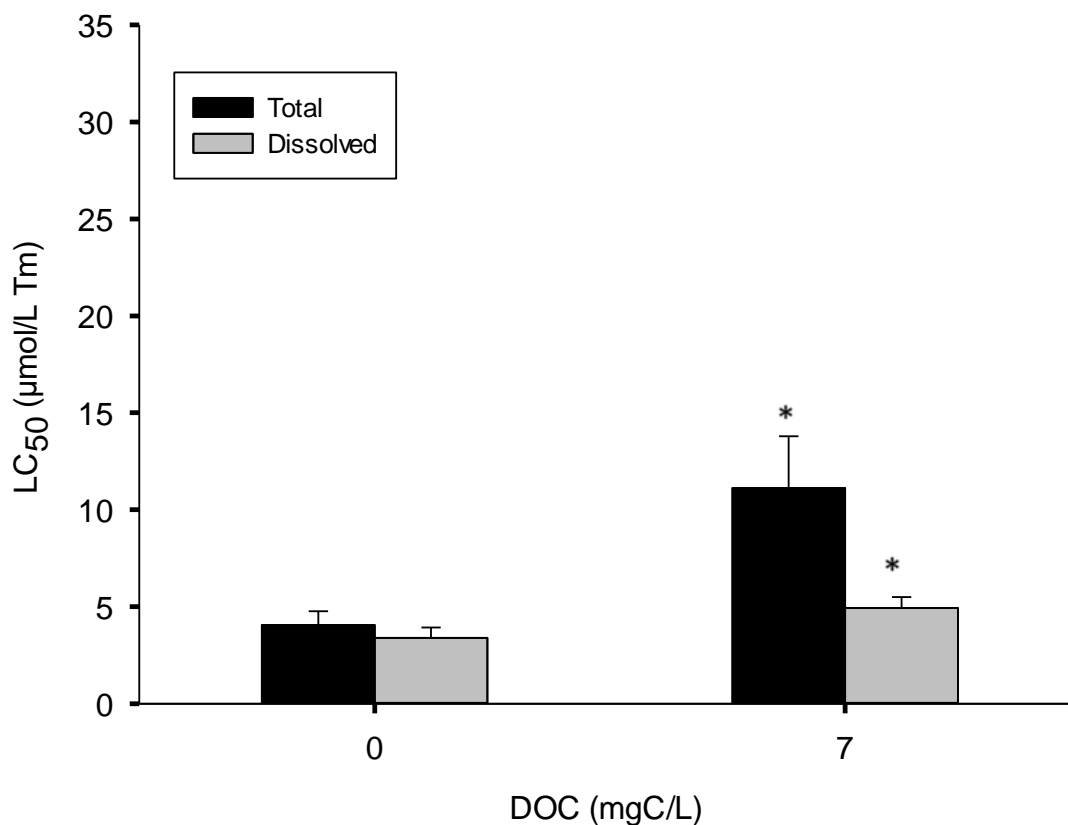


Figure 2.7: LC₅₀ values (with upper 95% confidence intervals) for *Hyalella azteca* exposure to Tm at a Koughibouguac DOC concentrations of 7mg C/L. The LC₅₀ values are based on 96h tests and measured total (Tm-T black bars) and measured dissolved (Tm-D grey bars) concentrations. The stars indicate LC₅₀ values significantly different for either Tm-T or Tm-D from acute test in RM at 0 mg C/L DOC.

Chapter 3: Chronic Tm Toxicity

3.1 Introduction to Chronic Tm Toxicity

3.1.1 Global Demand of Rare Earths and Potential Environmental Concern

Rare earth elements (REEs) are a group of 17 metals with similar chemical and physical properties that include 15 lanthanides as well as yttrium and scandium (Environment Canada, 2013). REEs are not rare and crustal abundance is similar to other metals such as copper or lead (Castor and Hedrick, 2006). However, enriched REE deposits are uncommon (Humphries 2013; Paul and Campbell, 2011). Global demand for REEs has increased dramatically with predictions of increased production nearly tripling over the next 25 years (Humphries 2013). Increases in demand are attributed to a growing number of uses related to their optical, magnetic and catalytic properties (Alonso et al., 2012). Canada is home to some of the largest REE deposits, particularly heavy REEs (elements 64 to 71; Y, Tb, Dy, Ho, Er, Tm, Yb and Lu) potentially making it a leading global producer (Humphries 2013).

There is limited information about potential environmental impacts of REEs in aquatic systems. This is particularly the case for Thulium (Tm). To my knowledge however, there is only one study where Tm toxicity was evaluated. Borgmann et al. (2005) found Tm to be highly toxic to *Hyalella azteca* with a reported measured dissolved LC₅₀ value of 0.01 µg/L or 5.9 nmol/L (reported nominal LC₅₀ value of 721 µg/L). This result was from exposures in very soft waters (12.4 mg CaCO₃/L) Borgmann et al. (2005). Borgmann et al. (2005) also conducted tests in water with an elevated hardness (Lake Ontario, hardness of 124 mg CaCO₃/L) and reported a LC₅₀ of 739 µg/L. However, this value was a nominal value and measured values were only reported for tests done in the soft water. The difference in nominal and measured LC₅₀ values for soft water tests, indicates that precipitation was occurring with Tm exposure solutions. With both nominal results being similar and no measured result reported for Tm exposures in the hard

water medium, it is hard to interpret if water hardness had any mitigating effect for Tm toxicity. Since there is only limited information available, to gain a greater understanding of Tm toxicity, more research is needed.

3.1.2 Toxicity Modifying Factors (TMFs)

For many well studied metals, acute toxicity results from the uptake of free metal ions into the organism and the resulting disruption of essential ion balance (Niyogi and Wood, 2004). Water chemistry is a major influence on the toxicity of metals. Cations (eg. H^+ , Ca^{2+} , Na^+ and Mg^{2+}), dissolved organic matter (DOM) and inorganic ligands (e.g. Cl^- and carbonates) have been known to modulate responses (El-Akl et al., 2015). These factors can be grouped into two general categories: complexation where a negatively charged ligand complexes the metal free ion and reduces bioavailability; competition where cations compete for uptake at the site of toxicity (Santore et al., 2001). Complexation and competition can dramatically alter toxicity. For example, in a study by Vukov et al. (2016), the addition of Ca^{2+} in solution with Dy exposure to *H. azteca* found to decrease toxicity significantly. In that same study, increased concentrations of DOM positively correlated with decreased toxicity. To develop data for water quality criteria and guidelines for Tm, not only is it important to gain an understanding of its toxicity but also how TMFs might influence responses. An improved understanding of the site specific toxicity of Tm will be useful for application in risk assessment.

DOM is ubiquitous in aquatic systems and plays an important role in mitigating metal toxicity (Wood et al., 2011). It arises from both autochthonous and terrigenous inputs and there are significant differences in composition among sources (Al-Reasi et al., 2011). This variability in composition can result in significant differences in the protective capacity of DOM for metals (Al-Reasi et al., 2013; Wood et al., 2011). DOM is a large, heterogeneous, complex molecule

and metal will bind to functional groups such as carboxylates and phenols (Al-Reasi et al., 2013). With growing amounts of research on DOM, it has become well understood of its importance in metal toxicity mitigation and therefore, has become a key variable in predicting site specific metal toxicity (Wood et al., 2011).

Toxicity reduction through cationic competition occurs because the free metal ion form of the dissolved metal is generally considered to be the most toxic form (Di Toro et al., 2001). This is particularly the case for monovalent and divalent metals such as Cu^{2+} , Zn^{2+} , Pb^{2+} , Ag^+ and Co^{2+} (Niyogi and Wood, 2004). However, it is not known if this is the case for REEs, which occur as trivalent ions. In previous research, La toxicity to *D.carinata* was shown to decrease with increased hardness (Barry and Mehan, 2000). A positive correlation between Ca^{2+} and LC_{50} was also seen in Dy toxicity to *H. azteca* (Vukov et al., 2016). Borgmann et al. (2005) also saw decreases in some REE toxicity with an increase in water hardness although in that study water chemistry was altered by dilution and therefore changes in hardness co-occurred with changes in other TMFs including DOM. These acute toxicity studies illustrate that cations such as Ca and Mg may compete antagonistically with REE^{3+} cations for uptake and/or binding to the site of toxic action.

3.1.3 The Biotic Ligand Model Approach

The Biotic Ligand Model (BLM) is a tool that has become widely accepted for the site specific assessment of toxicity (Niyogi and Wood, 2004). This tool predicts toxicity of a metal based on the bioavailability of the free metal ion and incorporates the influences of TMFs such as cationic competition and complexation with inorganic and organic ligands (Paquin et al., 2000). Essentially the BLM defines toxicity through accumulation thresholds of the free metal ion at the site of action (the biotic ligand). Since very little is known about the toxicity of REEs,

we have applied a BLM approach to understand how TMFs influence Tm toxicity and if indeed the BLM is a tool that can be used to predict the bioavailability of REEs.

3.1.4 Hyalella azteca: A Sensitive Invertebrate

In this study *H. azteca* was the species used for Tm toxicity tests and it was chosen so for a number of reasons. *H. azteca* are a species of amphipods that are commonly found in fresh water lakes, streams and marshes across North America (Environment Canada, 2013). *H. azteca* reproduction in lab is continuous and therefore harvest of neonates for testing is predictable (Environment Canada, 2013). They have been used widely for toxicity tests for many decades due to their sensitivity to contaminants. Environment Canada has a standard biological tests method for culturing and testing of this species for both water only toxicity and sediment toxicity tests (Environment Canada, 2013). Within this standard method, based on the study of the ion requirements of *H. azteca* (Borgmann, 1996) a standard artificial medium is provided. This standard artificial medium allows consistency and comparability between *H. azteca* toxicity tests.

