CHRONIC TOXICITY OF LITHIUM TO THE WATER FLEA *DAPHNIA PULEX* AND FINGERNAIL CLAM *PISIDIUM DUBIUM*

AWA MAMEY MALIKA ALEXANDRINE OUEDRAOGO

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Department of Biology
Faculty of Science
University of Ottawa

ABSTRACT

Lithium (Li), a metal used in batteries and pharmaceuticals, is increasingly in demand as part of a greener economy. However, its extraction, use, and disposal may elevate environmental concentrations, raising concerns about aquatic ecosystems. This study assessed Li chronic toxicity and bioaccumulation in two freshwater invertebrates: *Pisidium dubium* (28-day exposure) and *Daphnia pulex* (21-day exposure). Organisms were exposed to environmentally relevant Li concentrations (0.05–10 mg/L for *P. dubium*; 0.5–3 mg/L for *D. pulex*). Results showed reduced survival (EC_{50} = 1.37 mg/L) and impaired burrowing behavior (EC_{50} = 1.59 mg/L) in *P. dubium*, with tissue analysis suggesting internal Li regulation. In *D. pulex*, reproduction (EC_{50} = 1.77 mg/L) and growth (EC_{50} = 3.01 mg/L) were significantly affected. While natural Li levels in surface waters are usually below these thresholds, elevated concentrations near mining and urban areas could harm aquatic invertebrates, highlighting the need for improved waste management strategies.

RÉSUMÉ

Le lithium (Li), utilisé dans les batteries et produits pharmaceutiques, connaît une demande croissante pour une économie verte. Son extraction et utilisation peuvent accroître les concentrations environnementales, posant un risque pour les écosystèmes aquatiques. Cette étude a examiné la toxicité chronique et la bioaccumulation du Li chez *Pisidium dubium* (28 jours) et *Daphnia pulex* (21 jours), exposés à des concentrations écologiquement pertinentes (0,05–10 mg/L pour *P. dubium* ; 0,5–3 mg/L pour *D. pulex*). *P. dubium* montre une baisse de survie (EC_{50} = 1,37 mg/L) et un enfouissement altéré (EC_{50} = 1,59 mg/L), avec une régulation interne du Li. Chez *D. pulex*, la reproduction (EC_{50} = 1,77 mg/L) et la croissance (EC_{50} = 3,01 mg/L) sont affectées. Bien que les niveaux naturels de Li soient inférieurs à ces seuils, des concentrations élevées près des zones minières et urbaines pourraient impacter ces invertébrés, soulignant l'importance d'une meilleure gestion des déchets.

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Abbreviations

BLM	Biotic Ligand Model
CI	Confidence Interval
CIx	Concentration inhibitrice de x% des organismes testés
CLx	Concentration léthale pour x% des organismes testés
BCF	Bioconcentration Factor
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
ECx	Concentration affecting x% of organisms tested
EPA	Environment Protection Agency of the United States
ERA	Environmental Risk Assessment
HC_5	Hazardous Concentration for 5% of Species
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
IQR	Interquartile Range
LCx	Concentration lethal to x% of organisms tested
Li	Lithium

Na	Sodium
PNEC	Predicted no-effect concentrations
RSW	Reconstituted Soft Water
SSD	Species Sensitivity Distribution
TOC	Total organic carbon
YCT	Yeast Cerophyll Trout feed suspension

Chapter 1: General Introduction

Since the beginning of industrialization, metal contamination in surface waters has been an environmental concern, necessitating the management of various point and non-point sources, such as industrial discharges, agricultural runoff, and atmospheric deposition (Gagnon & Vigneault, 2013). Metals can cause toxic effects in aquatic ecosystems through environmental dispersion, bioaccumulation and the disruption of essential cellular metabolism in aquatic organisms, leading to cascading impacts on biodiversity (Campbell, 2022).

To better understand these toxic effects and effectively manage metal contamination in aquatic ecosystems, it is crucial to examine the speciation of metals, a determining factor in their bioavailability and toxicity (Bourg, 1995).

1.1 Metal speciation

Speciation refers to the occurrence of an element in different chemical forms or oxidation states within a system (de Paiva Magalhães et al., 2015). In aqueous media, metal speciation is more specifically defined as the various chemical forms of a metal, including both free and complexed states (Gagnon & Vigneault, 2013).

Several factors influence metal speciation in aquatic environments. Intrinsic properties of metals, such as ionic size, valence state and electronegativity, influence their sorption capacity with dissolved or particulate ligands present in natural waters (Gagnon & Vigneault, 2013). The valence state determines the charge and coordination environment of the metal, affecting its ability to form complexes, while ion size affects electronegativity, which impacts the metal's affinity for different ligands, influencing its interactions with organic and inorganic particles in the water.

Extrinsic properties that affect metal sorption into dissolved and particulate phases include ionic strength, pH, redox potential, and water hardness (i.e., the concentration of divalent cations, such as calcium (Ca^{2+}) and magnesium (Mg^{2+})). Cations and protons present will compete for sorption sites on surfaces, reducing the availability of binding spots for metals. Additionally, these ions can screen the electric fields of sorbing phases, thereby decreasing the potential for metal sorption. Other important extrinsic factors relate to the presence of sorbing phases in the environment, such as clays, iron (Fe) and manganese (Mn) oxides, and organic matter (OD), which can significantly affect metal binding due to their high surface area and reactivity, providing a variety of binding sites for metals in aquatic systems (Lofts & Tipping, 1998).

In aquatic environments, metals are distributed typically into three main fractions: dissolved form (< 1 kDa), colloidal form (between 1 kDa and 0.45 µm), and particulate form (> 0.45 µm) (Bjerregaard et al., 2022) . The dissolved fraction is operationally obtained by filtration through a 0.22 or 0.45 µm membrane (Gagnon & Vigneault, 2013). This dissolved form of metals is generally considered the most available, and thus the most bioreactive and potentially toxic to aquatic organisms. The distribution of metals between these fractions can vary considerably based on their solubility and the physicochemical characteristics of the water, such as pH, ionic strength, and the presence of other substances (Figure 1, Bjerregaard et al., 2022). This variability can pose challenges for trace metal toxicity assessments, necessitating appropriate controls and precises analytical measurements to accurately evaluate metal impacts on aquatic ecosystems.

To fully understand the impact of metals on aquatic ecosystems, it is essential to explore how the different chemical forms resulting from speciation influence their bioavailability and toxicity.

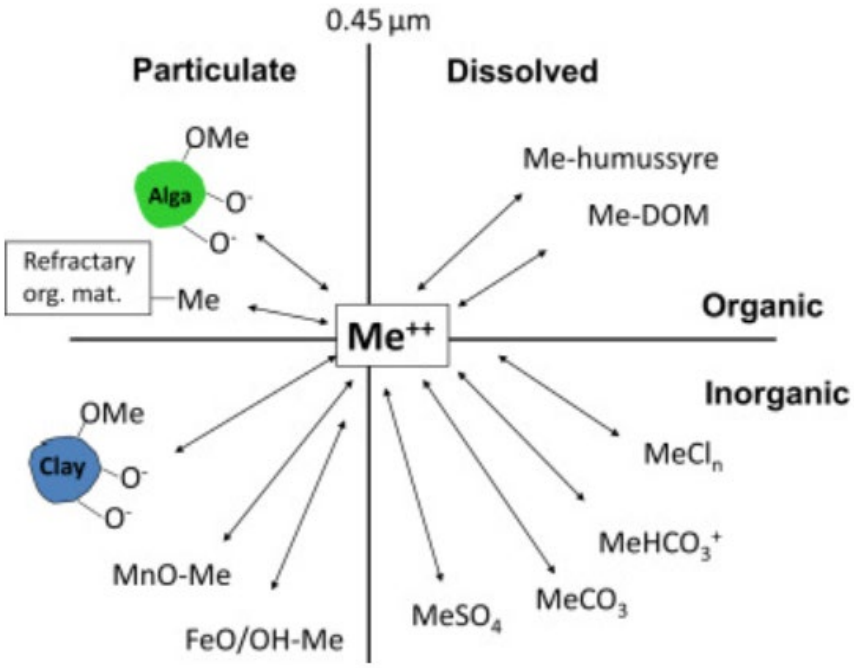

Figure 1. Schematic representation of equilibria between free metal ions (Me^{++}) and various organic and inorganic chelators, including both particulate and dissolved ligands (Bjerregaard et al., 2022).

