Intra-lake and inter-lake variation in copper tolerance in *Daphnia* from clean and metal-contaminated lakes

A thesis presented to

The Faculty of Graduate Studies

of

Lakehead University

by

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In partial fulfillment of requirements
for the degree of
Master of Science in Biology

September 2013

Abstract

The freshwater invertebrate *Daphnia* can persist in historically metal-contaminated lakes due to its ability to adapt to metal contamination. Since different *Daphnia* isolates have been shown to have varying degrees of tolerance to metals, metal toxicity testing in *Daphnia* is a useful model for studying intra-population variation. However, many ecological studies of *Daphnia* use single isolates to represent the population, with an assumption of minimal diversity.

Copper LC50 tests were performed among *Daphnia* isolates from clean and metal-contaminated lakes in order to analyze variation in metal tolerance. *Daphnia* from metal-contaminated lakes were found to be significantly more tolerant to copper than clean lake isolates ($p = 3.5 \times 10^{-4}$), and had a slightly lower degree of variation of LC50 (COV = 19.9%) compared to that of clean lakes (COV = 21%). However, pairwise LC50 comparisons found more significantly different comparisons in metal-contaminated lakes (40.7%) than clean lakes (25.9%). Of the within-lake pairwise LC50 comparisons, 33.3% varied significantly, compared to 65.9% for among-lake comparisons. Overall variances within lakes were not significantly different than among lakes ($p = 0.147$), demonstrating high intra-lake variability in metal tolerance despite significant overall differences in average values. The Lactate dehydrogenase gene (*Ldh*) and NADH dehydrogenase 5 gene (ND5) were used to distinguish *Daphnia pulicaria*, *Daphnia pulex* and hybrid species and analyze genetic diversity in metal tolerance. Analysis of *Ldh* genotypes revealed that *Daphnia* with S and F alleles were significantly more tolerant than *Daphnia* with two F alleles ($p = 1.8 \times 10^{-4}$). Phylogenetic reconstructions based on ND5 gene sequences showed that *Daphnia* isolates from metal contaminated lakes clustered together showing a reduction in genetic diversity, while clean lake isolates clustered among four

different haplotypes (three *pulex* and one *pulicaria*).

These results suggest that metal contamination reduces genetic diversity among *Daphnia* isolates within lakes by selecting for metal-tolerant phenotypes, which correlates with the *Ldh* SF genotype. This reduction in diversity can negatively impact *Daphnia*'s ability to adapt to future environmental stress, and the high variability within the populations from clean lakes suggests multiple isolates should always be used when collecting representative *Daphnia* isolates for studies.

Lay Summary

Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms. The purpose of this study was to understand diversity in the form of variation in metal tolerance in *Daphnia* isolates within lakes and among lakes. Natural populations of *Daphnia* in relatively uncontaminated environments have high levels of diversity with regard to tolerance of toxic stress, and it may be difficult to interpret the results of some ecotoxicology tests without characterizing and understanding the genetic sources of this diversity. The research conducted in this study found that metal contamination affects the diversity of *Daphnia* in lakes by reducing the number of unique haplotypes through the selection of resistant *Daphnia* and particular *Ldh* genotypes. Metal contamination also has widespread effects on other species, but these results show a reduction in inter-population variation as a result of metal contamination.

Acknowledgements

I would like to thank my thesis supervisor, Dr. Greg Pyle, for taking me on as a student and for his guidance during the process. Thank you to my committee members, Dr. Peter Lee, and Dr. Carney Matheson for their support and knowledge throughout my thesis. I would also like to thank my external examiner Dr. Melania Cristescu for reviewing my work. Thank you to Dr. Kam Leung for sparking my interest in research and for providing me with my first research project. Additionally, I would like to thank all of the lab members of ABEL, both past and present, for their support and assistance. I would especially like to thank Ali Azizishirazi for his assistance and patience in collecting study material for my project (twice!) as well as Patrick Gauthier for his R statistical knowledge. I would also like to thank Roland Vergilino, Steve Injic, and Tiffany Chin for all their help with phylogenetic analysis and molecular techniques. Thank you to Dr. Celis-Salgado and Dr. Joseph Shaw for supplying *Daphnia* for my project. Thank you to NSERC for funding this project.

I am truly grateful for all the support I have received from both family and friends over all my years of education and especially during my thesis. First and foremost, I would like to thank my parents, Paulette and James, for always keeping me motivated and inspiring me to follow my dreams, no matter how difficult they seemed. Thank you to my sisters, Paula, Crystal, and Katie for always lending an ear and giving me advice during the ups and downs of my thesis. Thank you to my brother-in-law, Bruce, for all his knowledge and editing expertise. Thank you to my lab-sharing friends Jessie Jones, Karen Giffin, and Felicia Joseph for all the lunch dates, pep talks, vent sessions, and most of all, for making the lab a joyous place (even at 10 P.M....on a Saturday). Thank you to good friends Deanna Bessel, Sarah Niccoli, Rob Jackson, and the

Arkells for always being there for me. Last but not least, a special thank you to my cat friend, Hermione, for her emotional support and indifference to my entire project.

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Chapter 1

Introduction

1.1 Variation

It is unknown how well a single *Daphnia* isolate represents an entire population in a lake with regard to metal tolerance. Even though studies have shown that *Daphnia* can have varying tolerance to metals (Baird et al., 1990; Lopes et al., 2004), few studies have looked at intra-lake variation and inter-lake variation with regard to metal tolerance in order to explain this variation. Even fewer studies have looked at the genetic diversity present in those populations and the impact that metal tolerance can have on it. The goal of this study was to analyze intra-lake and inter-lake variation using multiple *Daphnia* isolates in order to understand variation in metal tolerance, as well as look at genetic diversity in order to determine how variation in metal tolerance can affect genotype diversity.

The genetic variation present in organisms can be introduced by mutation, genetic drift, gene flow and maintained by natural selection. Genetic variation can be defined as diversity in the alleles of genes and can occur both among populations and within populations of the same species, and may give rise to significant phenotypic variability even within a relatively confined geographical area (Hamrová et al., 2011). Variation among individuals in a population is important to study, as observed phenotypic traits in one single organism may not be an accurate representation of the entire population, and may result in significant sampling bias. However, many ecological studies examine individuals of a single species to serve as a model for a population, with an assumption of minimal phenotypic diversity within the population (Duffy, 2010). Ecotoxicology laboratory studies typically use populations with low variability for toxicity testing and in some cases, a single isolate, which leads to high reproducibility of the

results (De Schamphelaere et al., 2010). However, natural populations can have high levels of genetic variability with regard to tolerance of toxic stress and therefore using populations that have been under selection and had the genetic diversity eroded makes extrapolations from laboratory to field unreliable (Barata et al., 2002; De Schamphelaere et al., 2010). Therefore, taking variation into consideration is extremely important especially when dealing with species that reproduce clonally such as *Daphnia*. This study focused on investigating intra-lake and inter-lake variation in tolerance to copper in *Daphnia* from clean and metal-contaminated lakes.

1.2 **Daphnia** *as a study organism*

Daphnia are freshwater invertebrates that can be found worldwide (Lampart, 2006). *Daphnia* play an important role in aquatic ecosystems at the primary consumer trophic level (Lampert, 2006). In addition to the important trophic position that they occupy in aquatic ecosystems, their sensitivity and rapid response to environmental changes makes *Daphnia* ideal indicator organisms for water quality and toxicity studies. For example, previous studies have found *Daphnia* to be sensitive to metals (Winner and Farrell, 1976; Pane et al., 2003; Von der Ohe and Liess, 2004; Shaw et al., 2007; Fernández-González, 2011), pesticides (Hanazato, 2001), antimicrobial/antifungal agents (Peng et al., 2013), and temperature changes (McFeeters and Frost, 2011). Moreover, their life history makes them excellent study subjects since they exhibit a number of favourable traits such as the production of dormant eggs, vertical migration, phenotypic plasticity, anti-predator defence, and their ability to be cultured in a laboratory with ease (Stollewerk, 2010).

Variation in the *Daphnia* lifecycle (Figure 1) is relevant to the current study because it can result in an increased or decreased amount of genetic diversity depending on the time of

season and the environmental conditions. Most *Daphnia* undergo cyclic parthenogenesis, which allows them to produce parthenogenetic embryos during normal conditions and resting eggs during unfavourable conditions such as cold and drought (Kerfoot et al., 2004; Stollewerk, 2010). In a normal growth season, *Daphnia* undergo asexual reproduction, producing diploid eggs that develop in the brood chamber, resulting in low genetic diversity during favourable conditions (Stollewerk, 2010; Allen and Lynch, 2011). However, if unfavourable conditions arise, *Daphnia* undergo sexual reproduction, producing haploid eggs that require fertilization by a male and a period of dormancy for development (Stollewerk, 2010). These resting eggs contain can survive harsh conditions and they contain a hard outer chitinous envelope called an ephippium which aids in their dispersal (Vanvlasselear and De Meester, 2010). Resting eggs can remain dormant in lake sediment for years. Over time, an egg bank is created in the lake sediment, and when favourable conditions return, these eggs can resume development and hatch (Kerfoot et al., 2004). Each hatchling from an ephippium produced by a cyclically parthenogenic female is genetically unique (due to sexual reproduction), and hatchlings from many different years may hatch at the same time due to extended dormancy, which gives rise to a large amount of genetic diversity at the beginning of the hatching season (Geedey et al., 1996; Hamrová et al., 2011). Later on in the season, selection processes and genetic drift can lead to more favourable genotypes dominating the population and some clonal lineages can be lost (Geedey et al., 1996; Hamrová et al., 2011). This change in population dynamics is important to take into consideration when studying *Daphnia*, as the time of the season and the environmental conditions can affect the amount of variation in the clonal isolates of a lake. Additionally, some *Daphnia* are asexual obligates which produce genetically identical daughters (even during ephippia production) through parthenogenesis (Crease et al., 2011). Therefore, even if the

reproductive lifecycle of an individual isolate of *Daphnia* is well characterised, it cannot necessarily be used to predict reproductive behaviour in another isolate.

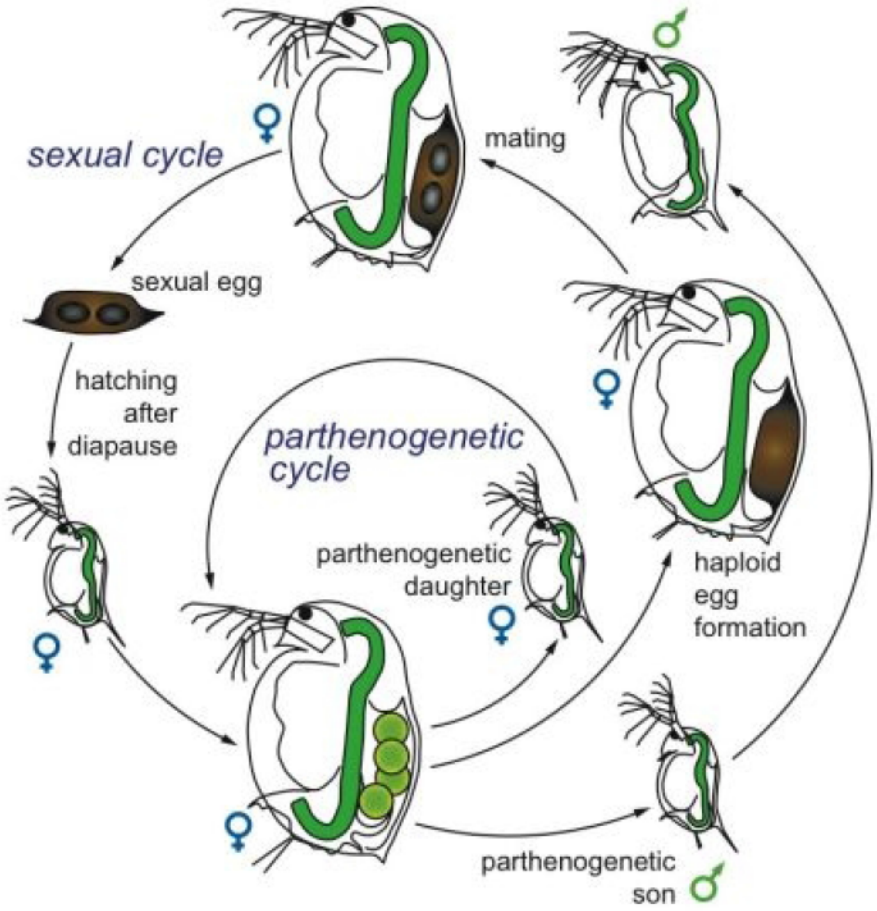

Figure 1: Diagram of the cyclical parthenogenetic life cycle of *Daphnia*. During the sexual cycle, female *Daphnia* form haploid eggs that are fertilized by males and hatch after a period of dormancy. During the asexual cycle, female *Daphnia* produce diploid eggs that develop in the brood pouch into parthenogenetic daughters and are released. Drawn by Dita B. Vizoso, Fribourg University (Ebert, 2005).