3.1.5 Chronic Testing Relativity

Acute toxicity tests provide information about responses to high concentrations of a contaminant. Chronic tests are over much longer periods of time and at much lower doses which provide information about sublethal responses such as the inhibition of growth or reductions in brood size. While acute tests can give us information about the level of toxicity of a metal, chronic tests are important to do to reflect toxicity that is more relative to contamination that would occur in natural waters. For these reasons, chronic studies were conducted on the Tm toxicity to *H. azteca*.

3.2 Objectives

The objective of this study was to develop chronic toxicity data on Tm to a sensitive aquatic invertebrate and to also gain an understanding of the influence of TMFs. The main goals are to assess if Tm acute toxicity is influenced by changing water hardness by changing Ca^{2+} concentrations as well as to assess if Tm complexes with DOM, therefore decreasing its bioavailability. By assessing the chronic toxicity of Tm, we also aim to determine linkages between bioaccumulation and growth and survival during chronic exposures.

We hypothesize that Tm will not only have adverse acute effects on *H.azteca* but that the tested TMFs will reduce the adverse effects of Tm toxicity. We also hypothesize that toxicity will affect growth and that bioaccumulation of Tm can be linked to these effects.

3.3 Methods

3.3.1 *H. azteca* culturing

Culture and test procedures followed the Environment Canada method for *Hyalella azteca* (Environment Canada, 2013) and organisms were originally collected from the shore of Eabamet Lake, ON and maintained in the lab for 2 y. Culture and testing was in a reconstituted medium (RM) as described by Vukov et al (2016) and made with analytical grade CaCl_2 , NaHCO_3 , MgSO_4 , KCL and NaBr (Sigma-Aldrich, Mississauga, ON) at 500, 500, 125, 25 and $5\mu\text{M}$ respectively to give a hardness of 60 (mg CaCO_3/L) and pH of 7.6 ± 0.2 . Cultures of 20-30 adults were kept in 2L beakers with 1600 ml of RM and held at $23^\circ\text{C} \pm 2$ in an incubator (LTCB-19 BioChamber, BioChambers Inc., Winnipeg MN) with full spectrum lighting at 400 to 1,000 lux and a 16:8hr light: dark photoperiod. *H. azteca* were fed on Mon., Wed. and Fri. with 5 mg of finely ground tropical fish food (TetraMin, Tetra, Blacksburg, VA). Neonates between 0 and 7 d of age were separated from cultures at the weekly media renewal using 650 and 275 μm

mesh polyethylene mesh. At RM renewal, a fresh piece of cotton gauze (approx. 10 x 5cm) was added to the beakers (Borgmann et al., 1989).

3.3.2 Chronic Tm Toxicity Tests

Standard 14 d water only static renewal tests (Environment Canada, 2013) were done at 3 different concentrations of Ca and with added NOM. Mortality, dry weight of survivors and bioaccumulation were the endpoints and tests were done maintaining the temperature and photoperiod of the cultures. Tests were initiated with 0-3 fold neonates exposed to one of 7 concentrations (including control) of Tm. A 30 mg/L Tm stock solution was made from an analytical standard (Inorganic Ventures Inc., Christiansburg, VA) that was adjusted to a pH of 7.6 ± 0.2 . Exposure solutions (4.5 L of each conc.) sufficient for the 14 d test were equilibrated for 24 hours prior to test start. Exposures were done in triplicate with 10 neonates in 400 mL polyethylene beakers with 250 mL of test solution. Each beaker had a 10 cm by 5 cm piece of gauze added following equilibration in a 40 mL plastic cup with 10 mL of the exposure solution. Water changes were done three times weekly at which time mortalities were removed and 2.5 mg of food was given. Water samples (15 mL) were collected at the beginning (day 0) and end (day 14) of the test as well as each water change. Two samples were collected as each of these times, for both renewal solutions and old solutions for a total of 4 samples at each water change. One sample was not filtered (total Tm: Tm_T) and the other was filtered (dissolved Tm: Tm_D) through a 45 µm filter (HT Tuffryn polysulfone membrane, Acrodisc, Pall). All water samples were acidified to 2% (v/v) with concentrated (16N) HNO₃ (trace metals grade, Fischer Scientific, Nepean, ON). All samples were stored in 15mL tubes (Celltreat, Mandel Scientific, Guelph, ON). On day 14 surviving neonates were rinsed briefly with deionized water gently, blotted with a tissue to removed excess water then dried in an oven at 60°C for 12 h before being weighed

(Sartorius Ultramicrobalance SE2, NSERC). Individual amphipods were then grouped by replicate for each concentration and then digested in 100 µl of concentrated nitric acid for one week prior to the addition of 10 µL of 30% H₂O₂ (Sigma-Aldrich, Mississauga, ON) and then dilution prior for analysis for Tm content (see below).

3.3.3 Chronic tests and TMFs

Based on 96h acute toxicity tests with Tm which showed that Ca and NOM (but not Na or Mg) mitigated toxicity we tested the effects of Ca and DOC in chronic Tm tests. Tests for the effect of Ca were done by preparing volumes of medium sufficient for all test solutions at either 0.25mM, 0.5 mM (i.e. unmodified RM) or 1.5 mM CaCl₂. Similarly, the effect of DOC on Tm toxicity was tested by the addition of NOM collected from Luther Marsh (Luther Marsh, Grand Valley, ON) at a nominal concentration of 7 mg DOC/L (Table A2). For tests with NOM additional samples (50 mL of a 0.45 µm filtered solution, as described for Tm-D but not acidified) were collected at day 0, at each water change and at day 14.

3.3.4 Sample Measurements and Calculation and Statistics

Tm concentrations for water samples (Tm-T, Tm-D) as well as grouped whole body digests were measured using inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 8000, Perkin-Elmer Inc., Woodbridge, ON) and solution cations (Ca, Na, Mg) in water samples were measured using an atomic absorption spectrophotometer (AAS, SpectAA-880, Varian Inc., PaloAlto, CA) in flame mode. Analysis parameters and wavelengths followed the manufacturer guidelines and recommendations with appropriate standards (Inorganic Ventures Inc. Christianburg VA). DOC concentrations were measured using a total organic carbon analyzer (TOC-LCPH, Shimadzu Corporation, Mandel Scientific, Guelph, ON) as non-

purgeable organic carbon. Exposure concentrations associated with 50% lethality (LC₅₀ and 95% confidence interval) were calculated by using the average of measured Tm concentrations across the entire 14d test. An LC₅₀ was calculated using these average concentrations for both Tm-T and Tm-D using probit analysis in SPSS Statistics software (IBM Corp. Version 22, 2013).

Significant differences between LC₅₀ values were assessed using the Litchfield and Wilcoxon (1949) method as given in Environment Canada (2005). One-way ANOVA test with Dunnett's post hoc comparison was run on SPSS Statistics (IBM Corp. Version 22, 2013) to find significant differences between dry weights of exposed neonates and control neonates in all exposure tests. EC₂₀ estimates were completed using regression lines and equations made by Excel (Microsoft, 2016).

3.4 Results

3.4.1 Chronic Tm Toxicity and Effects of TMFs

Much like the acute tests, the results for effects of Ca showed significantly lower LC₅₀ values for both Tm-T and Tm-D at the 0.5 mM Ca treatment (unaltered RM medium) than LC₅₀ values for the 0.3mM treatment (Fig. 1). For the 1.5 mM Ca treatment, the LC₅₀ values did increase however, there were no significant differences. The Luther Marsh DOC treatment with 7 mg C/L showed an significant increase in LC₅₀ for both Tm-T and Tm-D (Fig. 2)

3.4.2 Tm Exposure and Growth Inhibition of H.azteca

For all the chronic exposure tests, growth inhibition in dry weight of *H. azteca* was observed in surviving neonates (Fig. 3). One-way ANOVA's indicated that there were significant differences between groups in all of the tests. However, a multiple comparisons test indicated that in the 14d chronic exposure in the RM, only dry weights of neonates at 1.6µM of dissolved measured Tm, was significantly different from the control (Fig. 3a). In the test exposure with

1.5mM Ca, all exposed neonate dry weights were significantly different from the control (Fig. 3b) while at 0.3mM Ca, only exposed neonates at 0.26 μ M Tm and above were significantly different from the control (Fig. 3c). However, neonates in the 7mgC/L exposure test, showed a general decrease in dry weight with increasing exposure concentration but the multiple comparisons test showed that only dry weights of exposed neonates at a concentration of 2.1 μ M Tm were significantly different from controls (Fig. 3d).

Percent inhibition showed that in all tests, the dry weights of the neonates at the highest surviving concentrations were always between 47-50% the weight of the control (Fig 40). No significant differences in EC₂₅ and EC₅₀ values were seen in any of the Ca treatments (Table 2). However, there was a significant increase in EC₂₅ and EC₅₀ values in the 7 mg C/L DOC treatment when compared to the test done in RM (Table 2).