1.2 Metal bioavailability and toxicity

Measuring metal concentrations in water or sediment alone is insufficient to determine their bioavailability or toxicity. A comprehensive assessment of metal toxicity involves not only measuring concentrations but also analyzing their chemical forms and environmental context, in other words, their speciation. In metal ecotoxicology, the term bioavailability has two meanings: environmental and toxicological. Environmentally, bioavailability refers to the fraction of metal available to be taken up by a living organism and integrated into its metabolic processes. Toxicologically, bioavailability is defined as the fraction of the metal concentration that is absorbed through food and/or adsorbed onto respiratory surfaces by the organism, interacting with

receptors and physiological sites essential for metabolism, thereby triggering potentially toxic effects (de Paiva Magalhães et al., 2015).

The bioavailability of metals is determined by their speciation into dissolved and particulate phases, as defined above (Bjerregaard et al., 2022; de Paiva Magalhães et al., 2015; Gagnon & Vigneault, 2013). Free metal ions are the most bioavailable and potentially toxic because they can directly interact with the respiratory organs of aquatic organisms. In aquatic environments specifically, the bioavailability of metal contaminants depends on numerous factors such as the route of exposure, the mechanism of metal sequestration and transport by organic ligands, and the exposed organism itself (de Paiva Magalhães et al., 2015).

1.3 The Free Ion Activity Model

The Free Ion Activity Model (FIAM), proposed in the 1970s, is a pioneering framework used to explain metal-organism interactions in aquatic environments (Campbell, 1995; de Paiva Magalhães et al., 2015). This widely adopted model simplifies the understanding of metal speciation by focusing on the free ion concentration of metals, which is often the form that interacts most directly with biological systems and thus determines the metal's toxicity (Campbell, 1995). By emphasizing the importance of the free ion activity, the FIAM provides a practical approach for predicting and assessing the biological effects of metals in aquatic environments. The model assumes a rapid equilibrium between free metal ions and their complexes with ligands in the solution, allowing the concentration of free ions to serve as a proxy for the potential biological effects of the metal (Campbell, 1995). This simplified approach has been instrumental in developing our understanding of metal behavior in aquatic systems.

While the FIAM provides a useful simplification, it has notable limitations. The model does not account for the effects of complexation with organic matter, the presence of colloids, or the interactions between different metal ions (de Paiva Magalhães et al., 2015; Lofts & Tipping, 1998). Additionally, it may not accurately predict metal behavior in systems with high concentrations of organic ligands or in the presence of competing ions.

Despite its limitations, the FIAM remains a fundamental concept in aquatic toxicology and serves as a foundation for more complex models. It continues to be a valuable tool for initial assessments of metal toxicity in aquatic systems, providing a starting point for more detailed analyses (Lofts & Tipping, 1998).

1.4 Bioaccumulation

Once metals become bioavailable to organisms, a certain degree of bioconcentration and bioaccumulation generally occurs. The bioconcentration factor (BCF) is the ratio of the metal concentration in an organism (C_b) on a per unit tissue weight basis to that in water (C_w) being the only exposure phase. The bioconcentration factor (BCF) is calculated by dividing the concentration of a metal in the organism (C_{org}, μg/g on a per unit tissue fresh weight basis) by its concentration in the environment ($C0$, μg/L or μg/g, assuming the approximation 1 L = 1 kg) (Arnot & Gobas, 2006) as follows:

$$\mathbf{BCF} = \frac{Corg}{Co} \qquad \mathbf{(1)}$$

In contrast, the bioaccumulation factor (BAF) is the ratio of the metal concentration in an organism or biota (C_b) from all exposure pathways (including water, sediment, and dietary pathways) on a per unit tissue fresh weight basis to that in water (C_w).

Metal bioaccumulation in fish, invertebrates and bivalves is an environmental concern. Fish can accumulate metals by direct absorption through the gill (Campbell, 2022) , as well as through the consumption of contaminated prey (Bjerregaard et al., 2022). Metals are taken up at the cell membrane through several mechanisms, including passive diffusion, endocytosis and active transport (Puckett et al., 2010). Passive diffusion allows some neutral metal species to move across the membrane along concentration gradients without energy input. Endocytosis is another method where the cell membrane engulfs extracellular material, forming vesicles that are internalized (Puckett et al., 2010). However, many metals require active transport, that facilitates the movement of metal ions into the cell, often against a concentration gradient and requiring energy (Campbell, 2022). These processes are influenced by the metal's chemical properties, the presence of specific transport proteins, and the cell's physiological state.

Bioaccumulation cannot be successfully predicted from dissolved total extracellular concentrations alone. It has been demonstrated that the concentration of free ions is best correlated with uptake, but competition with other cations, such as Ca, Mg, Na and protons, also affects absorption (Vijver et al., 2004). Calcium (Ca^{2+}) and protons (H^{+}) can compete with metal ions for uptake at the cell membrane. Calcium ions often compete with metal ions for binding sites on transport proteins, as many metal ions share similar transport pathways (Campbell, 2022). The presence of Ca^{2+} can either enhance or inhibit the uptake of other metals depending on the specific interactions and the concentrations of the ions involved (Pasricha et al., 2021).

For instance, if Ca^{2+} binds more strongly to the biotic ligands than the target metal, it can effectively block the uptake of that metal (Campbell, 2022; Pasricha et al., 2021). Protons influence metal uptake by affecting the membrane potential and the activity of transport proteins. The H^{+}-ATPase activity in the plasma membrane can be inhibited by heavy metals, altering the proton gradient and impacting the uptake of other ions, including metals (Janicka-Russak et al., 2008). High concentrations of protons can also directly compete with metal ions for transport sites, affecting the overall uptake efficiency (Janicka-Russak et al., 2008).

1.5 Metal toxicity

It is important to note that the toxicity of a metal depends on the nature of the response measured. By definition, lethal concentrations of a compound will be higher than those causing sublethal effects, such as reduced growth (Campbell, 2022). Similarly, the concentration or dose required to induce an effect during a short-term (acute) exposure is generally higher than that needed to produce the same effect during a prolonged or chronic exposure. Therefore, no expression of toxicity is complete without modifiers indicating the duration of exposure and the measured effects (Campbell, 2022). For instance, acute lethality tests on animals often measure the median lethal concentration (LC_{50}) or dose (LD_{50}) over 96 hours. For chronic and sublethal effects, results might be expressed as "CE_{50} for 30 days of growth," indicating the median effective concentration affecting growth over a 30-day period. It should be emphasized that the terms "acute" and "chronic" are relative and lack precise definitions. A 96-hour test might be considered to measure acute toxicity for a long-lived species but could be deemed chronic for a species with a short life cycle (Campbell, 2022). Finally, an important diagnostic feature of toxicity is a monotonic relationship between exposure and lifespan (Campbell, 2022). This relationship

underscores the importance of considering both concentration and exposure duration when assessing the toxic effects of metals on organisms

Heavy metal toxicity in organisms results from a complex combination of mechanisms, often interacting synergistically. The main mechanisms include: generation of reactive oxygen species (ROS) inducing cell-damaging oxidative stress; disruption of essential metal homeostasis; enzymatic inhibition and alteration of protein structure by binding to sulfhydryl and imidazole groups; direct or indirect genotoxicity; disruption of cell signalling pathways and ionic balance; mitochondrial dysfunction affecting energy production; induction of apoptosis or necrosis; and disruption of the endocrine system(de Paiva Magalhães et al., 2015). Sensitivity to metals varies considerably between species, reflecting their different accumulation, detoxification and resistance strategies (Haq et al., 2003).

While understanding the general principles of metal toxicity is crucial, it is equally important to explore how organisms manage and regulate metals at the cellular level, as this process significantly influences the overall impact of metal exposure on aquatic life.

1.6 Metal regulation at the cellular level

The biodynamic model of metal bioaccumulation for aquatic species includes regulation (balance between uptake and excretion) and net accumulation (continuous storage in detoxified form) (Rainbow, 2002). The presence of metal in tissues does not necessarily indicate harm, as metals can accumulate while remaining metabolically isolated and non-bioavailable. Toxicity depends on metabolically available metal concentration, not total accumulation (Campbell, 2022).

Metal compartmentalization is characterized by different accumulation and elimination patterns. Easily eliminated excess metals create saturation-type absorption curves, while inert storage results in linear accumulation patterns. During chronic exposure, the formation of metal-rich granules provides protection against metal stress, as these granules sequester metals with slow or negligible elimination rates (Vijver et al., 2004). Species vary in accumulation and detoxification capabilities, and environmental factors (pH, salinity, seasonal variations) influence bioaccumulation and toxicity (Rainbow, 2002).