1.3 ND5 mitochondrial lineages and Ldh genotypes

Additional genetic complexity in studying *Daphnia* arises among the 12 member species in the *Daphnia pulex* species-complex, which have distinct mitochondrial lineages based on the NADH dehydrogenase 5 (ND5) gene (Cristescu et al., 2012). The two main lineages of *Daphnia* that are present in North America are *Daphnia pulex* (Leydig) and *Daphnia pulicaria* (Forbes) (Colbourne et al, 1998). In addition to differences in the ND5 gene, habitat is a discriminating factor separating these taxa, where *Daphnia pulex* are typically located in temperate ponds and *Daphnia pulicaria* are found in stratified lakes. Their habitat has been linked to the genotype of the lactate dehydrogenase (*Ldh*) enzyme carried by both species (Crease et al, 2011); homozygosity of slow alleles at the *Ldh* locus are conserved in *Daphnia pulex*, while *Daphnia pulicaria* is homozygous for fast alleles conserved at the *Ldh* locus. The "slow" and "fast" designation is based on the speed at which the alleles separate during allozyme eletrophoretic assays. Complications in the genetic characterization of *Daphnia pulex* members arise from the fact that these species are able to hybridize, creating asexual offspring that are heterozygous in *Ldh* alleles containing one slow and one fast allele (Crease et al, 2011). Hybridization makes it very difficult to distinguish *Daphnia pulicaria* (lake species) from *Daphnia pulex* (pond species) based on morphology and mitochondrial lineages alone, since some *Daphnia pulicaria* share the mitochondrial genome of *Daphnia pulex*. Therefore, it is important to look at both molecular markers in order to make inferences about genotype diversity. Most toxicity studies do not take into account the *Ldh* genotype and even fewer have looked at mitochondrial lineages in combination with *Ldh* genotype. More research needs to examine the variability among pure *Daphnia pulex*, pure *Daphnia pulicaria,* and hybrids with regards to metal tolerance.

1.4 Effect of contamination on **Daphnia**

Changes in the environment, such as those caused by contaminants, can greatly affect the behaviour and life history traits of *Daphnia*. Metal contaminants, including copper (Winner and Farrell, 1976; Fernández-González, 2011), cadmium (Shaw et al., 2007) and nickel (Pane et al., 2003) have been shown to be toxic to *Daphnia* species. Exposure to metals can affect the response of *Daphnia pulex* to predators, by interfering with their response to larval *Chaoborus americanus* ("phantom midge") kairomone, making them vulnerable to predation (Mirza and Pyle, 2009). When the midge larvae consume young *Daphnia pulex,* the midge larvae release a kairomone, cueing other *Daphnia pulex* to produce defensive neckteeth that make it difficult for the midge larva to consume the young *Daphnia* (Laforsch and Tollrian, 2004; Penalva-Arana et al., 2009). Studies have shown that *Daphnia pulex* with neckteeth have 60% higher survival rates in the presence of *Chaoborus* (Havel and Dobson, 1984; Parejko, 1991; Mirza and Pyle, 2009). Environmental concentrations of copper and nickel have been found to reduce neckteeth induction in young *Daphnia pulex* in the presence of *Chaoborus* kairomone (Hunter and Pyle, 2004), and environmental concentrations of copper have been found to cause young *Daphnia pulex* to have fewer and shorter neckteeth, as well as lower survival rates in staged predator prey trials (Mirza and Pyle, 2009). Impairment of neckteeth can be severely damaging to *Daphnia pulex* populations. The potential over-predation associated with necktooth impairment threatens to disrupt the aquatic community as a whole. The effects of metals on the ability of *Daphnia pulex* to sense and evade predators have been demonstrated (Hunter and Pyle, 2004; Mirza and Pyle, 2009), but it remains unknown whether *Daphnia* populations in chronically metal-contaminated lakes have adapted to the effects of metals on their physiology or their anti-predator response, and how much these adaptations may vary within an isolated population.

1.5 Adaptation in Daphnia

Since the 1800's, metal contamination of lakes has occurred through industrial activities such as mining and smelting. One area that has been particularly affected is Sudbury, ON, Canada. An area of 17,000 km^2 including over 7,000 lakes was subjected to metal contamination for almost a century (Keller et al., 1999). Although more environmentally friendly practices have now been adopted, lakes have only slowly begun to recover from the damage (Keller et al., 1991). *Daphnia* have been able to persist in these lakes during recovery, which suggests that they may have acquired an ability to adapt to metal contamination. Because of this, multiple studies (Muyssen et al., 2002; Lopes et al., 2004; Agra et al., 2010; Saro et al., 2012) have looked at the ability of *Daphnia* to adapt to metal contaminants, resulting in tolerant individuals surviving over less tolerant ones. Populations exposed to contaminants can often undergo natural selection which results in a shift in genotype frequencies and reduced genetic variation in the population (van Straalen and Timmermans, 2002). This type of contaminant-induced selection can lead to a population with more resistant genotypes that are more suited to survive in contaminated environments (Agra et al., 2010). An increased fitness for one environment can lead to the same population having decreased fitness in other environments and is known as a "cost of adaptation" (De Schamphelaere et al., 2010). Therefore, adaptation to metals is extremely important when looking at toxicity studies, as using animals that have adapted to metals in these studies may not reflect the toxic effects that metals may have on populations that have not been previously exposed to metals.

1.6 Adaptation and natural selection

Metal toxicity testing is a useful model for studying intra-population variation in *Daphnia*, since it can be easily observed, and *Daphnia* have been known to adapt to metals in their environment (Muyssen et al., 2002; Lopes et al., 2004; Agra et al., 2010; Saro et al., 2012). Increased selective pressure within a population due to the presence of metals can lead to the emergence of *Daphnia* that have a greater chance of surviving the metal toxicity through adaptation, and decrease the amount of variation within the population. The more sensitive *Daphnia* will not be able to tolerate the conditions. Populations not experiencing metal toxicity will be able to support *Daphnia* with a variety of tolerances and therefore an increased level of variation within the population. *Daphnia* with a variety of tolerances can also be introduced to clean lakes via transportation of ephippia by animals, wind or anthropogenic practices such as recreational boating. Previous research has examined metal tolerance of wild *Daphnia* populations by using single isolates, but the use of single isolates does not account for clonal variation within a population, and cannot be used to study differences in the amount of intra-population variation among lakes.

1.7 Research objectives

Currently, it is impossible to know how well a single isolate represents an entire population in a lake. The objectives of this study were to conduct toxicity tests using three single isolates from three chronically metal-contaminated lakes and three single isolates from three clean lakes located in the Sudbury area in an effort to examine intra- and inter-lake variation in copper tolerance. It is unknown how much variation in copper tolerance exists among *Daphnia* isolates in within a lake and whether the amount of variation that exists exceeds the variation that

exists among lakes. In addition, the isolates were identified using their *Ldh* genotype as well as mitochondrial lineage based on the ND5 gene in order to determine their lineage and how they may develop differential copper tolerance.

Chapter 2

Methods

2.1 Experimental Approach

The relative metal tolerance of several unique *Daphnia* isolates was tested using standard toxicity-testing protocols. A total of 18 *Daphnia* isolates (three from each of six lakes) were isolated from three clean and three metal-contaminated lakes, and the isolates were identified by examining morphological features and characterized using genetic marker genes. Acute (48-hour) copper LC50 tests were performed on each isolate to determine whether there was significant variability in metal tolerance among *Daphnia* isolated from the same lake, and to compare that variability to isolates from different lakes. It is important to look at intra-lake and inter-lake variability in order to determine the degree to which they influence LC50 values, especially in comparisons involving clean and metal-contaminated lakes.

2.2 Collection

In July 2012, *Daphnia* were collected from Kelly Lake (N46°26', W81°04') and Geneva Lake (N46°45', W81°32') (Figure 2) using a conical handheld plankton net with a mesh size < 100 μm and a diameter of 12 inches. Samples were collected from ~ 1 m below the surface of the water and transported to the laboratory in their native lake water in clear polypropylene snap-lid containers. Clear containers were used in order to allow light to reach the samples and were rinsed three times prior to use by filling the containers with distilled water and emptying them. The samples were held at ambient temperature during transportation to the laboratory which lasted no more than 1 hour. Adult *Daphnia* were isolated from the samples using a plastic pipette and dissecting microscope (WILD Heerbrugg, M3C model) at 100x magnification.