3.4.3 Bioaccumulation of Tm in *H.azteca*

Since only surviving neonates were used to assess bioaccumulation, the highest exposure concentrations where mortality was 100% were not included. However, bioaccumulation in *H.azteca* was observed in all tests with bioaccumulation increasing as Tm-D concentrations increased (Fig. 5). The addition of Ca²⁺ decreased the amount of bioaccumulation in *H. azteca* with a slope of 121.98 at 0.3mM Ca, 65.783 at 0.5mM Ca and 27.525 at 1.5mM Ca. At a exposure concentration of 3 μ M Tm-D, estimates were made using linear regression line in Excel to find that the amount of Tm accumulated in *H. azteca* at 0.3mM Ca was 354.8 μ g Tm/g. At 0.5mM Ca, 194.8 μ g Tm/g accumulated and at 1.5mM Ca 105.4 μ g Tm/g accumulated. The experiment with Luther Marsh DOC showed accumulation to be 120.3 μ g Tm/g, also showing a decrease in accumulation in tissues compared to tests done in RM (unaltered medium). As Tm accumulation increased in *H. azteca* tissues, *H. azteca* growth (%) decreased (Fig. 3.6).

3.4.5 Water Chemistry during chronic tests

The pH of solutions on average remained between 7.4 ± 0.2 (Table 1). Mean Ca, Mg concentrations were consistently around the nominal concentration between tests and between Tm concentrations. However, Na concentrations increased as DOC concentrations increased. In the new solutions (fresh renewal solutions) the Tm-T and Tm-D were typically similar (within 90-100% of each other). However, Tm-T and Tm-D concentrations all decrease from new to old solutions except for at the highest concentrations. This effect seemed to reverse in the DOC test, where both Tm-T and Tm-D concentrations are consistent between old and new solutions, except at the highest exposure concentrations.

3.5 Discussion

3.5.1 Precipitation of Tm in Chronic Exposure Solutions

A significant amount of precipitation occurred in the 14d chronic tests, particularly within the first 24 h of making the solutions and this was similar to the precipitation that occurred in our acute tests (see Chap 2). Previous literature has shown significant loss of REE from solutions during toxicity tests (Barry and Meehan, 2000; El-Akl et al., 2015; Herrmann et al., 2016; 2000; Vukov et al., 2016) particularly at elevated concentrations and therefore we hypothesized that there might be an increase in precipitation as concentrations increased. Accordingly, sampling methods were adjusted from the standard Environment Canada methods and we measured the total and dissolved metal in the incoming solution (new solution) and then the replaced exposure solution (old solution) at each water change to account for this possible loss. From the Tm-T and Tm-D concentrations, it was evident that there were significant losses of Tm from solution during the initial equilibration and also during the tests (Table 1). Tm-T and Tm-D concentrations of the new solutions were very similar throughout the test. However, in old solutions both Tm-T and

Tm-D decreased compared to the new solutions. This proved to be surprising since because there was an increase in DOC (with feeding during test), we hypothesized more Tm would be brought into solution.

The loss of Tm in test solutions increased with increasing concentration and this could have been due to complexation causing low solubility. Previous studies suggest that a feature of REE tests done in artificial media is the formation of insoluble species such as hydroxides, carbonates and phosphate complexes (Gonzalez et al., 2015). While these results show the importance of characterizing water chemistry for REE toxicity tests, many studies which have analyzed the toxicity of different REEs to aquatic biota either did not report all measured concentrations for LC₅₀ values (Borgmann et al, 2005) or took a mean of measured values and nominal values to produce LC₅₀ values (Barry and Meehan, 2000). Due to the variability of measured concentrations throughout the test, we based our LC₅₀ values on an average of all measured Tm-T and Tm-D taken throughout the entire test to reflect the true exposure of Tm to *H. azteca*. Basing our results on nominal concentrations would underestimate the true toxicity of Tm (Gonzalez et al., 2014). Our results reflect the importance of water chemistry characterization in Tm toxicity tests. More research is required to understand Tm speciation in an artificial aquatic medium and how total and dissolved Tm effects toxicity.

3.5.2 Chronic Tm Lethality to H. azteca and the Effect of TMFs

In our 14 d chronic Tm exposure to *H.azteca*, we obtained a dissolved measured LC₅₀ of 0.84 µM/L which is much greater than the acute measured LC₅₀ of 5.9 nM reported by Borgmann et al. (2005). In the same study, the reported nominal LC50 values for both the hard and soft water treatments were similar, with nominal being 721µg/L for the soft water medium and 739 µg/L for the relatively hard water medium. This result shows that hardness may not have

an effect on Tm toxicity. Our study showed similar results where Ca did not have a protective effect when Ca concentrations were increased (Fig. 1). However, a measured LC₅₀ was not reported for Borgmann et al. (2005) results in hard water making interpretation difficult. In our results, there was no significant difference between LC₅₀ values when Ca concentrations were increased to 1.5 mM from the 0.5 mM but when Ca concentrations were decreased to 0.3 mM, we did see a significant increase in the LC₅₀ for both Tm-T and Tm-D. This result shows that decreased Ca concentrations in fact had a protective effect. There is little research that can explain such observations but by examining the data, it can be seen that while the water chemistry does stay relatively consistent throughout the tests, the amount of total and dissolved concentrations of Tm appear to change between the tests. Average concentrations of Tm-T and Tm-D in old solutions, are highly variable between tests with different Ca concentrations perhaps making Tm exposure to *H. azteca* also variable. However, further research on Tm speciation and effects of Tm-T and Tm-D on toxicity are required.

We chose to examine if increasing concentrations of Ca had a protective effect on Tm toxicity based on previous studies where results showed a competitive effect between REEs and Ca. Vukov et al. (2016) found that by increasing Ca 3-fold, he had an increase in LC₅₀ by 1.8-fold. Other comparisons are with a study by Barry and Meehan (2000) where increased hardness from 98 to 160 mg CaCO₃/L resulted in an almost 6-fold increase in EC₅₀. However, in this study, all three aquatic media had very different water chemistries. Borgmann et al. (2005) had a similar water chemistry to our tests and as mentioned above did not see a change in nominal LC₅₀ between tests with different hardness, however as mentioned above these were nominal results. Also, Borgmann et al. (2005) diluted his hard water medium to obtain the soft water medium thus, altering the entire water chemistry where in our study we specifically changed

waterborne Ca. All of these referenced studies were either acute 48 or 96 h studies. Chronic REE studies are limited and to my knowledge did not examine the effects of Ca.

Based on our acute and chronic results, we found that the acute to chronic ratio was 4:1, indicating a much greater toxicity in chronic tests. A chronic study done on *D. carinata* using Lanthanum (La) found 100% mortality in concentrations greater than 52 μ g/L (Barry and Meehan, 2000). No LC₅₀ was reported for these chronic tests making comparison of sensitivities and toxicity of metals difficult. More chronic research on REE is definitely required.

In the chronic tests with 7 mg C/L of Luther Marsh DOC, we found that DOC does have a protective effect for Tm toxicity. Significant differences were seen in both Tm-T and Tm-D LC₅₀ values when DOC was added with approximately a 2.5-fold increase in these values. It is well understood that metal complexation with DOC makes it unavailable to cause toxicity (Al-Reasi et al., 2013; Wood et al, 2011). Also, studies with Dy showed that there was a positive correlation between increasing DOC concentration and LC₅₀ for measured dissolved Dy (Vukov et al., 2016). However, there are studies with opposing results indicating that some REE complexes with organic ligands can be bioavailable and therefore perhaps also toxic (Zhao and Wlikinson, 2015). Further study is required to understand the effects of DOC and perhaps different DOC sources on REE toxicity and speciation.

In comparison to our acute Tm test result, we found that there was an acute to chronic ratio (ACR) of approximately 4. There is little literature, if any that describes ACRs for any REE. However, Mu et al., (2014) reviewed the ACRs of 9 metals or metalloids to predict criteria continuous concentrations (CCCs). In this study, an ACR of 4 was reported for Aluminum using *D. magna*. Al is also a trivalent ion, like Tm and with this similar reported ACR, it can be

hypothesized that Tm and Al may have similar toxicity. However, further study comparisons would have to be made to make any conclusions.

3.5.3 Growth inhibition in *H.azteca*

In general, inhibition of growth was seen in *H.azteca* across all tests (Fig. 3). At the highest exposure concentrations with surviving organisms the average dry weight after 14 d was between 40-50% of the dry weight of controls at 14 d (Fig 4). A One-way ANOVA was run along with a post-hoc multiple comparison of means test to show the significant differences between average dry weight of controls and those exposed within each test. Significant differences from the control in dry weights were observed only for *H. azteca* exposed to 1.6 μM Tm-D in the 0.5 mM Ca test (Fig. 3a). The test with 1.5 mM Ca however, significant differences in dry weight were observed in all exposure concentrations compared to the control (Fig. 3b). Significant differences were seen in *H. azteca* exposed from 0.26 μM Tm-D concentrations and above in the 0.3 mM Ca test (Fig. 3c). Although our mortality results did not show a protective effect with increased concentrations of Ca, we did find that the EC₂₀ increased with increasing concentration of Ca. While these results may infer that Tm³⁺ and Ca²⁺ may actually be competitive and increased concentrations of Ca may protect from Tm toxicity, there are other possible explanations. For instance, there is some evidence that in certain species of Arthropoda, exoskeleton growth and calcification has a positive correlation to ambient Ca²⁺ concentrations (Rukke, 2002; Waervagen et al., 2016). Increased calcification of the exoskeleton can increase dry weight of the organism (Waervagen et al., 2016). Therefore, it is possible that the correlation between Ca concentrations and EC₂₀ for growth inhibition was not necessarily due to a competitive effect between Ca²⁺ and Tm³⁺ but an effect of increasing Ca concentrations in

general. Previous studies on lanthanum (La) as a Ca blocker show that La can either mimic or displace Ca from its binding sites in human skin cells (Pillai and Bikle, 1992). Studies with such results indicate that La may mimic Ca at cellular sites of action. However, research specifically on Tm interaction with Ca and with invertebrates would be necessary to make definitive conclusions. The Luther Marsh DOC test had the highest EC₂₀ value of 2.19 μM Tm-D (Table 2). It is well understood that complexation of the metal with DOC can make it unavailable to bind at the site of action to cause toxicity (Niyogi and Wood, 2004; Paquin et al., 2000). Although growth inhibition was seen in this test, there was only one significant difference in dry weight from the control (Fig. 3d). This result could be due to possible hormesis observed in the first two exposure concentrations where low exposure concentrations actually increased growth of the neonates compared to controls. The growth inhibition results for the DOC test correspond with our lethality results and verifies that DOC indeed has a strong protective effect in Tm toxicity.