Cellular defense mechanisms against certain metals significantly impact their toxicity by reducing the availability of free metal ions and mitigating the oxidative stress they cause. The synthesis of phytochelatins and metallothioneins, triggered by the activation of metal-sensitive enzymes and transcription factors, sequesters these ions, limiting their direct harmful effects and helping to maintain cellular homeostasis (Cobbett & Goldsbrough, 2002). Although these systems offer protection and allow some cellular adaptation, they have not evolved specifically to cope with metal pollution, which explains their limited effectiveness in the face of high or chronic contamination. This sequestration also influences the bioavailability and distribution of metals in the organism, potentially modulating their long-term toxicity (Cobbett & Goldsbrough, 2002). These defense mechanisms thus play a crucial role in modulating metal toxicity, but their capacity to protect the organism may be exceeded under conditions of intense or prolonged exposure.

1.7 Lithium as a metal of interest

1.7.1 Environmental properties and behaviour

Lithium is an alkaline earth metal, appearing as a soft silvery-gray metal, with an atomic mass of 6.94 g/mole (Kszos & Stewart, 2003) . The fate of lithium in the natural environment is determined by the physicochemical characteristics of the water. It is highly reactive and oxidizing rapidly on contact with air and water (Shahzad et al., 2017; Kavanagh et al., 2018). It is mostly found in the form of ionic compounds, such as lithium carbonate (Li_2CO_3), lithium chloride (LiCl), and lithium hydroxide (LiOH) (Bolan et al. 2021). With low electronegativity, lithium has a single valence electron forming mainly ionic bonds (Kszos & Stewart, 2003). In this reaction, lithium (Li) reacts with water (H_2O) to form lithium hydroxide (LiOH) and hydrogen gas (H_2).

$$\mathbf{2Li + 2}\boldsymbol{H_2O} \rightarrow \mathbf{2LiOH +}\ \boldsymbol{H_2} \quad \mathbf{(2)}$$

The solubility of lithium compounds in water varies widely: phosphates (Li_3PO_4 = 0.039 g/100 mL at 20°C), fluorides (LiF = 0.127 g/100 mL at 20°C) and carbonates (Li_2CO_3 = 1.33 g/100 mL at 20°C) are insoluble, hydroxide (LiOH = 12,8 g/100 mL at 20°C) is sparingly soluble, while nitrates ($LiNO_3$ = 70.1 g/100 mL at 20°C) and chlorides (LiCl = 83.5 g/100 mL at 20°C) are highly soluble (Kszos & Stewart, 2003).

Lithium differs from other metals in that, once it is dissolved in water, it remains stable over a wide pH range, specifically from pH 1 to 11. This stability allows it to remain soluble in water and retain its chemical composition without precipitating at these varied pH values.

Furthermore, it exhibits minimal tendency to adsorb to surfaces when in solution (Smith, 1973). In nature, the dissolution of lithium-containing minerals is enhanced by rainfall and runoff, particularly under oxidizing and acidic conditions (Paquet & Mueller, 2022). Its concentration in water tends to correlate positively with hardness, the presence of magnesium, sulfates, and chlorides, while it correlates negatively with aluminum (Paquet & Mueller, 2022). Lithium concentrations are typically higher in waters containing chlorides and sulfates compared to those with bicarbonates (Paquet & Mueller, 2022). Lithium would then be more soluble in sea water than freshwater with low minerality.

1.7.2 Occurrences and Sources in the Environment

In Canadian natural waters, lithium concentrations in freshwater range from 0.001 to 0.190 mg /L (Paquet & Mueller, 2022). In seawater, concentrations can range from 0.100 mg/L (in the North Sea) to 0.220 mg/L (in the Atlantic Ocean), while coastal areas are more variable and can reach up to 0.300 mg/L (Barbosa et al., 2023). In groundwater, lithium concentrations can reach up to 0.5 mg/L. Lithium concentrations are particularly high in regions where lithium-rich brines and minerals are found. Lakes and rivers in lithium-rich lands such as in Chile can have concentrations as high as 6 mg/L (Barbosa et al., 2023). Lithium concentrations range from 13.7 mg/l in the Dead Sea to 1,500 mg/L in the brines of Chile's Atacama Salar (Aral & Vecchio-Sadus, 2008; Paquet & Mueller, 2022). Chloride- and sulfate-rich waters generally have higher lithium concentrations than those rich in bicarbonate (Araoka et al., 2014).

Although the natural concentration of lithium reported in Canada appears quite low, these concentrations can become elevated in contaminated areas reported elsewhere (Table 1). Due to

increasing demands for lithium, its extraction from mineral sources has increased and consequently, so has its concentration in nearby waters. For instance, near lithium mine sites, concentrations range from 0.7 to 1.0 mg/L. In stormwater management ponds, Li ranges from 0.003 to 0.200 mg/L (Table 1).

The reactivity of lithium makes it useful in various industries, especially the manufacturing of modern batteries. Forms of lithium most used in batteries are lithium carbonate (Li_2CO_3), lithium hydroxide (LiOH), and lithium chloride (LiCl) (Heredia et al., 2020). The production of rechargeable batteries accounted for 80% of total lithium demand in Canada in 2020 (Natural Resources Canada, 2022). These batteries are extensively used in electric and hybrid vehicles, as well as in electronic devices. These batteries also find applications in the storage of solar energy, playing an essential role in the transition to renewable energies and environmentally friendly technologies. Thus, the demand for batteries for energy storage has led to a growing demand for lithium, consumption of which is set to increase and is expected to escalate from the 23-33 kilotons/year used in 2010-2017 to 240 kilotons annually (Ambrose and Kendall, 2020).

Currently, only 3% of Lithium batteries are recycled (Barbosa et al., 2023). Recycling is impeded by processing constraints and high costs, resulting in less than 1% of the lithium contained in batteries being recovered (Barbosa, et al., 2023). Without effective recycling measures in place, it is predicted that the annual production of batteries for electric vehicles will result in 340 kilotons of waste by 2040 (Richa et al., 2014).

Without improvement in Li recycling, discarded lithium batteries will require specialized disposal in engineered facilities to limit groundwater contamination, as up to 42.5% of the lithium content in a battery can be leached out (Richa et al., 2014). Engineered landfills are designed to

minimize the impact of contaminants on the surrounding environment, utilizing advanced barrier systems to prevent leachate migration and protect groundwater (Touze-Foltz et al., 2021). However, since engineering barriers in landfills have limited effective lifetimes, releases of lithium to the environment may occur in the long-term.

Another industry that utilizes lithium is pharmaceuticals, with lithium carbonate (Li_2CO_3) and lithium acetate ($LiCH_3COO$) being the most widely used lithium salts in pharmaceutics (Aral & Vecchio-Sadus, 2008). Orally ingested lithium is mainly absorbed from the gastrointestinal tract, rapidly reaching a maximum concentration in plasma after ingestion. Due to its ability to substitute for sodium and potassium in certain cellular transporters, lithium readily penetrates cells and is evenly distributed in body fluids. Lithium is primarily excreted via the kidneys, through the urine where it enters the sewage systems and makes its way into municipal wastewater treatment plants (Bernard, 2015). Treatment plants, however, are not always able to eliminate all traces of pharmaceuticals, including lithium. Flocculation and filtration processes are less effective at eliminating lithium compared to other contaminants, and lithium is generally poorly adsorbed on activated sludge (Baudino et al., 2022; Massima Mouele et al., 2021). As a result, trace amounts of lithium can end up in treated water that is discharged into the environment, or even into drinking water supplies. This raises concerns about the potential environmental and health impacts of long-term exposure to low levels of lithium in water (Kessing, 2024).

Other anthropogenic sources of lithium in the environment include lithium mining and extraction activities, as lithium will end up in tailings, waste rock, and treated mining effluent. Lithium is found in large quantities in deposits of spodumene [$LiAl(Si_2O_6)$], lepidolite [$K(Li,Al)_3(Si,Al)_4O_{10}(F,OH)_2$] and other sedimentary rocks (micas, phosphate, silicates) (Aral &

Vecchio-Sadus, 2008). Lithium is also found in the form of carbonate and bicarbonate in limestone and sandstone (Tanveer et al., 2019). Australia is the top producer of lithium (47%) globally, with Canada being the 7th biggest producer of lithium at 0.4% or 0.5 kilotons in 2022 (Natural Resources Canada, 2022). The main producers of lithium in Canada are the Tanco Mine in Manitoba and the Sayona Operations in La Corne, Québec (Figure 2). The industry is expanding with additional proposed mining projects as well as exploration into unconventional sources like oil and industrial waste to diversify production capabilities.

Table 1. Lithium concentrations in various surface waters and nearby sources such as municipal stormwater, municipal effluent and mining effluent. Data from Ouedraogo & Pick, 2023 are unpublished .