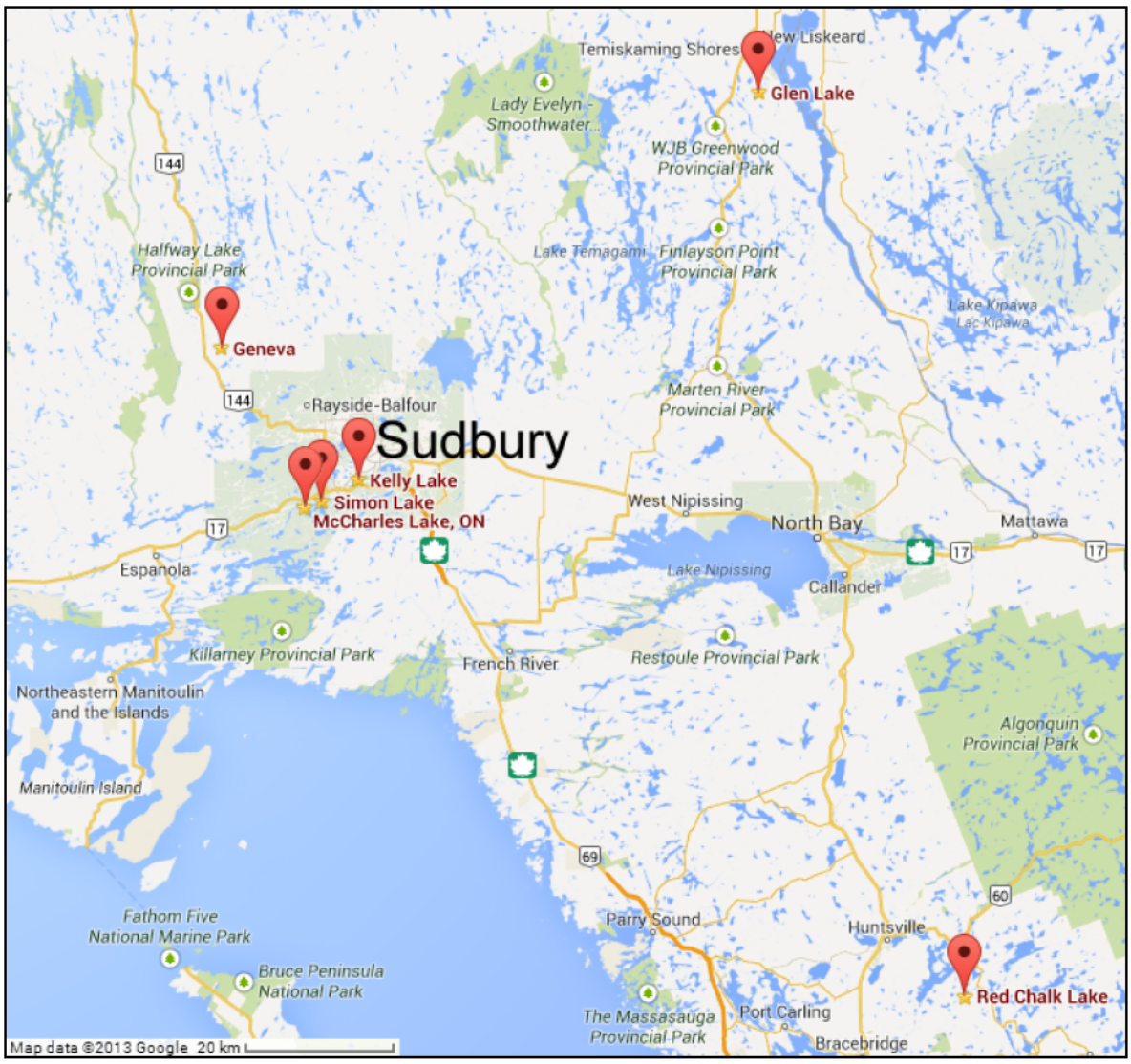

Figure 2: Map of the study lakes provided by Google Maps (2013).

The *Daphnia* were isolated by transferring the adults from the initial collection containers to new containers. The isolated *Daphnia* were maintained on a bench-top in the laboratory at room temperature in their native lake water until further processing. *Daphnia* were transported from Sudbury, ON, Canada to Thunder Bay, ON, Canada in a cooler with a digital thermometer to monitor the temperature, which stayed at 20 – 22 °C. Isolated *Daphnia* from Red Chalk (N45°11', W78°56') and Glen Lake (N45°08', W78°30') were provided by Dr. Martha Celis-Salgado of York University in Toronto, ON, Canada. Isolated *Daphnia* from McCharles Lake and Simon Lake were provided by Dr. Joseph Shaw of Indiana University in Bloomington, IN., United States. All isolates from both locations were shipped in 50 mL centrifuge tubes containing ~ 45 mL FLAMES medium (Celis-Salgado et al., 2008) and 10 – 20 adult *Daphnia*.

2.3 Study Lakes

Three metal-contaminated lakes and three clean lakes were chosen for this study with water quality parameters as presented in Table 1. The three metal-contaminated lakes selected were Kelly Lake, McCharles Lake and Simon Lake (Figure 2). These lakes are situated within the zone of impact of mining activities in Sudbury, ON, Canada. The three clean reference lakes selected were Geneva Lake, Red Chalk Lake and Glen Lake and are located outside the zone of impact in the Sudbury area (Figure 2). Geneva Lake is situated 70 km Northwest from the contaminated lakes, while Red Chalk Lake and Glen lake are situated 200 km East of Sudbury. The reference lakes and contaminated lakes are geologically similar and numerous studies have used these lakes as reference lakes due to their historically uncontaminated nature (De Schamphelaere et al., 2010).

Table 1: Water quality parameters taken from the literature for each of the study lakes.

Lake	Area (ha)	Maximum Depth (m)	Copper Concentration (µg/L)	pH	Hardness (mg/L as $CaCO_3$)	DOC	Alkalinity (mg/L as $CaCO_3$)
Kelly	340.8[a]	18[a]	15[b]	8.4[b]	582.7[b]	4.4[b]	33.7[b]
Simon	102[a]	12[a]	9.7[b]	7.4[b]	380.3[b]	6.6[b]	32.1[b]
McCharles	150.1[a]	15[a]	6[b]	7.5[b]	134.3[b]	5.8[b]	27.1[b]
Glen	16.3[c]	15[c]	0.2[d]	7.35[d]	8.21[e]	3.6[d]	62.8[d]
Red Chalk	44.08[c]	38[c]	<2.0[e]	6.83[f]	8.62[e]	2.4[f]	5.2[e]
Geneva	356.4[g]	25[h]	1.6[g]	6.85-6.98[g]	9.28-9.61[g]	4.2-5.7[g]	20-22[g]

[a] Lake Water Quality Department, City of Greater Sudbury (2013a,b,c)

[b] Pyle et al., 2005

[c] Girard et al., 2007

[d] Inglis, thesis, 2007

[e] Heneberry, thesis, 1996

[f] Arnott et al., 2003

[g] Azizishirazi et al., 2013

[h] Iles and Rasmussen, 2005

2.4 Identification

Daphnia were identified to species at 400x magnification using a compound microscope (Omano, OM36LED model) and an online key created by Haney, J.F. et al. (An Image Based Key to the Zooplankton of the Northeast, USA, version 4.0, 2010). To further ensure correct identification, analysis of the NADH dehydrogenase 5 (ND5) gene and lactate dehydrogenase (*Ldh*) gene were performed (see "Mitochondrial DNA Analysis" subsection below).

2.5 Daphnia Culturing

Daphnia were held under static renewal conditions in 1 L mason jars containing dechlorinated water from Lake Superior (20 °C, pH: 7.93, DOC: 3.3 mg/L) under full spectrum lights set at ~ 3000 lux with a 16:8 light:dark photoperiod at 22 - 24 °C ± 0.02. From these mass cultures, gravid females were selected and put into individual 50 mL centrifuge tubes (Fisher Scientific, Ottawa, ON, Canada) containing 50 mL of dechlorinated water for neonate collection. All *Daphnia* in mass cultures were fed 1 mL of algae (*Selenastrum capricornutum*) at a concentration of 3.3 x 10^6 cells/mL three times per week. Algae cell density was determined using spectrophotometry and a growth calibration curve as outlined in Rodrigues et al. (2011). Gravid females kept in 50 mL centrifuge tubes were fed 40 µL of algae three times a week. A 50% change of the culture water was done weekly for the mass cultures and 25% water changes were done every day on gravid female culture water to increase neonate production and to replace water removed during neonate collection.

2.6 Algae Culturing

Algae (*Selenastrum capricornutum)* were cultured using modified Bold's basal medium (BBM). Modified BBM was prepared as described by Stein (1973). A volume of 10 mL of 8.3 x 10^3 cells/L algae were added to a 1 L flask containing Bold's Basal Medium and kept under ~3000 lux light at a 16:8 light:dark photoperiod. The algae culture was left to grow for 1 week, when the cell concentration reached 3.3 x 10^6 cells/mL. The algae culture was stored at 4°C in the dark to prevent further growth thereafter.

2.7 Exposure Solution Preparation

A 1 L stock solution of 20 mg/L ACS reagent grade 98% copper chloride dehydrate (British Drug Houses LTD) was prepared by dissolving the metal in distilled and deionized water (ddH_2O). The 1 L solution was acidified using 40 µL of 70% trace metal grade nitric acid (Fisher Scientific, Ottawa, ON, Canada). Working solutions for toxicity tests were prepared in bulk to be used for all replicates by taking aliquots from the stock solution and putting them each into 1 L of dechlorinated water to make up the following nominal copper concentrations: 0, 2, 4, 6, 8, 10, 15, 20, 35, and 50 µg/L. Copper concentrations were measured after the toxicity tests (see "Water Sample Testing"). Copper concentrations were selected based on range-finder experiments that encompassed literature reported LC50 values ranging from 7 - 61 µg/L for copper for *Daphnia pulex* (Table 2) and prepared 24 hours in advance of each experiment to ensure solubility.

Table 2: The literature copper 48-hr LC50 values and their corresponding experimental parameters.

Species	Age	LC50 (µg/L)	Temperature (◦C)	pH	Hardness (mg/L CaCO3)	DOC (mg/L)	Source
Daphnia pulex	< 24 hrs	16-20	20	N/A	80-90	N/A	Roux et al. (1993)
Daphnia pulex	< 24 hrs	7-11	25	8.2	142	8.5-8.9	Griffitt et al. (2008)
Daphnia pulex	< 24 hrs	35-38	20	N/A	100-120	N/A	Dobbs et al. (1993)
Daphnia pulex	< 24 hrs	46-61	N/A	7.4	45	N/A	Mount et al. (1984)
Daphnia pulicaria	N/A	9.6-12	18	8.01	44	3.5	Lind et al. (1978)

2.8 Range-Finder Experiments

Range-finder tests were used to distinguish sensitive and tolerant isolates and to establish an appropriate range of copper concentrations for toxicity experiments. The range-finder experiments involved performing a toxicity test set up as described in "*Toxicity Testing*" on an isolate from a metal-contaminated lake and an isolate from a clean lake with a dilution series consisting of the following concentrations: 0, 1, 10, 20, 50, 100 µg/L. Once an LC50 was established for each isolate, the copper concentrations used in the toxicity experiments were adjusted to encompass those values. The copper concentration range used was dependent on the relative tolerance of the isolates. For sensitive isolates, a low-concentration range of 2 – 10 µg/L was used, and for tolerant isolates, a high-concentration range of 10 – 100 µg/L was used.

2.9 Toxicity Testing

For each of the three isolates collected from each of the six lakes (18 isolates total), a series of five copper concentrations were tested in triplicate in order to estimate the 48-h LC50 of each isolate. In each test, the highest concentration had 100% mortality and the control had 20% mortality or less. Neonates were collected using a transfer pipette every 24 hours. Neonates 24-48 hours old were used for toxicity tests. The neonates collected from the 24 hour and 48 hour time points were combined and placed into a common holding beaker. Neonates were drawn from the common holding beaker with culture water and randomly assigned along with their culture water to experimental replicates in 1.5 mL Eppendorff tubes. Culture water was then reduced in the Eppendorff tubes to approximately 200 µL before adding the neonates to copper solutions. Once the neonates were added to the copper solutions via pipetting, the Eppendorff tubes were rinsed with copper solutions using the same pipette to ensure the addition of all

neonates. A new pipette was used for each Eppendorff tube of neonates to avoid contamination. For each copper treatment, 10 neonates were used. Copper solutions were poured into 355 mL polypropylene cups to a volume of 150 mL. Each exposure treatment was performed in triplicate, including a control in which no copper was added. Experimental replicates were randomly arranged into a 3 X 6 matrix, and 10 neonates were randomly assigned to each replicate. The test was conducted under the same conditions as the cultures with full spectrum lights at ~3000 lux in a 16:8 light:dark photoperiod at 22 - 24 °C ± 0.02. Neonates were not fed during the test and kept isolated from external disturbances by surrounding the shelf that contained the toxicity test with a black sheet. After 48 hours, surviving neonates were counted. Neonates that were not moving upon agitation were considered deceased. Agitation involved sucking up water in a pipette and pushing the water at the neonates to cause them to move or swim away. Initial and final pH measurements for each test were taken using an Accumet Basic AB15 pH meter (Fisher Scientific, Ottawa, ON, Canada). The pH was found to not vary significantly ($p = 0.076$ according to a Mann-Whitney U test) between the initial measurements (which ranged from 7.63 to 8.13, with a median value of 7.93 and $n = 108$) and the final measurements (which ranged from 7.42 to 8.27, with a median value of 7.97 and $n = 108$). The pH was higher after testing in 67 of the 108 total samples measured, and lower in 41 of the samples. The pH meter probe was calibrated daily using three standards with a pH of 2, 7, and 10. Temperature was also monitored using a digital thermometer during the tests and found to fluctuate very little throughout the day and throughout experiments ranging from 22.0 – 24.2 °C, $n = 46$.