3.5.4 Tm Bioaccumulation in H. azteca and Links to Growth Inhibition

In all Tm exposure tests, bioaccumulation of Tm was measured in the surviving organisms. Also, we found a positive correlation between Tm-D and the amount of Tm measured in the *H. azteca* tissue (Fig. 5). However, contrary to survival results, increased concentrations of Ca reduced the amount of Tm that bioaccumulated in *H. azteca* tissue (Fig. 5). These results lead to the conclusion that bioaccumulation may not be a good predictor of survival. DOC also reduced the amount of bioaccumulation. Little research, if any has been done to investigate the chronic effects of REEs to any aquatic biota. Therefore, comparisons to our research are difficult. Barry and Meehan (2000) looked at chronic effects of La to *D. carinata* but did not

measure accumulation. Winner et al. (1986) measured chronic toxicity of Cu, Cd and Zn in *D. magna* and *D. pulex* and found that changes in toxicity when TMFs were altered did not correspond or have a consistent relationship with bioaccumulation in the organism. While this study agrees with our results there are other studies with different metals where bioaccumulation was a good predictor of toxicity. In a study on thallium (Tl) bioaccumulation, Borgmann et al. (1998) found that body concentrations in *H. azteca* were actually better predictors of toxicity where Tl uptake was proportional to Tl in the water and consistent between treatments. Further analysis of bioaccumulation data such as calculation of lethal body concentration (LBC) is required to fully understand bioaccumulation effects with Tm. Also, more research is required to understand mechanisms that may cause accumulation and toxicity causing lethality to not correlate.

3.6 Conclusions

In conclusion to our study on the chronic effects of Tm to *H. azteca*, we found that significant precipitation of Tm made water chemistry characterization important for REE study. More research is required to understand speciation of Tm and other REEs when in solution. We were able to conclude that DOC has a strong protective effect, reducing lethality, growth inhibition and even reducing the amount of Tm accumulated in *H. azteca* tissue. Results with Ca were much more complicated to interpret. In survival tests, it appears that there is no cationic competition between Tm^{3+} and Ca^{2+} since no significant differences were observed between LC_{50} values. However, we observed a positive correlation between Ca concentrations and EC_{20} values for growth inhibition of *H. azteca*. However, conclusions of whether there is a protective effect

of Ca or if increased ambient Ca^{2+} increases growth in general can not be made. Finally, bioaccumulation of Tm was observed in tissues in all exposure tests. A positive correlation between Tm-D and measured Tm in tissues was evident. Unexpected results showed that increased Ca concentrations were negatively correlated with the degree of bioaccumulation of Tm in tissue. This leads to the conclusion that bioaccumulation may not be a good predictor of survival. Linkages between bioaccumulation and chronic effects cannot be determined until further research is done. Future analysis will investigate this feature of the data.

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Table 3.1: Measured water chemistry data from chronic Tm toxicity tests after 14d and corresponding Tm toxicity to *H. azteca* as either total (T) or dissolved (D). All concentrations are in $\mu\text{g/L}$ except for pH, DOC (mg C/L) and Ca, Mg, and Na (mM). Differences (%) in total and dissolved concentrations are shown for old and new solutions during the tests as well as average total and dissolved concentrations taken from all water changes throughout the 14d tests where $n = 6$.

Test Type	Initial			Final			New			Old			Average Con. of entire test			Avg. % Mortality	
	Mg (mM)	Na (mM)	pH	pH	DOC(mgC/L)	Nominal	T	D	D/T%	T	D	D/T%	$T_{old}/T_{new}\%$	$D_{old}/D_{new}\%$	T		D
0.5mM Ca	0.49	0.26	0.56	7.50	7.43	1.18	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	18
	0.49	0.26	0.56	7.58	7.49	1.18	50	18.69	12.12	64.84	10.03	9.49	94.69	53.66	78.36	13.96	10.84
	0.49	0.26	0.56	7.58	7.51	1.18	100	64.89	63.92	98.50	41.80	23.97	57.34	64.41	37.49	52.76	41.12
	0.49	0.26	0.56	7.61	7.52	1.18	200	122.32	119.42	97.63	85.56	65.79	76.89	69.94	55.09	102.04	89.72
	0.49	0.26	0.56	7.59	7.52	1.18	400	338.00	325.14	96.20	300.08	236.41	78.78	88.78	72.71	318.53	275.15
	0.49	0.26	0.56	7.66	7.49	1.18	800	671.88	621.85	92.55	781.18	636.31	81.46	116.27	102.33	727.85	623.40
1.5mM Ca	1.49	0.26	0.52	7.41	7.32	1.06	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20
	1.49	0.26	0.52	7.48	7.34	1.06	50	46.37	43.75	94.35	13.62	5.61	41.21	29.36	12.82	29.45	24.08
	1.49	0.26	0.52	7.49	7.39	1.06	100	97.22	93.90	96.58	39.82	20.95	52.62	40.95	22.31	67.69	56.26
	1.49	0.26	0.52	7.48	7.42	1.06	200	178.82	175.55	98.17	107.94	74.10	68.65	60.37	42.21	142.01	123.14
	1.49	0.26	0.52	7.48	7.44	1.06	400	336.53	329.23	97.83	239.55	176.22	73.56	71.18	53.52	286.31	250.29
	1.49	0.26	0.52	7.41	7.48	1.06	800	599.47	595.99	99.42	466.68	353.22	75.69	77.85	59.27	534.37	479.92
0.3mM Ca	0.26	0.26	0.58	7.57	7.57	0.8	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10
	0.26	0.26	0.58	7.63	7.63	0.8	50	23.33	21.28	91.24	4.31	0.69	16.08	18.48	3.26	13.82	10.99
	0.26	0.26	0.58	7.61	7.61	0.8	100	83.13	80.11	96.36	44.52	8.12	18.23	53.55	10.13	63.83	44.11
	0.26	0.26	0.58	7.61	7.61	0.8	200	103.61	101.54	98.01	58.33	22.59	38.73	56.29	22.25	80.97	62.07
	0.26	0.26	0.58	7.64	7.64	0.8	400	384.30	371.43	96.65	275.41	228.13	82.83	71.67	61.42	329.86	299.78
	0.26	0.26	0.58	7.67	7.67	0.8	800	780.43	706.22	90.49	614.34	493.74	80.37	78.72	69.91	697.39	599.98
7mgC/L Lu	0.46	0.26	0.62	7.57	7.42	9.2	0	2.14	2.07	96.42	3.81	4.19	110.19	177.52	202.87	0.00	0.00
	0.46	0.26	0.62	7.59	7.44	9.2	200	98.69	95.26	96.52	100.78	96.92	96.17	102.11	101.75	99.83	96.11
	0.46	0.26	0.62	7.62	7.44	9.2	400	193.53	184.24	95.20	194.79	184.04	94.49	100.65	99.90	194.16	184.23
	0.46	0.26	0.62	7.63	7.47	9.2	800	393.74	361.81	91.89	395.50	362.88	91.75	100.45	100.30	394.09	362.45
	0.46	0.26	0.62	7.64	7.55	9.2	1600	673.36	464.59	69.00	578.39	492.57	85.16	85.90	106.02	820.91	640.25
	0.46	0.26	0.62	7.66	7.55	9.2	3200	2600.83	949.69	36.51	1532.23	1102.24	71.94	58.91	116.06	2005.25	1031.42

Table 2.2: Estimated EC₂₅ and EC₅₀ for Tm-D ($\mu\text{M Tm}$) for growth inhibition of *H. azteca* for 14d chronic toxicity tests.