Sampling location	Lithium concentration (mg/L)	References
Surface Freshwater		
Canada	0.001 – 0.190	Paquet & Mueller, 2022
Lake Tanganyika, Africa	0.015	Paquet & Mueller, 2022
Yangtze River, Tibet	0.004	Paquet & Mueller, 2022
Municipal Landfill Leachate		
Denmark	0.000 – 0.367	Paquet & Mueller, 2022
Urban Runoff and Treated Effluents		
Denmark (treated effluents)	0.011 –0.021	Barbosa et al., 2023
Canada (urban stormwater ponds)	0.003-0.200	Ouedraogo & Pick, 2023
Water Near Active Mines		
Canada	0.735 - 1.0	GENIVAR inc.,2013
Rwanda	0.0005-0.188	Nieder et al., 2014
Tailing Pore Waters for Pegmatite Deposit		
Canada (Quebec)	1-2	Roy et al., 2022
Water Close to Polluted Areas		
Han River, South Korea	1.57	Choi et al., 2019
Donbass river, Ukraine	1.18 - 1.37	Sobolev et al., 2019
Williston basin, USA	3.5	Lauer et al., 2016

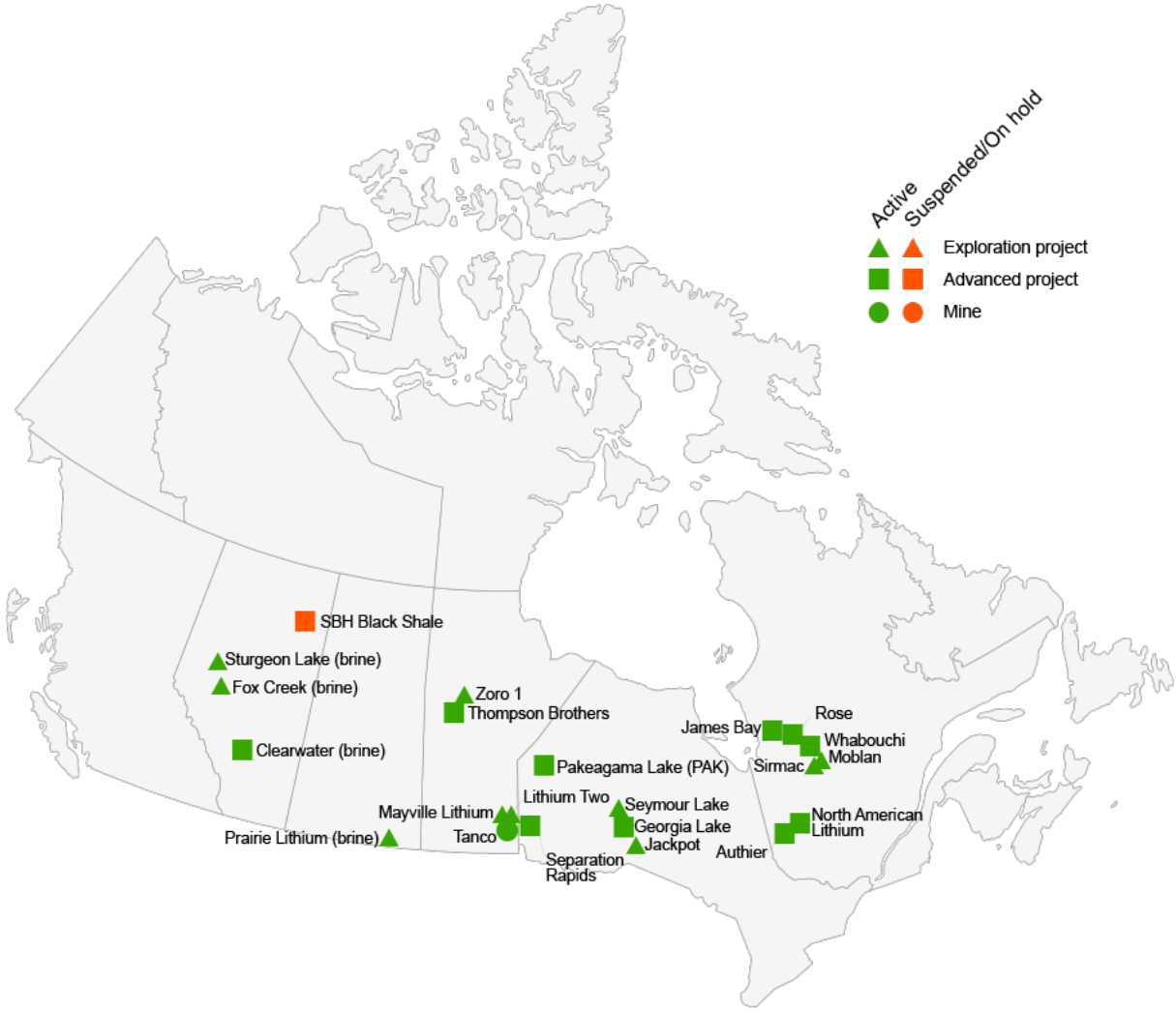

Figure 2. Map showing the location, status, and stage of Canadian lithium projects in 2022-2023(Natural Resources Canada, 2022).

1.7.3 Managing Releases of Lithium

To limit releases of lithium resulting from landfills, pharmaceuticals, and mining, environmental risk assessment models can be used as tool to implement mitigation measures. Mitigation measures can include water treatment systems, engineered covers, or regulatory restriction on uses. Environmental Risk Assessments (ERA) consist of comparing an exposure assessment to an effect assessment. The exposure assessment generally consists of an environmental dispersion model that predicts concentrations of substances in the receiving environment like a lake or a river. The effects assessment consists of deriving predicted no-effect concentration (PNEC), which can often be a water quality guideline. The environmental risk assessment compares exposure predictions to water quality criteria for the protection of aquatic life. If the predicted concentrations are below the guideline, the risk is low, and the environment is predicted to be protected. If it is above, there is a potential risk and it usually suggest the implementation of mitigation measures, if possible.

An example of the use of ERA models is when planning for mine waste management. In Canada's James Bay region, several lithium mines are proposing to dispose of tailings and waste rock together in aboveground facilities. Notable examples include the Galaxy Lithium, Whabouchi, and the Rose Lithium-Tantalum projects. For these mixed tailings and waste rock management facilities, ERA models are used to determine if a liner is required and to design the thickness and composition of the engineering cover on this waste management facility. The exposure assessment models the environmental dispersion of lithium under different mitigation scenarios and often through several iterations. The optimized mitigation measure is reached when the predicted concentration of lithium is ideally below the water quality guideline or as low as economically

achievable. Since there is no long-term Canadian Water Quality Guideline for the Protection of Aquatic Life (CWQGs-PAL) to use for Li in such ERA models, tailings/waste rock management facilities have been designed based on companies' assessment of Li toxicity. It means that several lithium mining projects have already been in operation or been approved without a federal or provincial Li water quality guideline.

1.7.4 Bioavailability and Bioaccumulation

Once in solution, lithium can be taken up from water by plants, microorganisms, and animals (Tanveer et al., 2019). Yuan et al., (2022) summarized studies showing that lithium is highly bioavailable to fish. Lithium can then bioaccumulate (Bolan et al., 2021) and there is evidence for bioaccumulation through food chains (Yuan et al., 2022). Four studies have also shown that lithium bioaccumulates in mussels in marine environments (Table 2) at tissue concentrations ranging from 0.73 to 61.86 µg/g dry weight, with bioconcentration factors (BCF) ranging from 1.4 to 4.2 (Viana et al., 2020).

Table 2. Lithium bioconcentration factor (BCF) in marine bivalves.

Species		Exposure duration (days)	Lithium Concentration		BCF (L/kg)	References
Common Name	**Latin Name**		**Water (mg/L)**	**Tissues (µg/g)**		
Blue mussel	*Mytilus edulis*	4	0.5-1.5	1.2-2.19	-	Thibon et al., 2021
Pullet carpet shell	*Venerupis corrugata*	14	0.69-1.94	2.9 -7.1	4.2 -3.7	Barbosa et al., 2023
Mediterranean Mussel	*Mytilus galloprovincialis*	21	0.1-10	2.47-61.86	-	Fraga et al., 2022
Mediterranean Mussel	*Mytilus galloprovincialis*	28	0.1-0.75	0.73-1.4	2.1-1.4	Viana et al., 2020

The lithium concentration in water appears to influence accumulation, with the highest tissue concentrations (61.86 µg/g) observed in *Mytilus galloprovincialis* after 21 days exposure to lithium concentrations in water reaching 10 mg/L (Fraga et al., 2022). Concerning the teleosts, there are fewer studies. However, in Rainbow Trout (*Oncorhynchus mykiss*), exposure to 0.1 mg/L Li for 60 days resulted in significant accumulation in brain (5.2 µg/g dry tissue) and plasma (3.8 µg/mL), associated with decreased concentrations of sodium (15%), potassium (20%), magnesium (18%) and total ammonia (25%) compared to controls (Tkatcheva et al., 2015). This ionic disturbance suggests that lithium interferes with active ion transport processes and disrupts ion homeostasis.