2.10 Water Sample Testing

Water samples of 15 mL were collected at the beginning and end of each toxicity test. Initial water samples were taken from working solutions and final water samples were pooled from all three replicates for a given concentration of metal. The final pH measurements were conducted prior to processing the water samples. All water samples were acidified to a pH of ~2 with trace metal grade nitric acid (Fisher Scientific, Ottawa, ON, Canada) and filtered through sterile 0.45 µm polyvinylidene difluoride filters (Pall Corporation, Quebec, Canada). Acidified water samples were kept at 4°C until metal analysis was performed by Lakehead University Instrumentation Laboratory (LUIL) using inductively coupled plasma atomic emission spectroscopy (ICP-AES). For metal analysis, an unmarked blank was provided with samples. The certified reference material used for the analysis was TMDA-64.2, a standard solution of trace metals in water used to test ICP-AES performance accuracy that is provided by the National Water Research Institute. The reference material only deviated by 5.89 µg/L across all samples (n = 5) from its expected value of 290 µg/L. For any given measurement the reference material did not deviate more than 5% from the expected value. Blanks of ddH_2O measured < 0.1 µg/L for copper across all samples (n = 5). Target nominal concentrations were very close to observed concentrations across all samples (n = 216). The water samples with no copper added (controls) had an average background copper concentration of 3.86 µg/L, while the observed concentration in the other samples were usually only slightly higher than the target nominal concentrations (by an average of 1.53 µg/L, with a standard deviation of 0.40 µg/L across all samples, n = 216). Hardness and pH were measured for all samples and remained consistent throughout experiments. Hardness was calculated as 50 ± 12 mg/L as $CaCO_3$ across all samples, n = 216. The pH measurements for prepared copper working solutions at the beginning of the

tests ranged from 7.63 to 8.13, with a median value of 7.93 (n = 108) and the pH measurements taken after the tests ranged from 7.42 to 8.27, with a median value of 7.97 (n = 108). Dissolved organic carbon was only measured for the control water source (but not for each prepared copper solution) and was found to be 3.3 ± 0.1 mg/L across 44 samples.

2.11 DNA extraction

Daphnia were washed with ddH_2O prior to DNA extraction in order to remove any excess algae. Washing was performed by placing 10-20 adult daphniids into a 1.5 mL Eppendorf tube with culture water, then removing all of the water from the tube using a pipette and replacing it with ddH_2O. *Daphnia* were washed three times and then all water was removed from the tube. *Daphnia* were frozen with liquid nitrogen to preserve the tissue. The tissue samples were stored in a -20 °C freezer until DNA extraction was performed. DNA extraction was carried out using the CTAB (Doyle and Doyle, 1987) method according to standard protocol.

2.12 Mitochondrial DNA Analysis

A section of the ND5 gene was amplified in each isolate using a 25 µL polymerase chain reaction (PCR) with the following concentrations of reagents: 1x PCR buffer, 1 mM $MgCl_2$, 0.08 mM dNTPs, 0.08 µM forward primer: 5'-GGGGTGTATCTATTAATTCG-3', 0.08 µM reverse primer: 5'-ATAAAACTCCAATCAACCTTG-3', 0.5 units of Taq polymerase (GenScript, Pistcataway, NJ, USA), and 2 µL of template DNA. The PCR temperatures and cycling times were as follows: 5 cycles of 94 °C for 35 seconds, 54 °C for 45 seconds, and 72 °C for 40 seconds and 30 cycles of 94 °C for 35 seconds, 50 °C for 45 seconds, and 72 °C for 40 seconds. PCR product was run on a 1% agarose gel containing 1% ethidium bromide at 100 V with 100 bp ladder. Characterization of the mitochondrial lineages, based on the ND5 gene, was done by

constructing a maximum likelihood phylogenetic tree as well as constructing a Bayesian inference phylogenetic tree with the sequences (see 'Sequencing' section below) to identify the haplotypes present in the sample of *Daphnia* used in the study. Partial ND5 genes were sequenced from the 18 isolates that were collected and were pooled with previous ND5 gene datasets from Vergilino et al. (2009, 2011) and Cristescu et al. (2012) (Table 6, Appendix 1) resulting in a dataset of 137 sequences. These partial ND5 sequences were aligned using the ClustalW module of the genetic analysis program MEGA v5.1 (Tamura et al, 2011). A model of sequence evolution was identified using the server based package Topali v2.5 (Milne et al., 2009), the hierarchical likelihood ratio test (hLRT) and the Bayesian Information Criterion (BIC). MrBayes (Ronquist and Huelsenbeck, 2003), available on the Topali v2.5 package, was used to perform phylogenetic Bayesian inference on the sequences. The Bayesian inference that was performed allowed three codon positions (1st, 2nd, and 3rd codon positions) to evolve freely and the DNA substitution model used (selected by Topali v2.5) was a "General Time" reversible model with a Gamma shape (GTR+G) with a gamma substitution parameter (α) of 0.483, with substitution rates such as r_{AC} = 1.488, r_{AG} = 13.128, r_{AT} = 1.095, r_{CG} = 2.613, r_{CT} = 20.849, r_{TG}=1.000, and fixed base frequencies of A = 0.195, C = 0.182, G = 0.212, T = 0.411. Markov chain Monte Carlo analyses were conducted using two runs for 5,000,000 generations, where sampling from the chain occurred every 100 generations. Chain convergence was determined (average standard deviation of split frequencies <0.01), and the initial 25% of trees were discarded as the "burn-in period". A 50% majority-rule consensus tree with posterior probability values for each node was constructed from the remaining Bayesian trees.

DnaSP v5 software (Librado and Rozas, 2009) was used to analyze the haplotypes visualized in the maximum likelihood tree and Bayesian inference tree. A new maximum

likelihood tree and Bayesian inference tree were constructed to show the number of unique haplotypes among the isolates from the present study compared to those from the literature (Vergilino et al., 2009, 2011; Cristescu et al., 2012).

2.13 Nuclear DNA Analysis

The *Ldh* gene (248 nucleotides) was PCR-amplified using the same method as previously described in the "*Mitochondrial DNA Analysis"* section. At the *Ldh* locus, *Daphnia pulex* contain two conserved slow alleles, while *Daphnia pulicaria* contain two conserved fast alleles. The primers used to amplify the slow allele were 5'-GAGCGATTTAACGTTGCGCCC-3' (forward) and 5'-GGACGACTTGTGTGTGAATTTG-3' (reverse) (Cristescu et al., 2012). The primers used to amplify the fast allele were 5'-GAGCGATTTAACGTTGCGCCT-3' (forward) and 5'-GGACGACTTGTGTGTGAATTTC-3' (reverse) (Cristescu et al., 2012). PCR products for each allele were run on separate 1% agarose gels containing 1% ethidium bromide at 100 V with a positive control.

2.14 Sequencing

ND5 gene PCR products were sent to Lakehead University Paleo-DNA Laboratory in Thunder Bay, ON, Canada for purification using the ExoSap purification method and Sanger sequencing. Kelly Lake and Geneva Lake *Daphnia* PCR products were purified using the Solid Phase Reversible Immobilization (SPRI) purification method and Sanger sequenced at the University of Guelph A.A.C. Genomics Facility in Guelph, ON, Canada.

2.15 Statistical Analysis

All LC50s were estimated using a 4-parameter log-logistic model of the drc package (Ritz and Streilbig, 2005) in R (R Development Core Team, 2012). The coefficient of variation (COV) was calculated by dividing the standard deviation by the mean in order to determine the degree of variation. Within-lake and among-lake variation was examined using a ratio-based statistical procedure designed for comparing LC50 values ("LC50 ratio tests") (Wheeler et al, 2006).

Chapter 3

Results

Figure 3 shows all estimated LC50s with their respective confidence intervals. An ANOVA with "Lake" considered as a random effect revealed that LC50 values from metal-contaminated lakes ($\bar{x}$ = 16.5, standard deviation ± 3.3 µg copper/L, n = 9) were greater than those from clean lakes ($\bar{x}$ = 6.6 µg copper/L ± 1.4, n = 9) by nearly a factor of three ($p = 3.5 \times 10^{-4}$) (Figure 4).

The degree of variation was calculated among LC50s within all clean lakes, all metal-contaminated lakes and each individual lake (Table 3). It was found that the COV was slightly higher for the clean lakes compared to the metal-contaminated lakes (21% and 19.9% respectively; Table 3). The COV for each lake were very consistent across all lakes and revealed that Red Chalk Lake, a clean lake, had the lowest COV of all of the lakes (16.7%), while Glen Lake, also a clean lake, had the highest COV of all the lakes (26.7%). McCharles Lake and Simon Lake and Kelly Lake had a similar degree of variation (20%, 19.8%, and 23.6% respectively).

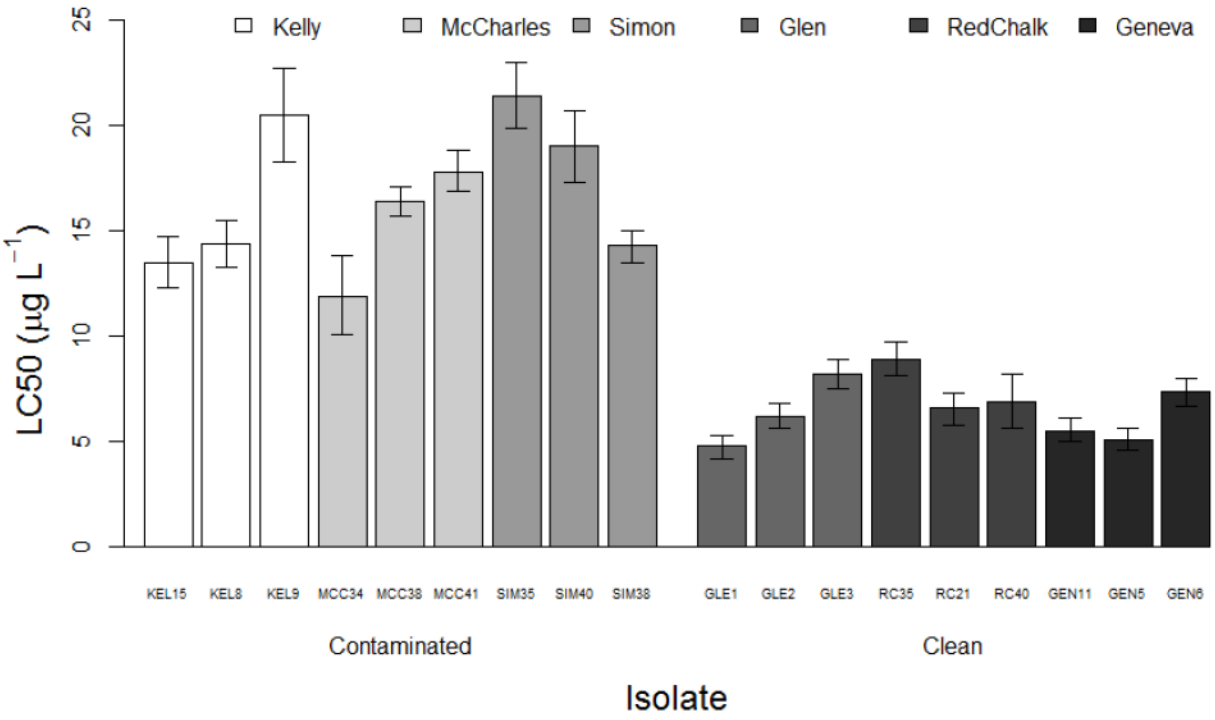

Figure 3: Copper 48-h LC50 values (n = 18) according to site contamination, lake, and isolate. Whiskers represent 95% confidence intervals for estimated 48-h LC50s.