Tests	EC₂₅ 95% C.I. ($\mu\text{M Tm}$)	EC₅₀ 95% C.I. ($\mu\text{M Tm}$)
0.3mM Ca	—	1.96 (1.63 – 2.41)
0.5 mM Ca	0.85 (0.44 - 1.18)	2.68 (2.30 - 3.23)
1.5 mM Ca	0.49 (0.09 – 0.80)	2.31 (1.92 – 2.93)
7 mg C/L L.M DOC	2.39 (2.03 – 2.70)	3.54 (3.22 – 3.96)

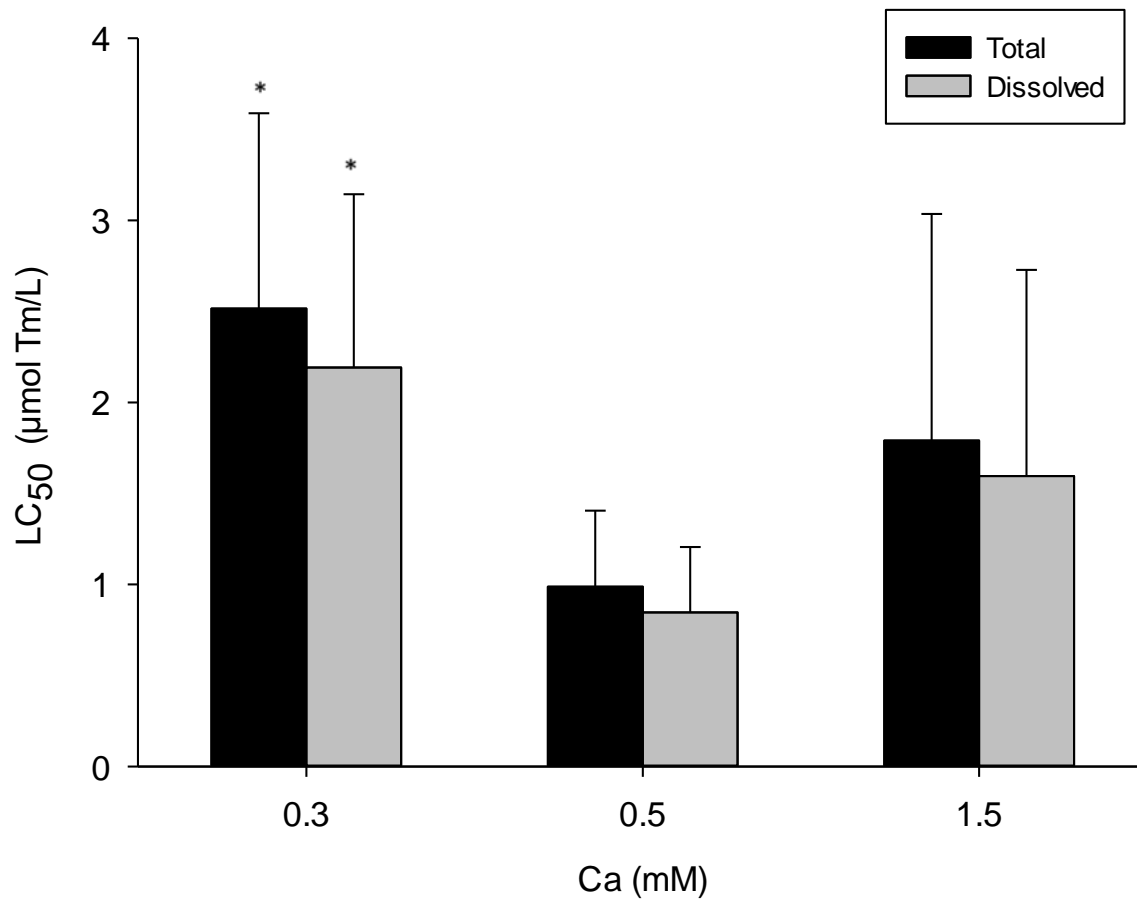


Figure 3.1: LC₅₀ values (with upper 95% confidence intervals) for *Hyalella azteca* exposure to Tm at different Ca concentrations. The LC₅₀ values are based on 14 d chronic tests and measured total (Tm-T black bars) and measured dissolved (Tm-D grey bars) concentrations. The stars indicate LC₅₀ values significantly different for either Tm-T or Tm-D from acute test in RM at 0.5 mM Ca.

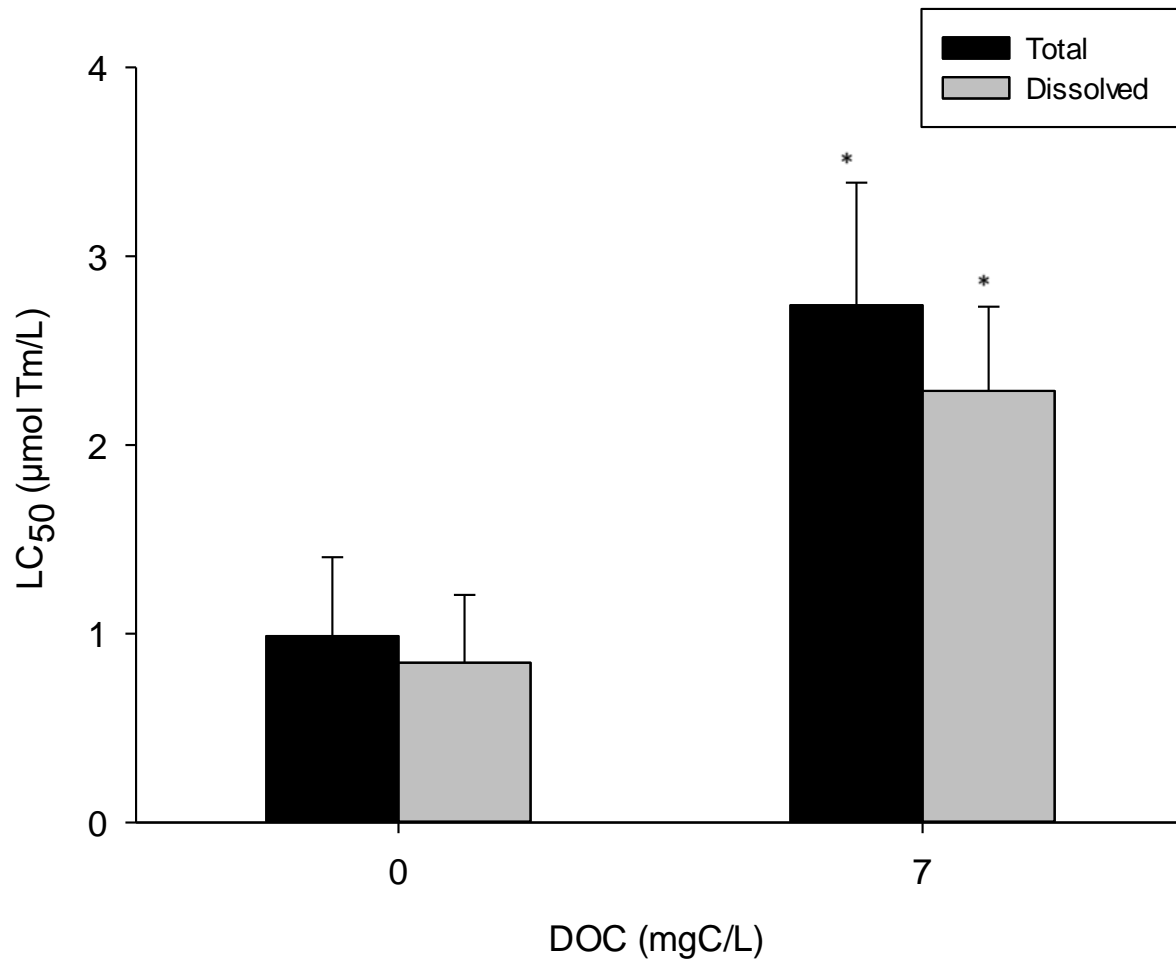


Figure 3.2: LC₅₀ values (with upper 95% confidence intervals) for *Hyalella azteca* exposure to Tm comparing tests done in RM at 0 mg C/L DOC and tests with 7 mg C/L Luther Marsh DOC. The LC₅₀ values are based on 96h tests and measured total (Tm-T black bars) and measured dissolved (Tm-D grey bars) concentrations. The stars indicate LC₅₀ values significantly different for either Tm-T or Tm-D from acute test in RM at 0.5