1.7.5 Mechanisms of toxicity

Lithium is generally considered less toxic than numerous other metals, particularly those classified as heavy metals. Lithium possesses relatively low electronegativity (approximately 1.0 on the Pauling scale), resulting in weaker bonds with ligands compared to metals such as cadmium or lead, which have higher electronegativities (Paquet & Mueller, 2022). This influences lithium's ability to interact with biomolecules, rendering its complexes generally less stable and less likely to disrupt biological functions. As an alkali metal, lithium has a small atomic size and a +1 charge. These characteristics enable it to behave distinctively in biological systems, where it can readily interact with ions and water molecules (Paquet & Mueller, 2022). In contrast, heavy metals such as cadmium or mercury, which have higher charges and larger atomic sizes, can induce more severe toxic effects due to their ability to bind more strongly and specifically to critical biological sites (Alonso & Girifalco, 1979). Furthermore, heavy metals like cadmium and mercury can interfere with essential biological processes, such as protein synthesis and enzyme function, by binding to thiol groups or replacing essential metals in enzymes. Lithium, conversely, is utilized medically to

treat psychiatric disorders, and its mechanism of action is primarily linked to the modulation of neurotransmitters rather than direct toxicity (Bernard, 2015; CADE, 1949). Finally, heavy metals tend to accumulate in biological tissues, leading to chronic toxic effects (Campbell, 2022). Although lithium can also accumulate, it is generally eliminated more efficiently by the kidneys, thereby reducing the risk of long-term toxic accumulation in the body (Bernard, 2015).

Among the lithium effects observed, Tkatcheva et al. (2015) reported significant ionic disruption, indicating that lithium interferes with active ion transport processes and disrupts ion homeostasis in aquatic organisms. In the latter study of Rainbow Trout, lithium disrupted the activity of Na^+, K^+-ATPase, triggering compensatory responses to maintain ionic homeostasis, such as increased mitochondrial density and changes in the lipid composition of gill membranes. Lithium can replace some essential elements such as Na^+, K^+, Ca^{2+}, and Mg^{2+} due to its smaller radius and high polarization (Tanveer et al., 2019). This interference can have significant repercussions on various cellular functions. Bioaccumulation of lithium can also induce oxidative stress, compromising energy balances and metabolism. This disturbance may impair the allocation of energy to crucial biological functions such as growth and reproduction, potentially impacting population dynamics (Barbosa, et al., 2023; Chen et al., 2024; Nagato et al., 2013). Studies on estuarine clams have revealed concentration-dependent energy depletion, oxidative stress, and oxidative protein damage. The clams accumulated lithium in their soft tissues, with a decrease in energy reserves and an increase in protein carbonylation at the highest concentrations (Tkatcheva et al., 2015) . Generally, other studies also highlight the oxidative stress induced by lithium, as well as its detrimental effects on the energy, reproduction, and growth of the studied species (Martins et al., 2022).

Furthermore, research has shown that lithium chloride (LiCl) promotes lipid accumulation through increased generation of reactive oxygen species (Lee et al., 2020). However, the effects of lithium exposure on hepatic lipids vary among species and depend on exposure duration (Yuan et al., 2022), with underlying mechanisms remaining unclear. In zebrafish, a dose-dependent relationship was observed, where increasing LiCl concentrations from 0 to 1 mg/L resulted in elevated lipid content (Lee et al., 2020) .

1.7.6 Acute and chronic toxicity

Most ecotoxicity data for lithium concerns acute effects in various aquatic species. These studies show considerable variability depending on the species and different endpoint. Table 3 provides a comprehensive summary of LC_{50} values for various aquatic species, offering a comparative overview of the sensitivity of different organisms to acute lithium exposure.

Table 3. Acute lithium toxicity - lethal concentrations (LC_{50}) on various aquatic species**.**

Organism	Species		Duration (hours)	Effect concentration (mg/L)	Reference
	Common Name	Latin Name			
Bivalve	Zebra Mussel	*Dreissena polymorpha*	24	185-232	Fraga et al., 2022
Fish	White Cloud Mountain Minnow	*Tanichthys albonubes*	48	9.2-62	Aral & Vecchio-Sadus, 2008
	Colorado squawfish	*Ptychocheilus lucius*	96	41	Kszos & Stewart, 2003
	Razorback sucker	*Xyrauchen texanus*	96	156	Shahzad et al., 2017
	Fathead minnow	*Pimephales promelas*	96	42	Kszos & Stewart, 2003
	Bonytail	*Gila elegans*	96	65	Kszos & Stewart, 2003
	Striped bass	*Morone saxatilis*	96	105	Kszos & Stewart, 2003

The acute environmental effect concentration (measured as EC_{50}) of Li on *Daphnia magna* has been reported to range from 33 to 197 mg/L, which is at least 1,000 times higher than the level present in freshwater (Aral & Vecchio-Sadus, 2008a; Paquet & Mueller, 2022). However, exposure to Li can have detrimental effects on this species even at lower concentrations. Another study conducted on *Daphnia magna* reported that exposure to 1.2 mg/L of lithium for 64 hours resulted in immobilization of the organisms (Kszos & Stewart, 2003).

1.7.7 Chronic Toxicity in Aquatic Species

Although most ecotoxicity data on lithium relate to acute toxicity, the effects of long-term chronic exposure are also of concern for aquatic ecosystems. Studies have shown that chronic exposure to low concentrations of lithium can have adverse effects on various organisms, with effects and toxicity thresholds differing according to species and life stages (Kszos & Stewart, 2003; Tkatcheva et al., 2015; Viana et al., 2020). Table 4 provides a comprehensive summary of L/EC_{50} values for various aquatic species, offering a comparative overview of the sensitivity of different organisms to chronic lithium exposure.

Chronic effects of lithium exposure include growth retardation, compromised reproduction, developmental abnormalities and behavioral changes and also mortality. For example, in fathead minnows *Pimephales promelas*, Li chronic exposure to concentrations as low as 0.35 mg/L resulted in reduced larval growth in 26 days (Kszos & Stewart, 2003). In Rainbow Trout (*Oncorhynchus mykiss*), exposure to 0.1 mg/L lithium for 60 days resulted in changes in swimming behavior and reduced feeding rates. In Rainbow Trout (*Oncorhynchus mykiss*), exposure to 1.0 mg/L lithium resulted in compensatory responses, including increased mitochondrial density and changes in the lipid composition of gill membranes (Tkatcheva et al., 2015) .

Martins et al. (2022) demonstrated that chronic lithium exposure in *Daphnia magna* lead to significant adverse effects, including a 90% reduction in reproduction and a 67% decrease in population growth rate at the highest concentration tested (0.08 mg/L) after 21 days of exposure. Another study done on the same species, showed that Li exposure (LC_{50}=4.1 mg/L) at 0.55 mg/L for 48 hours can result in altered swimming behavior, reduced feeding activity, and decreased predator avoidance (Chen et al., 2024). This exposure also led to increased lipid peroxidation and neurotoxic effects on acetylcholinesterase and Na^+, K^+-ATPase activity, an enzyme crucial for maintaining ionic homeostasis.

In the Mediterranean mussel *Mytilus galloprovincialis*, chronic exposure to environmentally relevant concentrations (0.1-10 mg/L) led to histopathological changes in the digestive system, over 21 days (Fraga et al., 2022). Another study with the same species, revealed concentration-dependent energy depletion and oxidative protein damage (Viana et al., 2020). In addition, a significant decrease in the activity of antioxidant enzymes, such as catalase (of 30%) and superoxide dismutase (of 25%), was observed at lithium concentrations of 0.1 mg/L.

Toxicity tests carried out on different chemical compounds of Li revealed chronic effect concentrations ranging from 0.039 to 153.78 mg/L (Table 4) while lithium concentrations in unexposed areas range from 0.01 to 1.88 mg/L (Table 1) sometimes exceed levels found in freshwater environments. In affected areas, the Li concentration ranges from 0.01 to 3.5 mg/L which means that species living in areas affected by industrial or mining activities could be at risk, as these areas may have Lithium concentrations similar to those observed in the tests

Table 4. Chronic toxicity of lithium to aquatic organisms - Long-term lethal concentrations (LC_{50}). Data from Environment and Climate Change Canada, 1980 are unpublished .