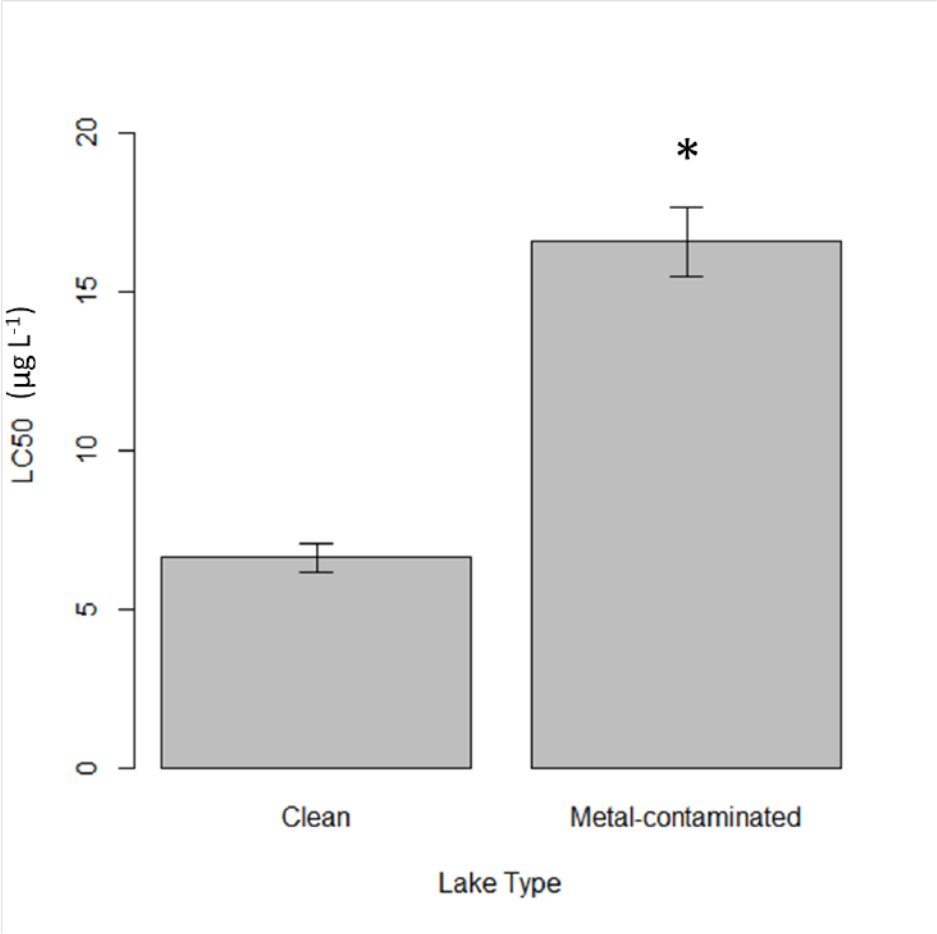

Figure 4: The average 48-h copper LC50s for *Daphnia* from clean (n = 9) and metal-contaminated lakes (n = 9).

Table 3: The coefficient of variation for the average copper 48-hr copper LC50 of each lake and for the average 48-hr copper LC50 of all clean lakes and of all metal-contaminated lakes.

Lake	Coefficient of Variation (%)
Glen	26.7
Geneva	20.5
Red Chalk	16.7
All Clean Lakes	21.0
Kelly	23.6
McCharles	20.0
Simon	19.8
All Contaminated Lakes	19.9

Within-lake and among-lake variation was examined using a ratio-based statistical test (Wheeler et al, 2006), which was performed on individual pairs of isolates (Figure 5). This test was used to determine the probability that two samples from the same lake statistically have the same LC50. Within-lake comparisons were found to be significantly different than one another for 33.3% of the sample pairs ($p \leq 1.0 \times 10^{-5}$ adjusted from 0.05 according to Bonferroni correction).

Among-lake variation was also examined using LC50 ratio tests (Figure 5). It was found that 65.9% of among-lake comparisons were significant, which is higher than for within-lake comparisons (Table 4). There were fewer significant differences among clean lakes (25.9%) than among metal-contaminated lakes (40.7%) (Table 4). However, when comparing metal-contaminated and clean lakes, there was a much larger difference, with 87.7% of samples having significantly different LC50 values according to the ratio test (Table 4). Levene's test for equality of variances (Levene, 1960) was used to examine whether within-lake variation was significantly different than among-lake variation. This test found that the overall variances within lakes was not significantly different than among lakes ($p = 0.147$).

Legend

Significant

Not Significant

			Metal-Contaminated									Clean								
			Kelly			Simon			McCharles			Geneva			Red Chalk			Glen		
			KEL8	KEL9	KEL15	SIM40	SIM38	SIM35	MCC34	MCC41	MCC38	GEN5	GEN6	GEN11	RC21	RC35	RC40	GLE1	GLE2	GLE3
Metal-Contaminated	Kelly	KEL8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		KEL9	7E-09	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		KEL15	4E-01	1E-09	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	McCharles	SIM40	2E-06	1E-01	4E-07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		SIM38	7E-01	3E-11	5E-01	1E-08	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		SIM35	1E-11	7E-01	6E-12	1E-01	4E-17	-	-	-	-	-	-	-	-	-	-	-	-	-
	Simon	MCC34	6E-02	4E-06	1E-01	1E-04	7E-02	4E-05	-	-	-	-	-	-	-	-	-	-	-	-
		MCC41	2E-05	1E-03	5E-06	9E-02	3E-09	1E-04	7E-04	-	-	-	-	-	-	-	-	-	-	-
		MCC38	1E-02	5E-06	7E-01	1E-03	2E-04	2E-08	6E-03	2E-02	-	-	-	-	-	-	-	-	-	-
Clean	Geneva	GEN5	2E-47	2E-66	6E-36	3E-68	3E-56	5E-88	5E-07	2E-82	4E-72	-	-	-	-	-	-	-	-	-
		GEN6	1E-30	2E-49	2E-20	3E-05	7E-40	4E-73	6E-03	1E-69	1E-57	6E-07	-	-	-	-	-	-	-	-
		GEN11	4E-49	1E-67	3E-35	2E-71	1E-61	6E-96	7E-06	1E-93	4E-81	2E-01	5E-05	-	-	-	-	-	-	-
	Red Chalk	RC21	3E-05	3E-09	1E-04	4E-08	3E-05	3E-09	1E-02	3E-07	2E-06	7E-01	2E-01	1E+00	-	-	-	-	-	-
		RC35	3E-19	2E-37	3E-12	1E-37	8E-25	1E-55	1E-01	5E-50	1E-39	2E-13	5E-03	2E-11	6E-02	-	-	-	-	-
		RC40	4E-34	6E-53	5E-24	2E-54	2E-42	2E-74	7E-04	2E-69	1E-58	7E-04	1E-01	2E-02	5E-01	3E-05	-	-	-	-
	Glen	GLE1	5E-40	3E-58	1E-31	6E-58	5E-45	2E-72	2E-07	7E-65	5E-57	6E-01	7E-07	9E-02	5E-01	2E-12	3E-04	-	-	-
		GLE2	9E-42	2E-60	2E-29	2E-63	7E-53	1E-86	1E-04	9E-84	1E-71	1E-02	8E-03	2E-01	7E-01	1E-07	3E-01	4E-03	-	-
		GLE3	2E-25	3E-44	2E-16	1E-45	6E-34	5E-67	3E-02	1E-63	1E-51	1E-10	5E-01	2E-08	1E-01	2E-01	2E-03	6E-10	2E-05	-

Figure 5: LC50 ratio test significance values (using a 1 x 10^{-5} significance cut-off) for the 48-h copper LC50 test.

Table 4: Percentage of significant differences calculated using an LC50 ratio test within and among lakes.

LC50 Ratio comparison group	Percentage Significant
Within Lakes	33.3%
Among All Lakes	65.9%
Among Metal-Contaminated	40.7%
Among Clean	25.9%
Among Metal-Contaminated and Clean	87.7%

The presence of *Ldh* genotypes was determined for each isolate by amplifying the fast (F) allele and slow (S) allele and noting the presence of the band (i.e., indicating a positive result). A positive control was used to ensure the accuracy of the reading. It was found that *Daphnia* from metal-contaminated lakes contained individuals with S and F alleles, while the clean-lake *Daphnia* used in this study contained only the F allele, aside from one isolate (GLE1) which contained both S and F alleles (Table 5). LC50s for the *Daphnia* with SF alleles (average of 15.4 µg copper/L, n = 10) were significantly higher than those containing two F alleles (6.9 µg copper/L, n = 8) ($p = 1.8 \times 10^{-4}$) (Figure 6).

Phylogenetic reconstruction was used to determine the haplotype of each collected isolate. Two types of phylogenetic trees were constructed to validate haplotypes. A maximum likelihood tree was constructed as well as a Bayesian inference tree (Appendix 2, Figures 10 and 11). Based on DnaSP v5 software (Librado and Rozas, 2009) analysis, a new maximum likelihood tree (Figure 7) and Bayesian inference tree (Figure 8) were constructed to show the unique haplotypes present in this study. The majority of the metal-contaminated lake isolates clustered together, with the exception of one McCharles Lake isolate (MCC34). Three of the haplotypes were found to have a *pulex* (PX) mitochondrial genotype and one had a *pulicaria* (PC) mitochondrial genotype based on their clustering with a number of reference sequences provided by Vergilino et al. (2009, 2011) and Cristescu et al. (2012) as well as their *Ldh* genotype. The majority of the clean lake isolates were found to have *pulex* mitochondrial genomes with *pulicaria* nuclear genomes (FF) and all metal contaminated lake isolates were found to have *pulex* mitochondrial genomes with hybrid nuclear genomes (SF). Only a single isolate from Red Chalk Lake (RC40) clustered with *pulicaria* haplotypes and had a *pulicaria* nuclear genotype and therefore *Daphnia* that were in the *pulex* haplotype were used for further

analysis. An ANOVA was performed to determine if haplotype significantly influenced *Daphnia* LC50s. There were significant differences in LC50s among *pulex*-A (17.2 µg copper/L, n = 8), *pulex*-B (6.6 µg copper/L, n = 4; $p = 4.5 \times 10^{-5}$) and *pulex*-C (6.0 µg copper/L, n = 3; $p = 6.8 \times 10^{-5}$) haplotypes (Figure 9).