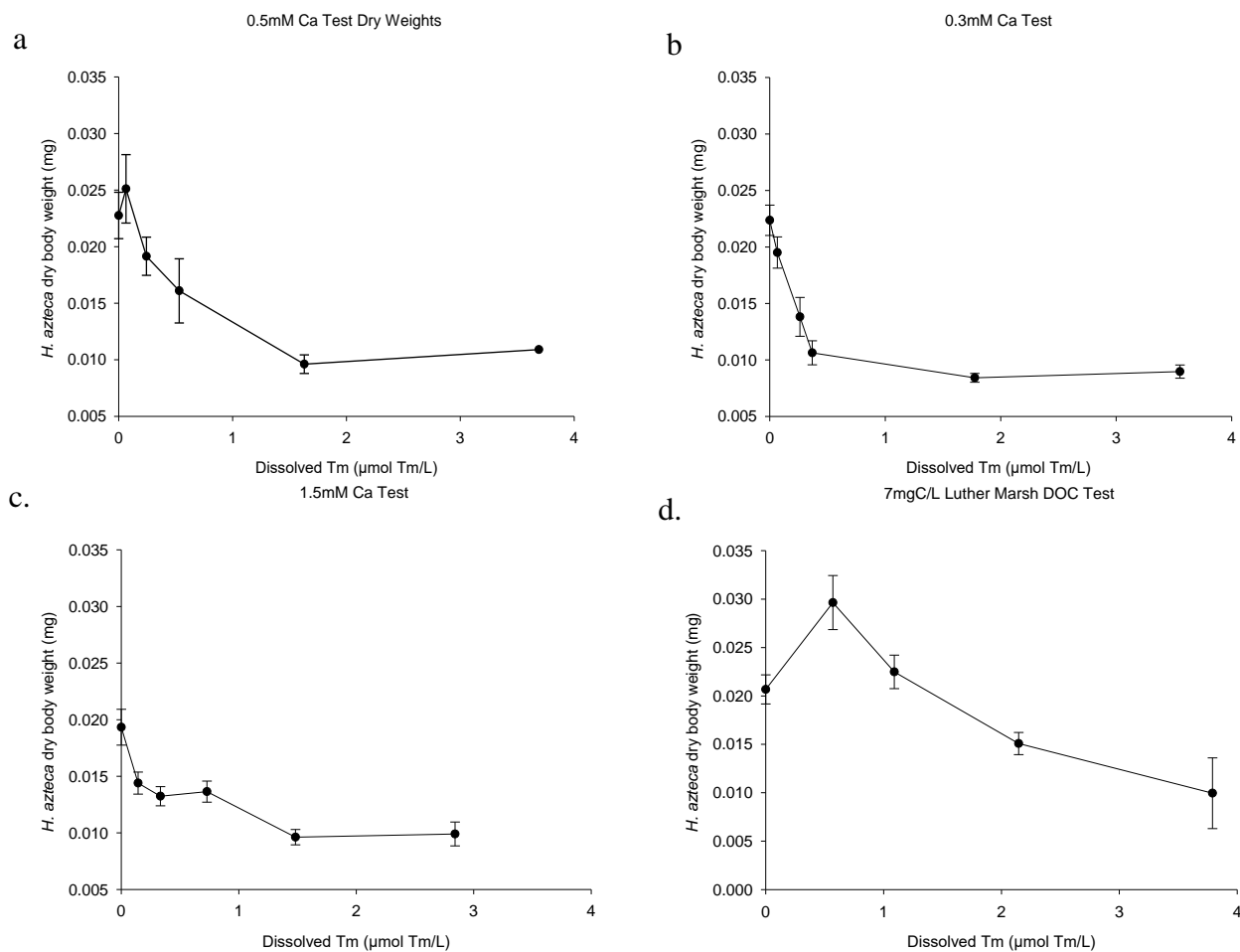


Figure 3.3: Dose response of *H. azteca* dry weights (mean \pm SE mg) as a function of dissolved Tm-D concentrations (average concentrations across 14d tests) for a) RM test with a Ca concentration of 0.5mM, b) at 1.5mM Ca, c) at 0.3mM Ca and d) with 7mgC/L

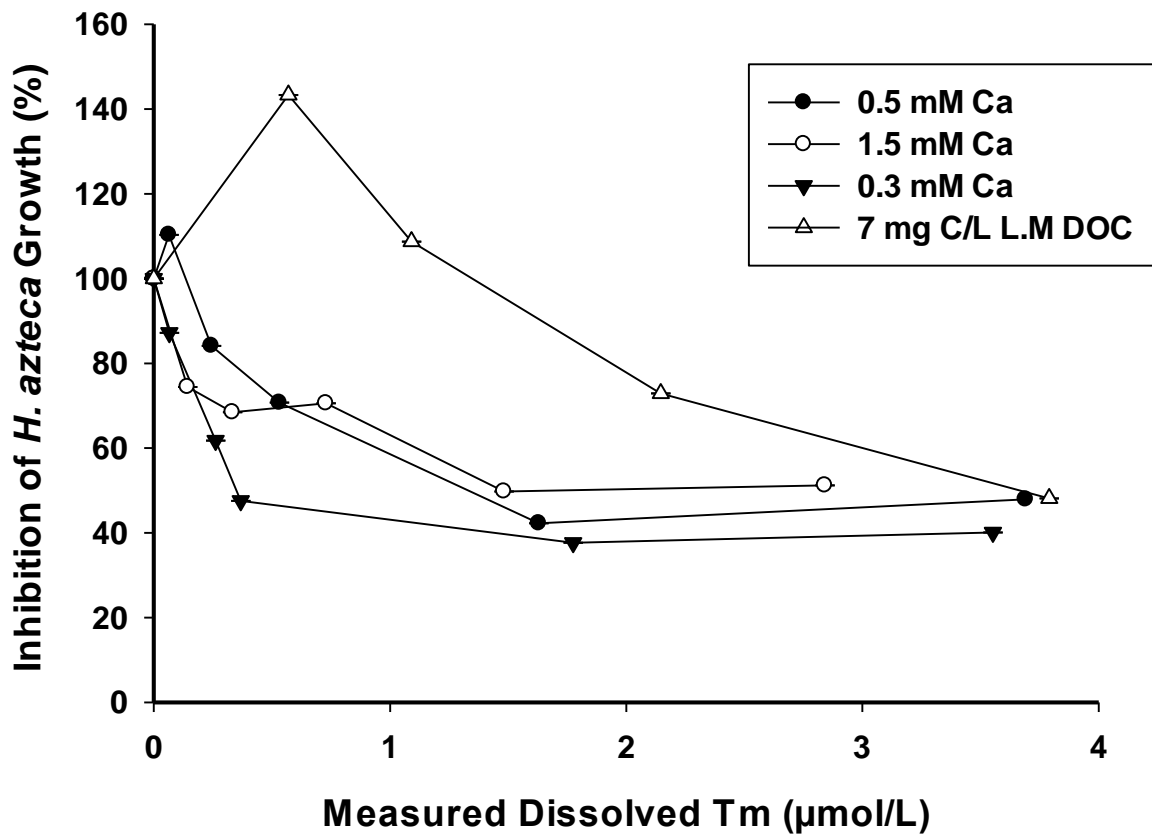


Figure 3.4: Growth inhibition (%) of *H. azteca* from control (100%) as a function of Tm-D concentrations (average of Tm-D concentrations over 14d tests) with error bars represented as ± 1 SEM.

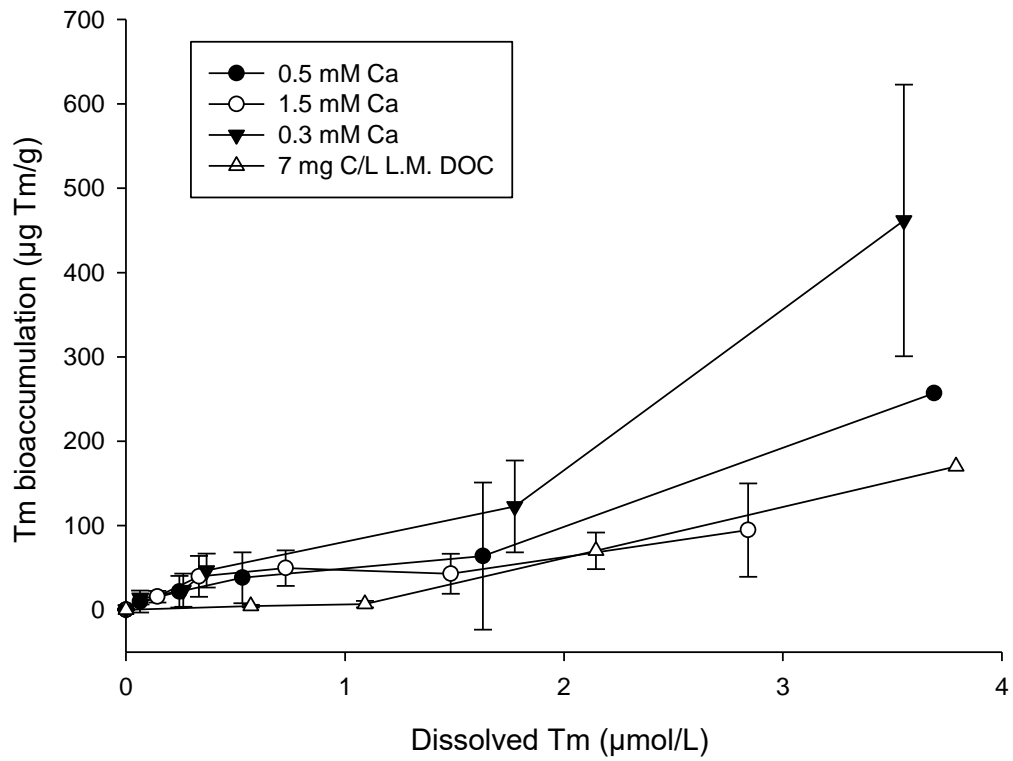


Figure 3.5: Mean bioaccumulation in tissues \pm 1 STD as Tm-D increases during 14d chronic tests at 0.5mM Ca, 0.3mM Ca, 1.5mM Ca and with 7mgC/L Luther Marsh DOC.

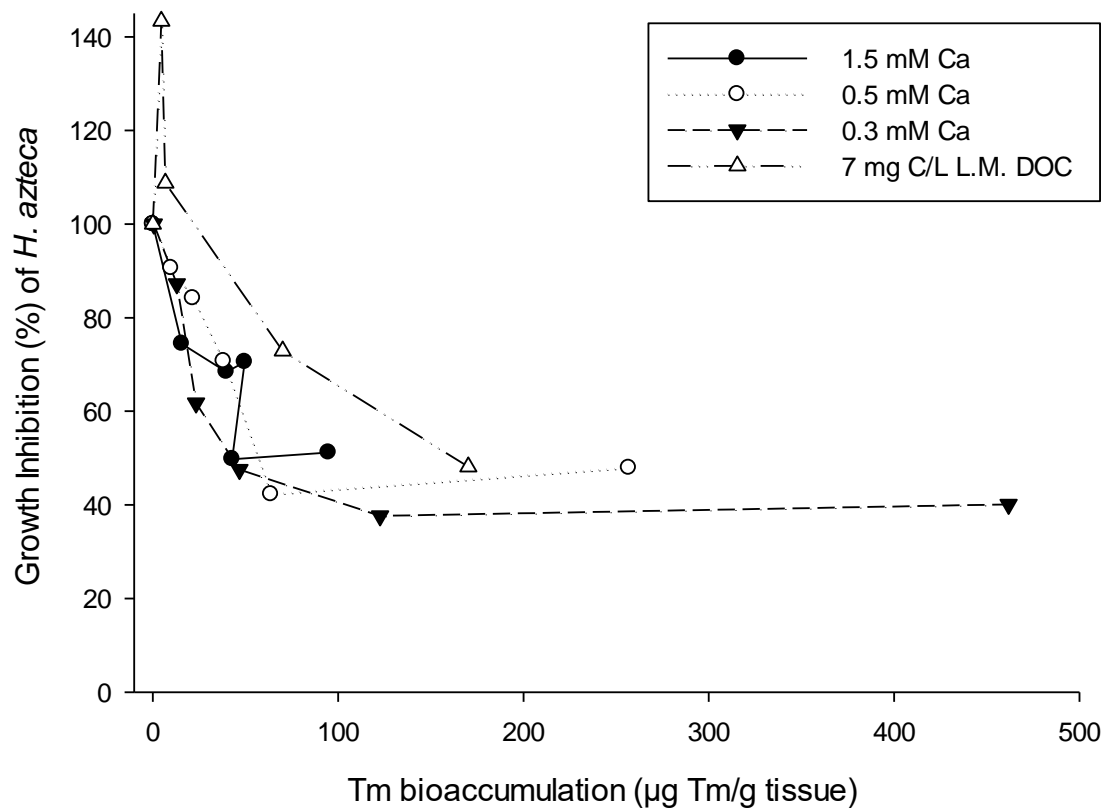


Figure 3.6: Effect of Tm accumulation in *H. azteca* tissues on *H. azteca* growth (%) in 14 d chronic Tm tests with 1.5 mM, 0.5 mM and 0.3 mM Ca and 7 mg C/L Luther Marsh DOC treatments.