Organism	Species		Duration (hours)	EC50 (mg/L)	LC50 (mg/L)	References
	Common Name	Latin Name				
Invertebrates	Water Flea	*Daphnia magna*	21	0.039	-	Martins et al., 2022
	Mediterranean Mussel	*Mytilus galloprovincialis*	21	-	0.25	Fraga et al., 2022
			9	-	153.78	
Fish	Zebrafish	*Danio rerio*	14	- -	25	Martins et al., 2022
	Fathead minnows	*Pimephales promelas*	26	1	1.4	Kszos & Stewart, 2003
	Carp	*Cyprinus carpio*	30	-	20	Liu et al., 2018
	Rainbow trout	*Salmo gairdneri*	28	-	9.28	Environment and Climate Change Canada, 1980

1.7.8 Derivation of Water Quality Guidelines

The derivation of water quality guidelines is a comprehensive process aimed at protecting aquatic life. In Canada, this process is guided by the Canadian Council of Ministers of the Environment (CCME) and involves several steps (CCME, 2015). Initially, data are gathered and evaluated, including physical, chemical, and biological information relevant to the substance in question. The process incorporates exposure and toxicity-modifying factors (ETMFs) to standardize toxicity data, ensuring guidelines are applicable under various environmental conditions (CCME, 2015). The guidelines are then derived based on the quality and quantity of available data, with a focus on protecting the most sensitive life stages of aquatic species. Once derived, these guidelines undergo rigorous internal and external reviews before final approval (CCME, 2015).

For substances such as lithium, understanding the chronic effects on aquatic life is crucial. However, there is a lack of comprehensive chronic data due to variability in species sensitivity and life stages, as well as the complex interactions within aquatic ecosystems that influence toxicity. While there are sufficient data on acute lithium toxicity, as illustrated by the sensitivity curve for freshwater organisms (Figure 3), chronic data remain limited. This gap in data is significant because current datasets may not fully capture lithium's long-term effects across diverse aquatic environments. Sufficient chronic toxicity data (more than 30 species) are essential for understanding long-term effects and establishing guidelines to protect vulnerable aquatic species (Del Signore et al., 2016; Health Canada, 2023; Posthuma et al., 2001). To derive a comprehensive guideline, chronic toxicity tests typically focus on representative species from at least three main trophic levels (algae, invertebrates and fish) (Posthuma et al., 2001) . As environmental conditions

evolve, particularly with climate change impacts, new data are necessary to ensure guidelines remain relevant and effective in safeguarding aquatic ecosystems.

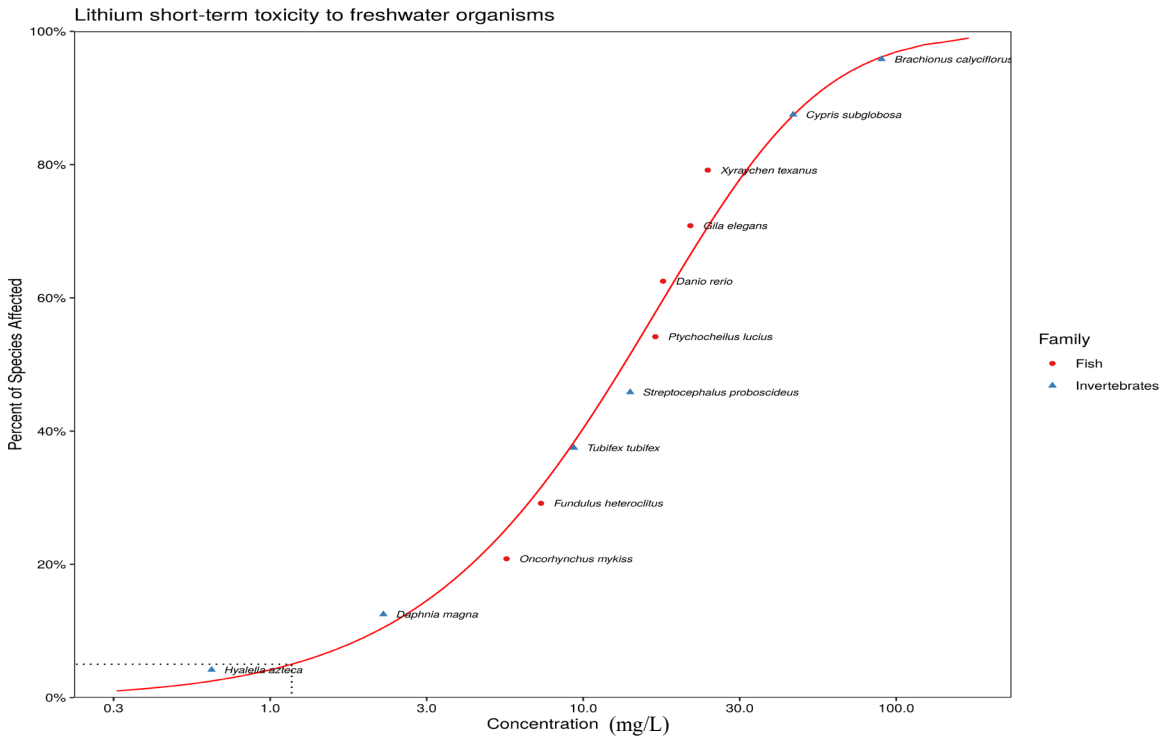

Figure 3. Lithium short-term toxicity sensitivity curve for freshwater organisms (unpublished data) (Environment and Climate Change Canada, 2021). Water quality guidelines or predicted no-effect concentrations (PNECs) are generally identified from the 5th percentile of the fitted species sensitivity distribution (SSD) curve (dot rectangle on Figure3). *Hyalella azteca* falling below the 5th percentile of the SSD curve indicates that this species is among the most sensitive to acute lithium toxicity in the aquatic ecosystem represented by the data.

Research Goals and Objectives

The rapid transition to a low-carbon economy necessitates a comprehensive understanding of lithium's potential environmental impact. At present there is limited information on the chronic toxicity of lithium to aquatic organisms. This lack of knowledge impedes the development of a water quality guideline for the protection of aquatic life and adds uncertainty to the environmental management of liquid and solid waste containing lithium.

My thesis objective was to quantify the chronic toxicity of lithium to the fingernail clam *Pisidium dubium* and the water flea *Daphnia pulex* using sensitive endpoints to quantify low-level effects. The specific research hypotheses and predicted are described below. These hypotheses and predictions guided the design of the study and the interpretation of the results, in order to better understand the potential impact(s) of lithium on freshwater aquatic invertebrates. *Daphnia pulex* and *Pisidium dubium* (Appendix: Figure A1; Figure A3) were selected as they are key species in freshwater ecosystems, playing important roles in benthic and planktonic food webs (Ebert, 2022; Mackie *et al*., 1980).

Daphnia, commonly known as water fleas, are model organisms in ecotoxicology due to their high sensitivity to pollutants, including metals. These planktonic freshwater crustaceans serve as excellent bioindicators, responding rapidly to contaminants and effectively signaling the presence of toxic substances in aquatic environments (Ebert, 2005). *D. pulex*, in particular, inhabits ponds, calm sections of streams, rivers, and lakes across North America, and is often a dominant herbivore (Ebert, 2005).

Native to North America, *Pisidium dubium* (fingernail clam) is a freshwater clam found throughout Canada, particularly in the Canadian Great Lakes, with the exception of Lake Erie

(Mackie et al., 1980). It also plays an important role in aquatic food chains, transferring nutrients from water and sediments to fish and ducks (Anderson et al., 1978) through filtration of algae, bacteria, and organic matter. Like other bivalves, their sedentary nature and filtering behavior enable them to process large volumes of water, reflecting long-term exposure to contaminants from both water and sediments. As a result, bivalves typically bioaccumulate metals, indicating the presence and concentration of pollutants in their environment. Bivalves are also sensitive to metal contaminants and make valuable bioindicators for assessing environmental health, as indicated by reduced taxonomic richness in metal-contaminated sediments (Kilgour et al., 2018).

Hypotheses:

Null hypothesis

- H_0: Lithium does not affect *Pisidium dubium* and *Daphnia pulex* at environmentally relevant concentrations.

Alternative hypotheses:

- H_1: Lithium has a chronic toxic effect on the survival and burrowing behavior of *Pisidium dubium* and on the growth and reproduction of *Daphnia pulex* at environmentally relevant concentrations.
- H_2: The sensitivity of *Pisidium dubium* and *Daphnia pulex* to lithium varies with concentration.

Predictions:

- Chronic toxic effects on the survival and burrowing behavior of *Pisidium dubium* and on growth and reproduction of *Daphnia pulex* will be observed at lithium concentrations higher than environmentally relevant concentrations.

- The magnitude of the chronic toxic effects observed in *Pisidium dubium* and *Daphnia pulex* will increase with increasing lithium concentration.