Table 5: *Ldh* genotypes and ND5 haplotypes for each isolate

Isolate	Lake	*Ldh*	Haplotype
GLE1	Glen	SF	*pulex*-B
GLE2	Glen	FF	*pulex*-B
GLE3	Glen	FF	*pulex*-D
GENC11	Geneva	FF	*pulex*-C
GENC5	Geneva	FF	*pulex*-C
GENC6	Geneva	FF	*pulex*-C
RC40	Red Chalk	FF	*pulicaria*-A
RC21	Red Chalk	FF	*pulex*-B
RC35	Red Chalk	FF	*pulex*-B
KELC15	Kelly	SF	*pulex*-A
KELC8	Kelly	SF	*pulex*-A
KELC9	Kelly	SF	*pulex*-A
MCC38	McCharles	SF	*pulex*-A
MCC41	McCharles	SF	*pulex*-A
MCC34	McCharles	SF	*pulex*-E
SIM35	Simon	SF	*pulex*-A
SIM38	Simon	SF	*pulex*-A
SIM40	Simon	SF	*pulex*-A

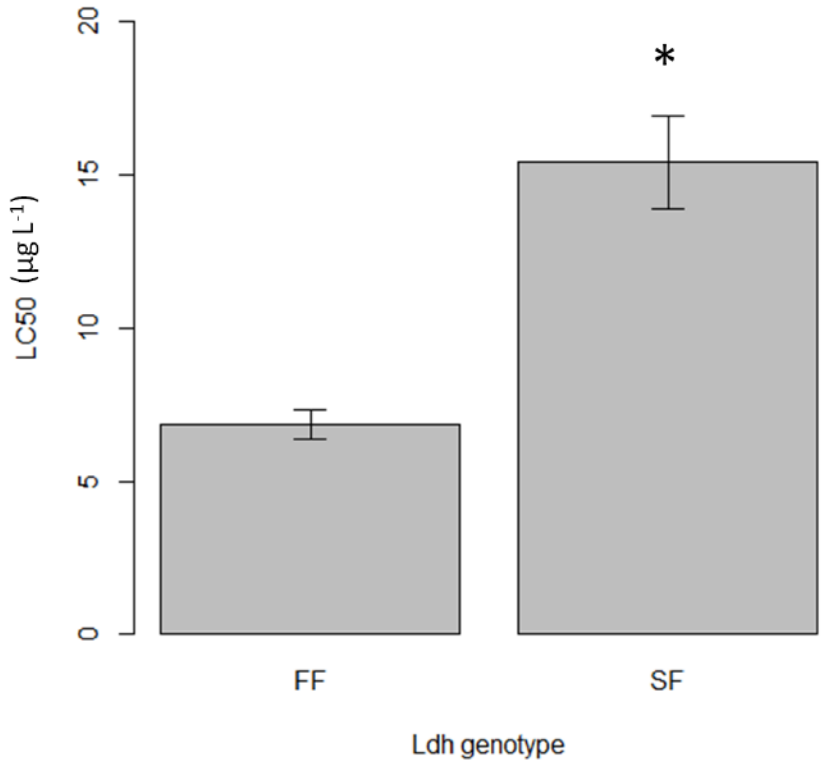

Figure 6: Average 48-h copper LC50s for *Daphnia* with SF alleles (n = 10) and *Daphnia* with FF alleles (n = 8).

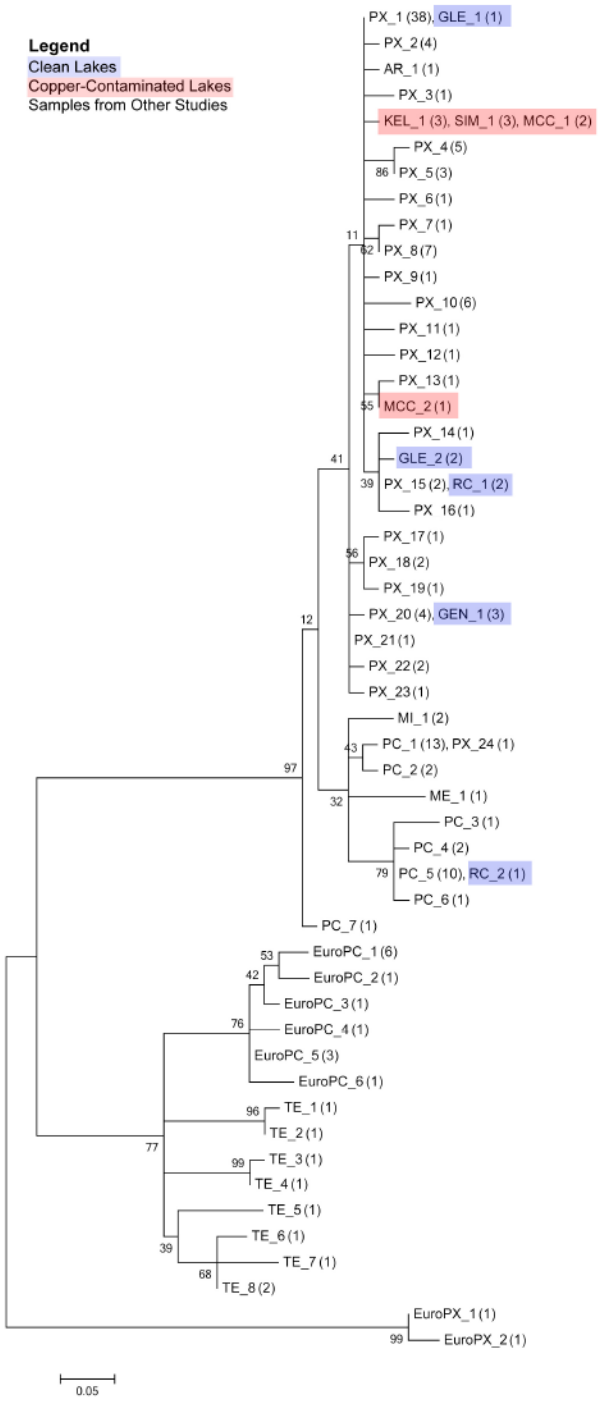

Figure 7: A maximum likelihood tree constructed using partial ND5 sequences from 18 isolates from the present study and PX (*pulex*), PC (*pulicaria*), TE (*tenebrosa*), AR (*arenata*), ME (*melanica*), MI (*middendorffiana*), EuroPX (European *pulex*) and EuroPC (European *pulicaria*) isolates from Vergilino et al. (2011) and Cristescu et al. (2012) (Appendix 1, Table 6) used to determine the haplotypes of the isolates used in this study. The numbers in brackets represent the amount of sampled haplotypes.

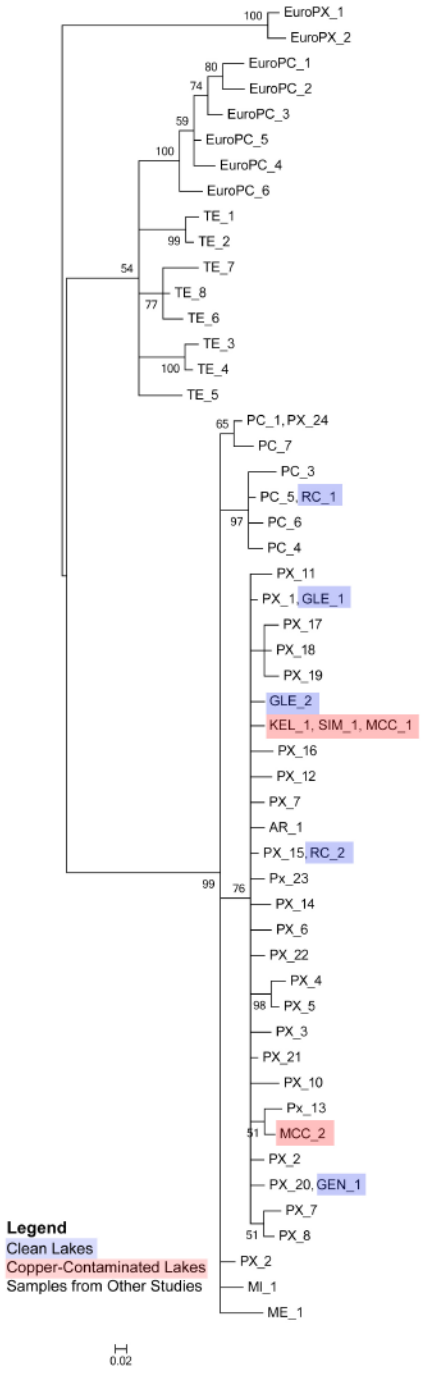

Figure 8: Bayesian inference-based phylogenetic clustering based on partial ND5 gene sequences from 18 isolates from the present study and PX (*pulex*), PC (*pulicaria*), TE (*tenebrosa*), AR (*arenata*), ME (*melanica*), MI (*middendorffiana*), EuroPX (European *pulex*) and EuroPC (European *pulicaria*) isolates from Vergilino et al. (2011) and Cristescu et al. (2012) (Appendix 1, Table 6) used to determine the haplotypes of the isolates used in this study.

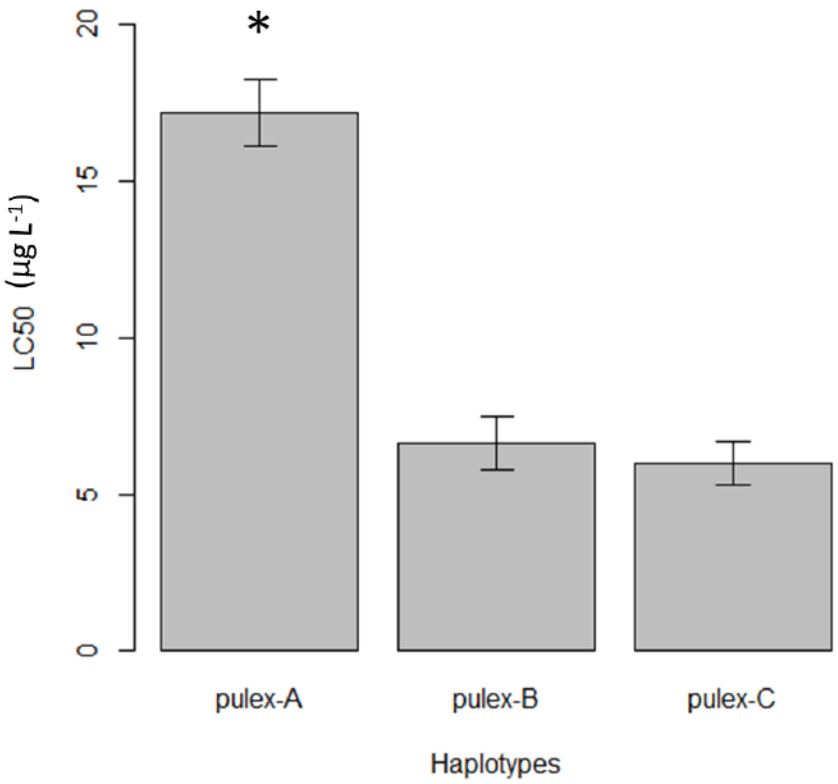

Figure 9: The average 48-h LC50 values for *pulex*-A (n = 8), *pulex*-B (n = 4) and *pulex*-C (n = 3) haplotypes.

Chapter 4

Discussion

Biological variation among organisms that breed clonally can lead to rapid adaptation to toxic environmental conditions, but is not very well understood and can lead to false assumptions about population phenotypes that are based on single individuals from a population (Duffy, 2010). This study investigated the relationship that exists between inter-lake and intra-lake variation among *Daphnia* isolates in order to understand the degree of variation that exists in the resistance to copper contamination among individual *Daphnids* and among populations.

Daphnia from metal-contaminated lakes were found to have almost a 3-fold greater tolerance for copper than *Daphnia* from clean lakes, according to LC50 measurements (Figure 4). This greater tolerance was expected for the *Daphnia* from metal-contaminated lakes, as previous studies have shown the ability of *Daphnia* species to adapt to metals in their environment (Muyssen et al., 2002; Lopes et al., 2006; Agra et al., 2010; Saro et al., 2012). One study, conducted by Lopes et al. (2006), used laboratory-acclimated and non-acclimated *Daphnia longspina* (collected from a site historically exposed to acid mine drainage) to determine whether a difference in sensitivity was present compared to a reference population. It was found that both laboratory-acclimated and non-acclimated populations from the acid mine drainage site were significantly less sensitive to acid mine drainage-contaminated water than those from reference populations, conferring an adaptation to metal stress (Lopes et al., 2006). A large difference in copper LC50 values resulting from resistance (as found in this study), was also found in a study by Agra et al. (2011), in which *Daphnia longspina* historically exposed to acid mine drainage had copper resistant clones that were 16 fold more tolerant than reference

clones in acute toxicity tests. The LC50 values for our variation study were found to be quite low compared to those in found in literature for the same toxicant (Table 2). This discrepancy may be due to the fact that the LC50 tests were not performed under direct compliance testing protocols, or due to the large degree of variation in literature LC50 values for the same species using the same toxicant (Table 2). Another reason for the discrepancy may be due to a great deal of variability existing among lakes and isolates within lakes (Table 3; Table 4), so there is no expected consistency for overall ranges of LC50 among samples collected from different areas.