Chapter 4: How this Research is Integrative and
Future Directions in Study

4.1 Why this Research is Integrative

4.1.1 Integration of Different Fields of Science

This research is inherently integrative in the foundation of its study and practice. It incorporates many different fields of science to attempt to understand the full story of the toxicity of Tm. In our research, we turned to the theory and principles of fields of sciences such as physiology, aquatic geochemistry, ecology and cell biology. For instance, in both our acute and chronic results we found that a significant amount of precipitation of Tm was occurring. To attempt to understand the possible mechanisms behind this occurrence, we used concepts from aquatic chemistry and even applied some concepts to further tests hypothesis about these mechanisms. The influence of different TMFs to the toxicity of Tm were central to this research since we focused on a biotic ligand approach to study this metal (Figure 1). The concepts around cationic competition, DOM complexation and inorganic complexation all require a basic understanding in water chemistry. Without these concepts and tools, we would not be able to fully understand how to test our contaminant nor understand the results obtained after testing. Furthermore, as this knowledge about how Tm interacts with differing water chemistry is built upon, it can be applied to how Tm may interact in site specific water chemistry of natural waters.

While we called upon concepts and principles of aquatic chemistry to gain an understanding of how Tm works chemically, we also required concepts from cell biology and physiology to understand how the metal interacts with the biological organism; in this study's case, *H. azteca*. While we did not directly study what the metal does at the site of action, we required basic understanding of principles on how the metal may compete at transporter sites at the biotic ligand with other cations in solution and binding affinities with transporter sites

(Niyogi and Wood, 2004). Furthermore, we required some knowledge on how different required ions, such as Ca, Mg and Na, can affect the organism's overall health when altered by a contaminant. For example, if Tm was found to compete with Ca, invertebrate calcification of exoskeleton, synaptic transmission, and other cellular functions could be disrupted (Spafford et al., 2003). It is this component of the BLM (Fig 1) that requires some knowledge of physiology to apply to the full understanding of Tm toxicity.

At a larger scale, the results of this study can have implications towards conservation and ecology. There have been cases in which a contaminant effects on a sensitive species created a cascade effect through different trophic levels, causing changes in an aquatic ecosystem (Fleeger et al., 2003). These indirect effects can be due to a number of reasons, such as directly effecting a keystone species, inducing changes in nutrient levels in an aquatic system or even by inducing changes in species behaviour that can in turn alter community composition (Fleeger et al., 2003). Conservation of ecological systems can rest on the understanding of contaminant impacts not only on the study species directly but indirectly as well.

Finally, and in the most obvious sense, this study required the principles and practice of different methods of statistics. Statistics was required for the analysis of all results including the calculation of means, obtaining significant differences through different statistical methods such as Litchfield and Wilcoxon (1949) methods and ANOVAs. We required probit analysis to determine LC_{50} values and regression equations for EC_{20} estimates for effects on growth. Toxicology may be the specific study that was practiced in this research, however the root of the research was biology, with which is inherently integrative in that it draws on the knowledge and concepts from all practices of many different fields.

4.1.2 Integration through Implications for Industrial and Government Use

This research is integrative by encompassing many fields of science in its study. However, it is also integrative due to its implications for use in industry, government and policy. Industrially, REEs are growing in demand (Humphries, 2013). This increase in production, use and disposal leads to environmental concerns of contamination. Industry and government alike require research and information about these contaminants to be able to develop and employ policies and regulations. Therefore, this research becomes integrative in a further sense; while the science initially is descriptive of the effects of the contaminant, by using the BLM approach, the science can become predictive of certain effects in site specific environments, thus making it useful in industry and policy development (McLaughlin, 2015). Little is understood about the aquatic toxicity of REEs and with a continuing effort to research, analyze and obtain data, knowledge can be built upon that can eventually help decide upon policy and regulation. With policy and regulation, there can be monitoring and risk assessment.

In summary, this research is fundamentally integrative because it requires knowledge and understanding of sciences from many fields including ecology, conservation, physiology and geochemistry. It is also integrative due to the practical applications and implications for government and REE industry, in government policy and regulation.

4.2 Future Studies

Some deficiencies of this project were that EC_{20s} for this study were not calculated with 95% confidence intervals for growth inhibition or bioaccumulation. Further analysis is required to understand the true relationship between bioaccumulation and toxicity to the organism. Tm³⁺ concentrations were not estimated and further analysis using Windermere Humic – Aqueous

Model (WHAM) to obtain these estimates. A Tm acute BLM of *H. azteca* has not been built and therefore more tests done on *H. azteca* with different TMFs would be required to build enough data to in order to develop a BLM for Tm. This can also be done for chronic toxicity. Study of the mechanisms of uptake for REEs is worth investigation and how total and dissolved Tm effect toxicity. Finally, investigating of Tm speciation in an artificial aquatic medium is certainly required to understand the unique water chemistry that studies are showing for REEs. This study is preliminary in the attempt to understand REEs and their aquatic toxicity. More research is certainly required.

4.3 References

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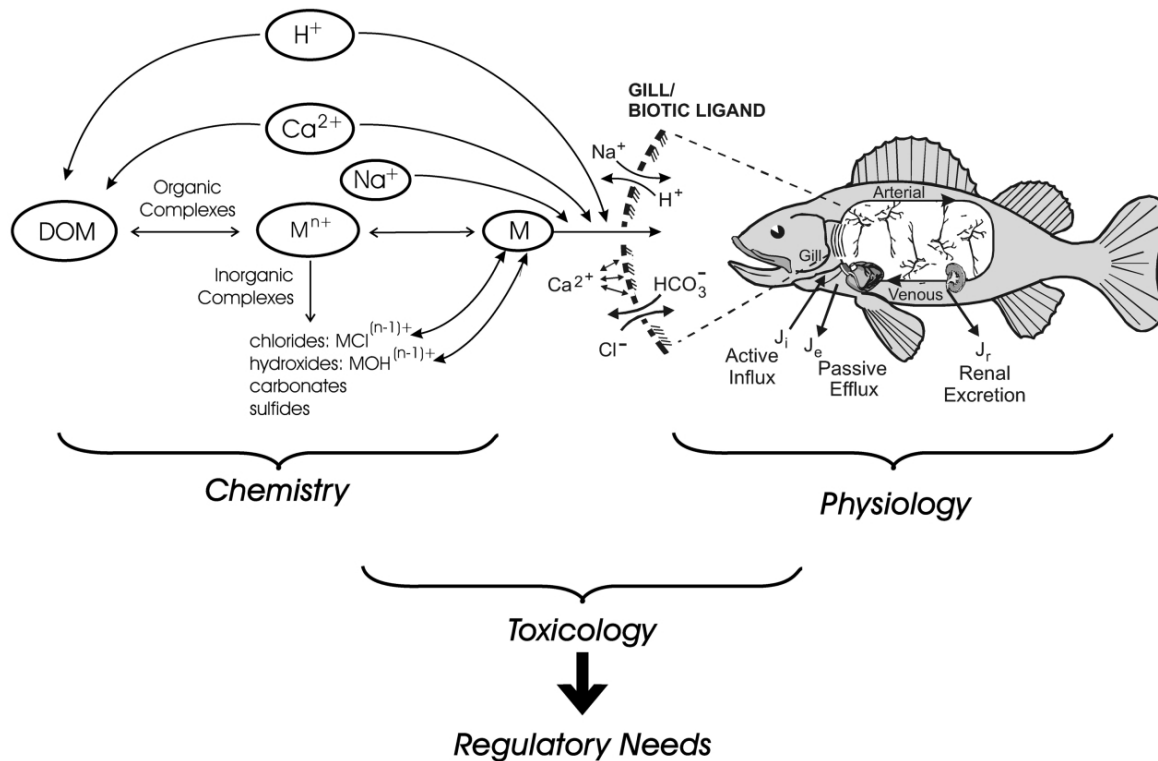


Figure 4.1: Schematic representation of the biotic ligand model and how it integrates the needs for many different field of study and eventually can lead to the requirements of regulation and policy (Paquin et al., 2003).

Appendix

Table A1: Exposure chemistry with mortalities for all *H. azteca* 96 h acute tests. Measured values for nominal, Tm-T (T) and Tm-D (D) in $\mu\text{g Tm/L}$ as well as the measured values for Ca, Na, Mg and DOC. Mortality and pH are averages of two replicates per concentration.