The chronic toxicity data generated in this thesis will enable the development of water quality guidelines for the protection of aquatic life against the effects of lithium.

Collaborations

This research was conducted in partial fulfillment of the requirements for a M.Sc. degree in biology, at the University of Ottawa. I received guidance from my supervisors Dr. Richard Goulet and Dr. Francis Pick. This research was conducted in collaboration with Natural Resources Canada (NRCan). The toxicity tests were conducted at the NRCan CANMET laboratory, with the guidance of Dr. Carrie Rickwood, Gauri Prabhakar, Emily Suominen and Catherine Proulx. The thesis was funded by a Critical Minerals Research program of CanmetMINING, Natural Resources Canada. These data will be used to derive a federal water quality guideline for the protection of aquatic life.

Chapter 2 of this thesis is in manuscript format for submission to the journal Environmental Toxicology and Chemistry.

Chapter 2: Chronic toxicity of lithium to the fingernail clam *Pisidium dubium* and water flea *Daphnia pulex*

2.1 Introduction

Over the past few decades, global lithium production has surged, with annual output rising from 14 to 82.5 kilotons (Ambrose & Kendall, 2020; Barbosa, et al., 2023) and projected to reach 600 kilotons by 2030, driven by the increasing demand for lithium-ion batteries, which is expected to reach 240 kilotons per year (Ambrose & Kendall, 2020). However, the low recycling rate of lithium-ion batteries poses environmental risks, as less than 1% of the lithium in these batteries is recovered, leading to the potential for substantial groundwater contamination from battery waste in landfills (Richa et al., 2014). Additionally, lithium pollution is exacerbated by its use in pharmaceuticals (Bolan et al., 2021), lithium mining (Parker et al., 2024), particularly in sensitive ecosystems (Haddaway et al., 2019). Without improved recycling and waste management practices, the continued growth in lithium production and use is likely to have environmental consequences.

In Canada, natural background Li concentrations in surface waters typically range from 0.001 to 0.190 mg/L (Paquet & Mueller, 2022), with groundwater and seawater concentrations up 0.3-0.5 mg/L worldwide (Barbosa et al., 2023; Chen et al., 2024). However, several industrial activities can expose or concentrate Li, above background levels. Examples of Li contaminated waters include the Han River in South Korea (Li = 1.6 mg/L) exposed to industrial pollution (Choi

et al., 2019), the Williston Basin in the United States (Li = 3.5 mg/L) exposed to discharge from petroleum wastewater (Lauer et al., 2016), and the Donbass River in Ukraine (Li = 11.8 to 13.7 mg/L) exposed to mine wastewater (Sobolev et al., 2019) . In Canada, surface water concentrations near lithium mining sites range from 0.735 to 1.0 mg/L (GENIVAR, 2013), while tailing pore waters in Quebec reach 1-2 mg/L (Roy, 2023) . These levels are concerning due to their similar values to toxicity thresholds for aquatic organisms. Toxicity testing is required to determine if these Li concentrations are of concern.

The acute toxicity of Li to freshwater species has been documented with a range from 4.1 to 232 mg/L (Aral & Vecchio-Sadus, 2008a; Chen et al., 2024; Fraga et al., 2022; Kszos & Stewart, 2003; Shahzad et al., 2017). However, there are limited chronic toxicity data available. Fathead minnow (*Pimephales promelas*) growth has been shown to be inhibited by 25% (IC_{25}) at concentrations of 0.38 mg/L, while reproduction of *Cerodaphnia dubia* has been inhibited by 25% at concentrations of 0.32mg/L (Kszos et al., 2003). A reduction in reproductive success has also been demonstrated in *Daphnia magna*, with an EC_{10} of 0.023 mg/L (Martins et al., 2022). In the same study, the highest concentration tested (0.08 mg/L) reduced *D. magna* population fitness by 67%. Since these effect concentrations are similar to Li concentrations near contaminated sources (0.735 to 1.0 mg/L) (GENIVAR, 2013), there is potential for Li to disrupt the reproductive success and population dynamics of other freshwater species of invertebrate .

The objective of this study was to investigate Li chronic toxicity to two freshwaters aquatic species from contrasting habitats, the fingernail clam *Pisidium dubium* and the water flea *Daphnia pulex*. This research aims to examine the potential long-term impacts of lithium on the survival, growth, and reproduction of these two key taxa. Such information is crucial when identifying

adequate landfill designs, mining and municipal effluent treatment, and tailings and waste rock management strategies to protect the downstream aquatic environment.

2.2 Material and methods

2.2.1 Collection and breeding of test species

Fingernail clams were collected from the Ottawa River at Britannia Bay, Ottawa, Canada (45°21'49.3 "N 75°48'16.7 "W) on August 24, 2022 (Figure 4). Clams were collected using D-nets by skimming the top ~ 5 cm of sandy substrate, along parallel transects at a water depth of around 1 m. Collected surface sediments were sorted in the field using sieves (0.0394 mm) and individual clams were hand-picked using tongs and transferred to 1 L with polytetrafluoroethylene (PTFE) plastic containers filled with Ottawa River water. Collected individuals were transported back to the laboratory (NRCan, Ottawa, ON) for identification to species and acclimation. Ottawa River water (8 L) was collected with polytetrafluoroethylene (PTFE) plastic containers as well. The Li level in the river water was $< 9\times10^{-4}$ mg/L (below the detection limit) and the Na was $8.69\times10^{-3}\pm0.18$ mg/L.

Figure 4. Photograph of the Britannia Beach collection site (A) of the fingernail clam, Pisidium dubium. The satellite image provided by Google Maps (B) indicates the collection site with respect to surrounding markers**.**

The collected clams were identified according to shell morphology, and *Pisidium dubium* was chosen as the test species as it was abundant. *P. dubium*, like other species of *P*isidium, has an asymmetrical shell with an off-center umbilical zone (Mackie et al., 1980). Adults of *Pisidium dubium* are at least 3 mm long with a slightly purple shell, which distinguishes them from other *Pisidium* species, which are relatively small. The identification process was guided and verified by an experienced biologist. The clams were then transferred into an aquarium containing 1000 g of fine aquarium sand (CaribSea® inc. Super Naturals Premium Aquarium Substrate, color: Marine Sand/Moonlight Sand) and 4L of media consisting at first of 50:50 Ottawa River water Reconstituted Soft Water (RSW, Table 5). RSW was produced using a modified EPA standard freshwater (EPA/600/4-90/027F) (Clesceri et al., 1998), prepared using ultra-pure water (18.2 Ohms) . Clams were transitioned to RSW over 2 days.

Daphnia pulex were obtained from Arofish (Hampton, USA) and maintained in culture in the laboratory following the guidelines of protocol EPS 1/RM/14 Second Edition (Sprague, 2000). *D. pulex* were also maintained in RSW media, with a modified vitamin recipe based on Lynch et al. (1986), with supplemented trace elements (Table 5).

Table 5. Chemical recipe of the Reconstituted Soft Water (RSW) (modified EPA standard freshwater (EPA/600/4-90/027F) (Clesceri et al., 1998; Lynch et al., 1986).

Test Species	Chemicals	Quantity(g)	Vitamins	Quantity (g)
Pisidium dubium	CaSO4*2H2O,	1.5	Thiamine hydrochloride	$3.75x10^{-4}$
	MgSO4*7H2O,	3.1	Cyanocobalamine(B12)	$5x10^{-6}$
	NaHCO3	2.4	Biotine	$3.75x10^{-6}$
	KCL	0.1		
Daphnia pulex	CaSO4*2H2O,	1.5	Calcium pantothenate(B5)	$1.05x10^{-3}$
	MgSO4*7H2O,	3.1	Cyanocobalamin(B12)	$4.5x10^{-8}$
	NaHCO3	2.4	Thiamin (B1)	$9x10^{-5}$
	KCL	0.1	Riboflavin (B2)	$6x10^{-5}$
			Nicotinamide (B3)	$1.95x10^{-4}$
			Folic acid (B9)	$4.95x10^{-4}$

2.2.2 Lithium partitioning and solubility

A 24-hr solubility test was conducted, using a 100 mg/L loading of LiCl powder (SIGMA, CAS number 7447-41-8, purity > 99.7%) added to five flasks containing pH 6 aquatic media and five flasks with pH 8.5, with triplicate blanks for each pH (Skeaff et al., 2011). The concentration of Li was measured at 0 and 24 hours to determine the net change, and ultimately, the extent of solubility of LiCl for the preparation of solutions for toxicity testing. To assess the rate of partitioning of Li from the water to sediment, a 28-day partitioning test, following the method described in Huntsman et al. (2019) was conducted. In this test, a 1 mg/L Li solution in pH 6 aquatic media was prepared and 10 g of a well- characterized river sediment introduced into each 1 L flask. Solution samples were drawn at specified time intervals (0, 2, 6, 24, 48, 96, 168, 336, 504, and 672 hours) to analyze for dissolved Li concentrations in the water. The reaction kinetic data was used to determine the rate and extent of partitioning of Li between the water and sediment. Additional, partitioning and solubility tests were conducted to confirm the high solubility of lithium chloride in the RSW medium and its partitioning to sand and food to be used in Li fingernail clam toxicity tests and to food in the *D.pulex* toxicity tests. Tests were carried out using two Li concentrations (5 and 10 mg/L) that were measured near anthropogenic sources of Li. Tests were conducted in 800 mL of RSW and consisted of three treatments: Li in RSW alone (W); Li in RSW + Sand (S), and Li in RSW + Sand + Food (SF). At the start of the experiment, 300 µL of an algae mixture of 1 mL Instant Algae Shellfish Diet 1800® and 100µL instant Algae Nano Diet 3600® in 9 mL RSW was added to each treatment. All treatments were conducted in triplicates. Tests extended for 48-hours to represent water renewal frequency during the toxicity tests.