The variability of LC50 values within lakes and among lakes was studied in order to determine whether there were relatively large differences in variability of metal tolerance among *Daphnia* individuals sampled from the same lake. Inter-lake variation was analyzed by calculating the COV for the average LC50 in each lake. It was found that Red Chalk and Glen Lake, two of the three clean lakes, had the highest degree of variation and Geneva Lake (a clean lake) had one of the lowest degrees of variation (Table 3). The lower degree of variation in Geneva Lake may be due to its relative isolation from the other two clean lakes. All metal-contaminated lakes had a lower degree of within-lake variation than the Red Chalk and Glen lakes. The COV was also calculated to compare the degree of variance in the LC50s of all *Daphnia* from clean lakes to *Daphnia* from contaminated lakes. *Daphnia* from clean lakes had a slightly higher degree of variation among LC50s (21.0%) compared to the LC50s of *Daphnia* from metal-contaminated lakes (19.9%; Table 3). There a was similar amount variability in the LC50 values among lakes, and the overall variances within lakes was not significantly different than among lakes ($p = 0.147$ according to Levene's test for equality of variances; Levene, 1960), indicating that despite large significant differences in overall metal tolerance among lakes, there

remained a high variability in metal tolerance among samples collected from the same lake, comparable to that of samples collected from other lakes with the same contamination status.

In order to further investigate intra-lake and inter-lake variation, an LC50 ratio test (Wheeler et al, 2006) to determine the probability that two samples from the same lake statistically have the same LC50. Less than half (33.3%) of the within-lake isolate LC50 comparisons were found to be significantly different than one another while 65.9% of the among-lake LC50 isolate comparisons were found to be significantly different (Table 4). The proportion of pairwise LC50 comparisons that were significantly different from one another was higher when comparing isolates from metal-contaminated lakes (40.7%) than when comparing isolates from clean lakes (25.9%) (Table 4). This suggests that the adaptation to metal is a progressive process, and isolates have a varied ability to tolerate metal stress even within the same lake.

Other studies have also shown that *Daphnia* isolates from the same area can have variable sensitivities to metals. One study by Baird et al. (1990), found a large difference in sensitivity among *Daphnia magna* clones when exposed to cadmium where LC50 values ranged from 0.06 µg/L – 100 µg/L. In our study, only two lakes (McCharles and Red Chalk) had significantly similar LC50 values for all three of the isolates, with the other lakes having significant differences among all of the samples (Figure 5). A large degree of variation was observed in the reference population used in a study by Lopes et al. (2004), in which some of the most tolerant isolates were found in the clean reference lakes, along with sensitive isolates. In our study, four of the six lakes lake had one outlier sample which was significantly different than the other two (including MCC34 which was not significantly different from several of the clean lakes), and there were more significant differences in LC50 values among metal-contaminated

lakes than among clean lakes (Table 4). However, among metal-contaminated and clean lakes, there was a much larger difference, where 87.7% of samples had significantly different LC50 values, suggesting that while there is a large variation within the same type of lake, there is an overall trend of higher metal tolerance in metal-contaminated lakes (consistent with the ANOVA test which found a significance level of $p = 3.5 \times 10^{-4}$ for this comparison).

Overall, these results highlight the fact that multiple isolates need to be collected when conducting toxicity tests and the average representative LC50 value for metal tolerance needs to be calculated, because while the overall average difference in LC50 values between clean and contaminated lakes may be significant, individual isolates may not be representative of the overall values for the lake due to high intra-lake variance. Ideally, the replicate samples should be collected from multiple points within a lake in order to capture the most intra-lake variation as possible.

Allozymes have been studied in multiple aquatic organisms such as fish and crustaceans (Couture and Kumar, 2003; Harper-Arabie et al., 2004) and several studies have found metals to have an effect on allozyme activity. A study by Couture and Kumar (2003) examined the effect of copper on liver *Ldh* activity in wild yellow perch (*Perca flavenscens*) and found that *Ldh* activity increased significantly with increasing concentrations of copper. Another study by Diamantino et al. (2001) found that *Ldh* activity measurement can be used as an effective indicator criterion in metal toxicity tests which supports the idea that metal can affect *Ldh* activity. Furthermore, studies on other *Daphnia* allozymes have found correlations between allozyme genotype frequency and a number of life history traits such as response to fish kairomones (Boersma et al., 1999), and diel migration patterns (King and Miracle, 1995). The *Ldh* genotype was identified for all of the isolates, and it was found that the *Daphnia* from metal-

contaminated lakes contained both the S and F alleles, while the clean lake *Daphnia* contained only the F allele, aside from one (GLE1) which contained both S and F alleles. As expected, due to the lake source of the strains, the LC50s for the *Daphnia* with the SF allele were significantly higher than those containing two F alleles (Figure 6). These results suggest that hybrids (*Daphnia* possessing S and F alleles) may be able to better adapt to metal contaminated environments, as *Daphnia* hybrids can obtain a competitive advantage over parental species due to increased genetic diversity from both parental lineages (Loaring and Hebert, 1981). Furthermore, since hybrids are primarily asexual, this may also reduce genetic variation within metal-contaminated environments. This study also found that hybrids can also inhabit lakes where they have previously only been found in intermediate habitats such as fishless lakes and ponds as well as disrupted habitats (Hebert et al., 1989). This finding is important as it suggests that gene flow may be occurring among the habitats which may be due to transfer of ephippia from one population to another via water fouling, wind or recreational anthropogenic means. This study found a significant difference in *Ldh* genotype but did not investigate *Daphnia* containing each type of allele allocation from both a clean and metal-contaminated source, which is an analysis that may be considered in future research.

ND5 haplotypes were determined by amplifying and sequencing the ND5 gene for each isolate, and then constructing a maximum likelihood phylogenetic tree and performing Bayesian inference clustering analysis. Reference sequences were used to determine the haplotype of the unknown sequences. It was found that six haplotypes were present in this study (five haplotypes of *pulex* mitochondrial genotype and one of *pulicaria* mitochondrial genotype). *Daphnia* that were in the *pulex* haplotype were used for further analysis. There was a significant difference in haplotypes based on the *Daphnia* isolates present in each haplotype and their respective LC50

measures (Figure 9). All except one of the *Daphnia* isolates from the contaminated lakes clustered together, with all of the Kelly Lake and Simon Lake isolates (as well as two of the three McCharles lake isolates) forming their own cluster (*pulex*-A) with 87% confidence (Figure 7, Figure 8). The other McCharles lake isolate (MCC34), however, did not cluster with isolates from their own contaminated lake. This result demonstrates that non-representative genotypes may be found in the population, despite selective pressure against them, since one McCharles Lake isolate had a low metal tolerance (with an LC50 of 11.9 µg/L compared to an average of 17.2 µg/L for the other contaminated lakes), and has a ND5 haplotype which is also different than the other isolates in the metal contaminated lakes. One isolate from Red Chalk Lake (RC40) was found to cluster with *pulicaria* species (Figure 7; Figure 8). This finding may contribute to the lower degree of variation in the lake (Table 3). These results also suggest that isolates from clean lakes have a high range of metal tolerance and ecologically, this may help the population to prepare for potential metal contamination or stressful situations. Experimentally, as shown in this study, this result means that at least three samples are needed from a population in order to get an idea of the overall tolerance level in the population. However, more samples will reduce sampling error and will also make it easier to detect effects that are expected to be relatively small compared to the natural variation in the population. The high baseline variation in metal-contaminated lakes may have evolved in regions where water contamination is common, and therefore it is important to be aware of the potentially higher variation in certain regions.

Daphnia are a widely used indicator species for environmental contamination and stress, and therefore understanding their adaptation and survival, as well as developing optimal sampling and measurement strategies, is essential for better understanding the environmental impact of pollution. This study has contributed to the understanding of variation among

organisms that breed clonally, as a high degree of intra-lake variance was found among isolates and among lakes. This source of variation may be attributed to genetic variation that exists in *Ldh* activity and ND5 mitochondrial lineages. Since *Ldh* activity has been linked with response to metals in *Daphnia* (Diamantino et al., 2001) as well as other organisms (Couture and Kumar, 2003; Harper-Arabie et al., 2004) it may mean that *Ldh* alleles correspond to contamination level in lakes and to LC50 (as seen in this study). Therefore the differences in type of *Ldh* allele may represent the metal contamination tolerance and evolve quickly in a population as an adaptive response to contamination. The same inferences may be made about ND5 haplotypes as this study found that isolates with high metal tolerance clustered together. This means that a greater overall genetic diversity in the clean lakes exists (where they are more variable in haplotypes), and that metal contamination leads to restriction of the number and diversity of haplotypes of *Daphnia* present. Regardless of the amount of variation present, there still exists a significant difference between copper tolerance from *Daphnia* isolated from clean lakes and from metal-contaminated lakes. While maintaining population variance among isolates is an effective strategy for developing tolerance to potential environmental stress, and in this case, allows *Daphnia* to adapt to metal-contaminated environments, contamination can ultimately lead to a reduction in overall genetic diversity among sensitive organisms due to the loss of haplotypes and genotypes that are unable to deal with the stress. The large degree of variance among found among isolates in this study shows that it is extremely important to use more than one representative isolate to get a more accurate understanding of the effects of contaminants especially when making assumptions about an indicator organism population.

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Appendix 1

Table 6: The GenBank accession numbers, phylogenetic tree labels, sampling locations, ecology of locations, and references for all reference sequences used in ND5 gene analysis.

GenBank Accession Number	Label	Sampling Location	Ecology	Reference
HQ434664	AR2-UN-1	Oak Patch, Oregon, USA	Pond	Vergilino et al. 2009
HQ434635	AR2-UN-2	Creswell Court, Oregon, USA	Pond	Vergilino et al. 2009
HQ434643	EuroPC-UN-2	Antermoia, Italy	Lake	Vergilino et al. 2011
HQ434642	EuroPC-UN-7	High Tatra Mountain, Slovakia	Lake	Vergilino et al. 2011
HQ434645	EuroPC-UN-12	Sweden	Lake	Vergilino et al. 2011
HQ434644	EuroPX-UN-2	Sweden	Pond	Vergilino et al. 2011
HQ434677	ME-UN-1	Oregon, USA	Pond	Vergilino et al. 2011
HQ434690	PC2-FF-1	Winnipeg, Manitoba, CAN	Lake	Vergilino et al. 2011
HQ434686	PC2-FF-2	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434684	PC2-FF-3	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434689	PC2-FF-4	Winnipeg, Manitoba, CAN	Lake	Vergilino et al. 2011
FJ591104	PC2-FF-5	IN, U.S.A.	Lake	Vergilino et al. 2009
FJ591112	PC2-FF-6	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2009
FJ591103	PC2-FF-7	IN, U.S.A.	Lake	Vergilino et al. 2009
FJ591124	PC2-FF-8	Kuujjarapik, QC, CAN	Pond	Vergilino et al. 2009
JN561035	PC2-FF-9	Big Gull, ON	Lake	Cristescu et al. 2012
JN561037	PC2-FF-10	Big Gull, ON	Lake	Cristescu et al. 2012
JN561036	PC2-FF-11	Big Gull, ON	Lake	Cristescu et al. 2012
JN561041	PC2-FF-12	Lawrence, MI	Lake	Cristescu et al. 2012