Test Type	Final			Initial			Final			Initial	Final	Fin/Init	%	Mortality
	Ca (mM)	Mg (mM)	Na (mM)	pH	DOC	Nominal	T	D	T					
RM	0.5	0.1	0.5	7.6	0.6	0	10.4	10.5	10.0	9.5	100.9	95.2	94.3	10
	0.5	0.1	0.5	7.7	0.6	375	328.4	320.9	261.4	256.4	97.7	98.1	100.4	20
	0.5	0.1	0.5	7.6	0.6	750	607.2	594.4	547.7	536.1	97.9	97.9	100.0	20
	0.5	0.1	0.5	7.6	0.6	1500	823.3	782.6	774.2	723.5	95.1	93.5	98.3	65
	0.5	0.1	0.5	7.6	0.6	3000	1162.5	827.9	1007.7	772.0	71.2	76.6	107.6	65
	0.5	0.1	0.5	7.5	0.6	6000	1753.5	832.1	1185.0	811.7	47.5	68.5	144.4	100
1.5mM CaCl ₂	1.5	0.2	0.6	7.5	0.6	0	4.7	10.3	5.9	7.7	220.8	130.3	74.4	5
	1.5	0.2	0.6	7.6	0.6	750	518.4	4.8	362.8	21.1	0.9	5.8	443.6	15
	1.5	0.2	0.6	7.7	0.6	1500	760.6	17.0	501.1	217.5	2.2	43.4	1281.9	20
	1.5	0.2	0.6	7.7	0.6	3000	2284.0	115.9	1766.3	504.7	5.1	28.6	435.5	80
	1.5	0.2	0.6	7.6	0.6	6000	2398.0	337.8	1696.0	812.6	14.1	47.9	240.6	95
	1.5	0.2	0.6	7.6	0.6	9000	10490.0	780.7	9607.5	745.7	7.4	7.8	95.5	100
0.3 mM CaCl ₂	0.2	0.1	0.5	7.8	0.6	0	7.2	8.1	7.8	9.4	111.5	120.5	117.1	5
	0.2	0.1	0.5	7.8	0.6	375	260.9	255.3	230.4	224.4	97.9	97.4	87.9	10
	0.2	0.1	0.5	7.8	0.6	750	576.3	550.4	523.2	495.6	95.5	94.7	90.0	30
	0.2	0.1	0.5	7.8	0.6	1500	796.8	725.3	793.5	666.6	91.0	84.0	91.9	60
	0.2	0.1	0.5	7.8	0.6	3000	1015.0	868.6	1433.8	742.3	85.6	51.8	85.5	50
	0.2	0.1	0.5	7.7	0.6	6000	1306.5	819.0	1947.3	753.2	62.7	38.7	92.0	55
1.5 mM Na	0.5	0.2	1.6	7.3	0.6	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
	0.5	0.2	1.6	7.5	0.6	750	404.2	368.6	376.9	352.3	91.2	93.5	95.6	25
	0.5	0.2	1.6	7.5	0.6	1500	719.4	596.5	741.4	568.3	82.9	76.7	95.3	85
	0.5	0.2	1.6	7.5	0.6	3000	1110.5	645.8	1109.8	647.3	58.2	58.3	100.2	85
	0.5	0.2	1.6	7.5	0.6	6000	1504.5	641.7	2761.5	711.0	42.6	25.7	110.8	100
	0.5	0.2	1.6	7.2	0.6	9000	6505.0	4572.5	3215.0	2055.0	70.3	63.9	44.9	100

Table 1A Continued.

Test Type	<u>Final</u>			<u>Initial</u>					<u>Final</u>					<u>%</u>	
	<u>Ca</u> <u>(mM)</u>	<u>Mg</u> <u>(mM)</u>	<u>Na</u> <u>(mM)</u>	<u>pH</u>	<u>DOC</u>	<u>Nominal</u>	<u>T</u>	<u>D</u>	<u>T</u>	<u>D</u>	<u>D/T%</u>	<u>D/T%</u>	<u>D%</u>		<u>Mortality</u>
0.3 mM	0.5	0.1	0.3	7.4	0.6	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
Na	0.5	0.1	0.3	7.4	0.6	375	85.4	73.2	9.8	1.2	85.7	12.7	1.7	5	
	0.5	0.1	0.3	7.4	0.6	750	384.8	366.3	302.3	288.5	95.2	95.4	78.8	45	
	0.5	0.1	0.3	7.4	0.6	1500	787.5	707.3	852.9	748.4	89.8	87.7	105.8	50	
	0.5	0.1	0.3	7.4	0.6	3000	1309.0	925.5	1604.3	965.2	70.7	60.2	104.3	70	
	0.5	0.1	0.3	7.3	0.6	6000	2308.0	1213.0	3065.5	1229.8	52.6	40.1	101.4	100	
0.4 mM	0.5	0.4	0.5	7.6	0.6	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15	
Mg	0.5	0.4	0.5	7.7	0.6	750	562.8	526.0	64.5	59.2	93.5	91.8	11.3	15	
	0.5	0.4	0.5	7.6	0.6	1500	875.8	759.5	358.6	352.7	86.7	98.4	46.4	35	
	0.5	0.4	0.5	7.6	0.6	3000	1142.0	810.7	488.5	483.0	71.0	98.9	59.6	55	
	0.5	0.4	0.5	7.5	0.6	6000	1571.5	852.9	1394.8	880.4	54.3	63.1	103.2	75	
	0.5	0.4	0.5	7.6	0.6	9000	1889.5	865.5	2112.8	881.2	45.8	41.7	101.8	100	
0.06 mM	0.5	0.1	0.5	7.7	0.6	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	
Mg	0.5	0.1	0.5	7.7	0.6	375	279.0	265.7	205.6	199.3	95.2	97.0	75.0	10	
	0.5	0.1	0.5	7.7	0.6	750	482.0	467.9	383.1	379.8	97.1	99.1	81.2	5	
	0.5	0.1	0.5	7.7	0.6	1500	936.0	798.4	932.0	658.1	85.3	70.6	82.4	60	
	0.5	0.1	0.5	7.7	0.6	3000	1235.0	818.5	1737.8	690.8	66.3	39.8	84.4	80	
	0.5	0.1	0.5	7.7	0.6	6000	1672.5	789.7	2449.8	713.6	47.2	29.1	90.4	100	
2 mg C/L	0.5	0.1	0.7	7.6	4.1	0	82.7	56.0	44.7	43.8	67.7	98.0	78.3	0	
DOC	0.5	0.1	0.7	7.6	4.2	750	615.0	505.2	565.4	537.0	82.1	95.0	106.3	0	
Luther	0.5	0.1	0.7	7.7	3.1	1500	1085.5	775.3	867.9	789.3	71.4	90.9	101.8	35	
Marsh	0.5	0.1	0.7	7.7	2.4	3000	1550.0	870.7	1071.3	797.3	56.2	74.4	91.6	65	
	0.5	0.1	0.7	7.6	2.2	6000	2092.0	907.7	940.3	806.1	43.4	85.7	88.8	100	
	0.5	0.1	0.7	7.5	2.0	9000	3322.0	884.1	1695.0	777.5	26.6	45.9	87.9	100	

Table 1A Continued.

Test Type	Final			Initial				Final				Initial	Final	Fin/Init	%
	Ca (mM)	Mg (mM)	Na (mM)	pH	DOC	Nominal	T	D	T	D	D/T%	D/T%	D%	Mortality	
7 mg C/L	0.5	0.1	0.7	7.6	10.9	0	9.0	9.1	7.6	8.1	101.7	107.2	88.9	0	
DOC	0.5	0.1	1.4	7.6	10.5	750	634.5	549.9	743.7	720.3	86.7	96.9	131.0	10	
Luther	0.5	0.1	2.3	7.6	9.8	1500	1195.5	967.7	1312.3	1258.5	80.9	95.9	130.1	0	
Marsh	0.5	0.1	4.0	7.6	9.2	3000	2295.0	1667.5	2207.3	1913.0	72.7	86.7	114.7	20	
	0.5	0.1	7.3	7.5	7.8	6000	4392.0	2151.5	3844.3	2565.0	49.0	66.7	119.2	30	
	0.5	0.1	10.7	7.5	9.0	9000	4456.5	2514.5	2635.5	2433.0	56.4	92.3	96.8	70	
12 mg	0.5	0.1	0.7	7.6	20.0	0	7.2	7.0	7.8	8.1	97.0	103.8	114.9	15	
C/L DOC	0.5	0.1	1.3	7.5	18.5	750	732.4	708.6	773.6	745.5	96.7	96.4	105.2	15	
Luther	0.5	0.1	1.9	7.5	18.2	1500	1390.0	1342.5	1421.8	1399.5	96.6	98.4	104.2	20	
Marsh	0.5	0.1	1.9	7.5	17.8	3000	2746.5	2506.0	2777.8	2591.3	91.2	93.3	103.4	5	
	0.5	0.1	6.0	7.4	15.6	6000	4230.0	3546.0	3728.0	3474.8	83.8	93.2	98.0	35	
	0.5	0.1	8.8	7.4	16.1	9000	5053.5	4065.0	4187.0	4167.5	80.4	99.5	102.5	100	

Table A2: Dissolved organic carbon location of collection with GPS coordinates and SAC340 values and Fluorescence Index (FI).

DOC Source	Location	Year of collection	GPS Coordinates	SAC₃₄₀	FI
Kouchibouguac (KB)	Kouchibouguac National Park, NB	2012	46° 48' N -64° 54' W	27.9	1.07
Luther Marsh (LM)	Luther Marsh, Grand Valley, ON	Not Found	43° 37' N 80° 26' W	39.3	1.19