JN561024	PC2-FF-13	Multiple locations	Lake	Cristescu et al. 2012
JN561062	PC2-FF-14	Warner, MI	Lake	Cristescu et al. 2012
JN561061	PC2-FF-15	Warner, MI	Lake	Cristescu et al. 2012
HQ434657	PC2-UN-1	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434658	PC2-UN-2	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434685	PC2-UN-3	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434683	PC2-UN-4	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434679	PC2-UN-5	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
FJ591099	PC3-SF-1	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2009
HQ434630	PC3-SF-2	Churchill, MB, CAN	Rock bluff	Vergilino et al. 2011
FJ591111	PC3-SF-3	Kuujjarapik, QC, CAN	Pond	Vergilino et al. 2009
FJ591110	PC3-SF-4	Kuujjarapik, QC, CAN	Pond	Vergilino et al. 2009
FJ591106	PC3-SF-5	Churchill, MB, CAN	Rock bluff	Vergilino et al. 2009
FJ591102	PC3-SF-6	Churchill, MB, CAN	Rock bluff	Vergilino et al. 2009
FJ591105	MI3-SF-1	Churchill, MB, CAN	Rock bluff	Vergilino et al. 2009
FJ591122	MI3-SF-2	Churchill, MB, CAN	Rock bluff	Vergilino et al. 2009
FJ591113	PC3-FF-1	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2009
HQ434680	PC3-UN-1	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434678	PC3-UN-2	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434626	PC3-UN-3	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434670	PX2-FF-1	Vermillion County, Illinois, USA	Lake	Vergilino et al. 2009
HQ434668	PX2-FF-2	Vermillion County, Illinois, USA	Lake	Vergilino et al. 2009
HQ434667	PX2-FF-3	Vermillion County, Illinois, USA	Lake	Vergilino et al. 2009
HQ434669	PX2-FF-4	Vermillion County, Illinois, USA	Lake	Vergilino et al. 2009
HQ434671	PX2-FF-5	Vermillion County, Illinois, USA	Lake	Vergilino et al. 2009
JN561043	PX2-FF-6	Lawrence, MI	Lake	Cristescu et al. 2012
JN561040	PX2-FF-7	Lawrence, MI	Lake	Cristescu et al. 2012
JN561042	PX2-FF-8	Lawrence, MI	Lake	Cristescu et al. 2012
JN561044	PX2-FF-9	Lawrence, MI	Lake	Cristescu et al. 2012
JN561023	PX2-FF-10	Multiple locations	Lake	Cristescu et al. 2012
JN561032	PX2-FF-11	Multiple locations	Lake	Cristescu et al. 2012
JN561059	PX2-FF-12	Sportsman, IL	Lake	Cristescu et al. 2012
JN561019	PX2-FF-13	Three Lakes 2, MI	Lake	Cristescu et al. 2012
JN561021	PX2-FF-14	Three Lakes 2, MI	Lake	Cristescu et al. 2012
JN561020	PX2-FF-15	Three Lakes 2, MI	Lake	Cristescu et al. 2012
JN561022	PX2-FF-16	Three Lakes 2, MI	Lake	Cristescu et al. 2012

JN561063	PX2-FF-17	Warner, MI	Lake	Cristescu et al. 2012
JN561065	PX2-FF-18	Warner, MI	Lake	Cristescu et al. 2012
JN561064	PX2-FF-19	Warner, MI	Lake	Cristescu et al. 2012
HQ434633	PX2-SF-1	Canard pond, ON	Pond	Vergilino et al. 2011
JN561025	PX2-SF-2	Canard pond, ON	Pond	Cristescu et al. 2012
JN561033	PX2-SF-3	Disputed road, ON	Pond	Cristescu et al. 2012
JN561027	PX2-SF-4	Multiple locations	Pond	Cristescu et al. 2012
JN561029	PX2-SF-5	Multiple locations	Pond	Cristescu et al. 2012
JN561031	PX2-SF-6	Multiple locations	Pond	Cristescu et al. 2012
HQ434660	PX2-SF-7	Rimouski, Québec, CAN	Pond	Vergilino et al. 2009
HQ434665	PX2-SF-8	Rimouski, Québec, CAN	Pond	Vergilino et al. 2009
HQ4346501	PX2-SF-9	Ste-Foy, QC, CAN	Pond	Vergilino et al. 2011
HQ434634	PX2-SF-10	Canard pond, ON	Pond	Vergilino et al. 2011
HQ434663	PX2-SF-11	Metis, QC, Canada	Pond	Vergilino et al. 2009
HQ434655	PX2-SF-12	MI, USA	Pond	Vergilino et al. 2011
HQ434651	PX2-SF-13	Ste-Foy, QC, CAN	Pond	Vergilino et al. 2011
HQ434649	PX2-SF-14	Canard pond, ON	Pond	Vergilino et al. 2011
HQ434631	PX2-SF-15	Churchill, MB, CAN	Rock bluff	Vergilino et al. 2011
FJ591096	PX2-SF-16	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2009
FJ591098	PX2-SF-17	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2009
HQ434674	PX2-SS-1	Restigouche, New Brunswick, CAN	Pond	Vergilino et al. 2011
HQ434666	PX2-SS-2	Povi Pond, MI, USA	Pond	Vergilino et al. 2009
FJ591107	PX2-SS-3	Churchill, Manitoba, CAN	Rock bluff	Vergilino et al. 2009
HQ434629	PX2-SS-4	Busey Woods, Illinois, USA	Pond	Vergilino et al. 2011
HQ434639	PX2-SS-5	Disputed road, ON	Pond	Vergilino et al. 2011
HQ434640	PX2-SS-6	Disputed road, ON	Pond	Vergilino et al. 2009
JN561034	PX2-SS-7	Disputed road, ON	Pond	Cristescu et al. 2012
JN561026	PX2-SS-8	Disputed road, ON	Pond	Cristescu et al. 2012
JN561039	PX2-SS-9	Grimey, MI	Pond	Cristescu et al. 2012
JN561038	PX2-SS-10	Grimey, MI	Pond	Cristescu et al. 2012
JN561030	PX2-SS-11	Multiple locations	Pond	Cristescu et al. 2012
JN561028	PX2-SS-12	Multiple locations	Pond	Cristescu et al. 2012
JN561051	PX2-SS-13	Solomon, MI	Pond	Cristescu et al. 2012
JN561053	PX2-SS-14	Solomon, MI	Pond	Cristescu et al. 2012
JN561055	PX2-SS-15	Solomon, MI	Pond	Cristescu et al. 2012
JN561057	PX2-SS-16	Solomon, MI	Pond	Cristescu et al. 2012
JN561046	PX2-SS-17	Solomon, MI	Pond	Cristescu et al. 2012
JN561048	PX2-SS-18	Solomon, MI	Pond	Cristescu et al. 2012
JN561050	PX2-SS-19	Solomon, MI	Pond	Cristescu et al. 2012
JN561052	PX2-SS-20	Solomon, MI	Pond	Cristescu et al. 2012
JN561054	PX2-SS-21	Solomon, MI	Pond	Cristescu et al. 2012

JN561047	PX2-SS-22	Solomon, MI	Pond	Cristescu et al. 2012
JN561049	PX2-SS-23	Solomon, MI	Pond	Cristescu et al. 2012
JN561056	PX2-SS-24	Solomon, MI	Pond	Cristescu et al. 2012
JN561058	PX2-SS-25	Solomon, MI	Pond	Cristescu et al. 2012
JN561045	PX2-SS-26	St. Michael, ON	Pond	Cristescu et al. 2012
JN561060	PX2-SS-27	St. Michael, ON	Pond	Cristescu et al. 2012
JN561067	PX2-SS-28	West Gull, MI	Pond	Cristescu et al. 2012
JN561066	PX2-SS-29	West Gull, MI	Pond	Cristescu et al. 2012
JN561068	PX2-SS-30	West Gull, MI	Pond	Cristescu et al. 2012
HQ434632	PX2-SS-31	Canard pond, ON	Pond	Vergilino-et-al_2011
HQ434636	PX2-SS-32	Chequamegon, Wisconsin, USA	Pond	Vergilino et al. 2009
HQ434648	PX2-SS-33	Eloise Butler, Minnesota, USA	Pond	Vergilino et al. 2009
HQ434659	PX2-SS-34	Listowel Pond, Ontario, CAN	Pond	Vergilino et al. 2009
HQ434661	PX2-SS-35	Long Point, Ontario, CAN	Pond	Vergilino et al. 2011
HQ434687	PX2-SS-36	Troy II, Maine, USA	Pond	Vergilino et al. 2011
FJ591108	PX2-SS-37	Closed Road, MI, USA	Pond	Vergilino et al. 2009
JN561018	PX2-SS_PX2-FF	Multiple locations	Pond and lake	Cristescu et al. 2012
HQ434672	PX2-UN-1	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434673	PX2-UN-2	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
FJ591109	PX2-UN-3	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2009
FJ591101	PX3-SF-1	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2009
FJ591114	PX3-SF-2	Disputed road, ON	Pond	Vergilino et al. 2009
FJ591097	PX3-SF-3	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2009
HQ434688	PX3-UN-1	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434682	PX3-UN-2	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434656	PX3-UN-3	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434681	PX3-UN-4	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434627	PX3-UN-5	Kuujjarappik, QC, CAN	Pond	Vergilino et al. 2011
HQ434647	PX3-UN-6	Avigliana, Italy	Lake	Vergilino et al. 2011

Appendix 2

Figure 10: A maximum likelihood tree constructed with the 18 isolates from this study as well as reference sequences as obtained from the literature (Vergilino et al., 2009, 2011; Cristescu et al., 2012) (Appendix 1; Table 6)

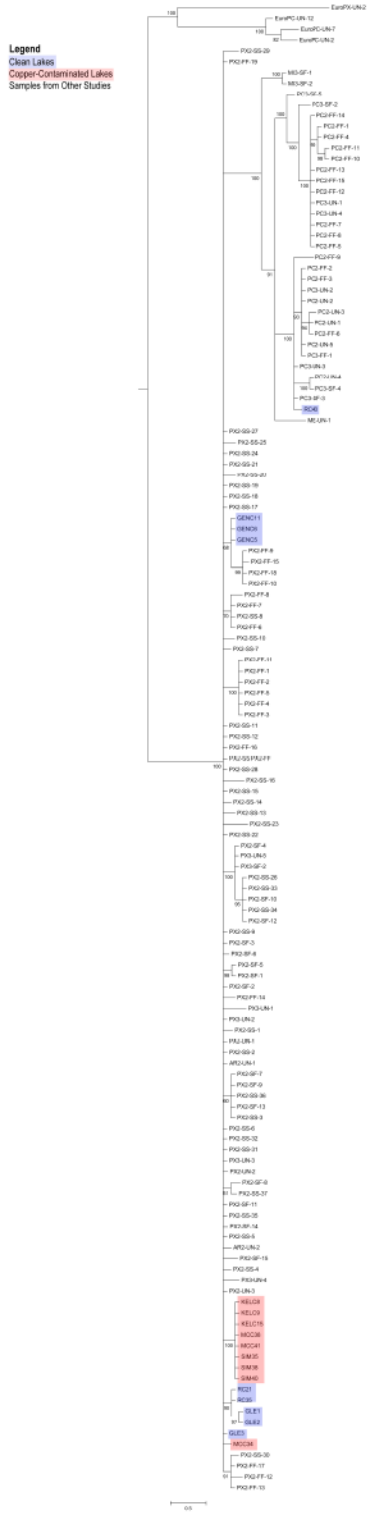

Figure 11: A Bayesian Inference tree based on partial ND5 gene sequences from 18 isolates from this study as well as reference sequences from the literature (Vergilino et al., 2009, 2011; Cristescu et al., 2012) (Appendix 1; Table 6)