Water samples (10mL) were collected at time 0, 1, 3, 6, 24 and 48 hours using a 20 mL Luer Lock plastic syringe (Fisher Scientific) for analysis of total and dissolved (filtered 0.45µm;

Acrodisc HT Tuffryn Membrane, PALL New York) Li fractions. Following collection, the samples were acidified with 100 µL trace metal grade HNO_3 from Fisher Chemical brand before analysis.

2.2.3 Toxicity Testing

The approach to the *P.dubium* toxicity test was modified from the protocol developed by Mackie (1980). Exposure concentrations were set at levels measured in natural unaffected waters and levels downstream of point sources. Exposure concentrations included a control (0 mg/L) and 6 Li treatments (0.05, 0.1, 0.5, 1, 5, and 10 mg/L) with 3 replicates per treatment. Exposure chambers consisted of 400 mL plastic beakers (Nalgene brand) filled with 100g of sand (Premium Aquatic Substrate, CaribSea), weighed using a Shimadzu balance (+/-0.0001) and filled with RSW at the 400 mL mark. Beakers were wrapped with black garbage bags (as opaque material) to minimize light penetration, and loosely covered with Parafilm to reduce evaporation. An 16:8 h light:dark photoperiod was maintained. Toxicity tests lasted 28 days, with Day 0 of the experiment defined as the day in which 10 test individuals of random age and size were randomly distributed into each beaker. During the experiment, water renewals were completed three times a week or every 48 hours. During water renewals, media was drained by introducing a tube at the surface and sucking the water out by gravity to the 200 mL mark, which minimized substrate disturbance. Fresh media was replaced by tilting the culture beaker to ~45º and gently adding the RSW from the 50L jug. Subsamples of 10 ml of water were collected weekly using a 20 mL Luer Lock plastic syringe (Fisher Scientific) for total and dissolved (filters 0.45 µ*m* Acrodisc HT Tuffryn Membrane, PALL New York) Li and Na analyses. Variables such as pH, conductivity and temperature were also measured at each medium renewal.

The *Daphnia pulex* toxicity test was carried out according to a modified protocol ((EPS 1/RM/11, Environmental Protection Agency, 2002), adapted to *Daphnia* species. The first concentration ranges tested were chosen based on those used in a previous study with *Daphnia magna* (Martins et al., 2022). Since no effects were reported on *D. pulex* at the ranges set by Martins et al. (2022), final concentrations were determined based on two additional 21-day range finding tests as *Daphnia pulex* appeared less sensitive than *Daphnia magna*. The final test consisted of exposing daphnids to 7 Li concentrations (0, 0.5, 1, 1.5, 2, 2.5, and 3 mg/L) for 21 days.

At the start of the toxicity experiment, one individual *D. pulex* (less than 24 hours old) was placed in 150 mL plastic beakers, with a solution volume of 100 mL. Each concentration treatment was replicated 10 times. Individuals were fed 700 µg/L of algal culture and YCT (yeast, Cerophyll™ and trout chow) daily, or every other day for the first 5 days. For the first 5 days, *D. pulex* were transferred every two days from one beaker to another, until neonates were produced. To prevent injuries during transfers, a 23 mL (30cm) transfer pipette (Avantor sciences central brand) with the extremity cut to widen the orifice was used. Then, from day 6 onwards, the daphnids were transferred every day and the neonates were removed. After the transfers, the beakers were covered with a light parafilm, allowing the air to pass through. Water temperatures during the test were maintained at 20ºC $^{+}$/- 1ºC.

The number of neonates in each beaker was counted daily using a Petri dish placed on a light table (VWR International). Subsamples of 10 ml of water were taken weekly using a 20 mL Luer Lock plastic syringe for single use from Fisher Scientific for total and dissolved (0.45 µm Choice™ Polypropylene (PP) syringe filters) Li and Na measurements. Subsamples for dissolved organic carbon (DOC) and total organic carbon (TOC) analysis were collected in 125 mL wide-mouth, amber HDPE (High-Density Polyethylene) bottles, 24 hours after the water change on day

5 of the test. DOC water samples were filtered through 0.45 µm Choice™ Polypropylene (PP) syringe filters and collected to three-quarters of the 125 mL bottle volume. TOC samples were collected unfiltered. All samples (DOC and TOC) were stored at 4°C prior to analysis. Variables such as pH, conductivity and temperature were also measured at each medium renewal.

2.2.4 Toxicity Endpoints

P. dubium toxicity endpoints included measurements of survival and behavior. Clam survival was assessed daily; individuals were considered dead when their shells were gaping and removed from the test beakers for confirmation. Signs of clam life were observed under a dissecting microscope, with the death guideline modified from Anderson (1977). The survival endpoint consisted of the percentage of clams alive at the end of the test (i.e., day 28). Burrowing behavior was assessed according to the criteria outlined by Nautilus Environmental Company Inc. (2019). Clams on the surface or partially visible on the sand were not considered burrowed. The percentage of burrowed clams was calculated using equation 1:

$$\%\ \boldsymbol{burrowed} = \frac{\boldsymbol{Total\ n\ living\ clams - n\ visible\ clams}}{\boldsymbol{Total\ n\ living\ clams}} \times \mathbf{100} \quad \textbf{(3)}$$

Where n is the number of clams.

D. pulex toxicity endpoints included measurements of survival, growth, and reproduction. Survival was assessed daily by observing daphnids individually. If they remained motionless for 15 seconds under bright light and did not move when given a light splash of water, they were considered dead. Growth was assessed as the length (mm) of the first exopodite to the second right antennae. Measurements were completed by first taking photos of 36 daphnids under 24 hours old using an Olympus SZ61 microscope equipped with Olympus DP37 camera, then importing

photographs into Image Pro (Media Cibernetics) for the measurements. Reproduction was quantified daily by averaging the number of broods produced by each live female for each treatment (number of neonates) per day.

2.2.5 Bioconcentration

After the clam experiment, the surviving clams underwent an overnight depuration period without food. Clams were transferred to clean water containing sand that had been prepared by rinsing 2-3 times with ddH_2O followed by RSW, using the same experimental set-up. After depuration, the clams were dissected to separate the soft tissues from the shells. For each treatment, specimens from the three replicates were pooled to create a shell sample and a tissue sample. These combined samples were then placed in labelled and pre-weighed 50 ml test tubes (Fisher) and dried (60 °C) to a constant weight, which took approximately 48 hours using an oven (Incubator shaker II from Boekel Scientific). Dried samples were then digested. First, tissue (1 mL) and shell (5 mL) samples were submersed with trace-metal grade nitric acid (HNO_3) for a period of 7 days. Then, hydrogen peroxide (H_2O_2) was added to tissue (0.75 mL) and shell (3 mL) samples for 48 hours. Digestion extractions were diluted ten times for tissues and twice for the shells. Detailed methodology is provided by Borgmann et al., 1991 and Leung et al., 2016.

2.2.6 Chemical Analyses

Water samples were analyzed by the CanmetMINING Analytical Chemistry Laboratory (Natural Resources Canada). Li and Na were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES Agilent 5110). The detection limits for Li and Na were 0.46 µg/L and 360 µg/L, respectively. Dissolved organic matter (DOC) was determined using a total organic

carbon analyzer (TOC-L, Shimadzu). Quality assurance/quality control included the collection of duplicates, spikes, and blanks.

Water quality variables (pH, dissolved oxygen (%), and specific conductivity (µS/cm)) were monitored daily using a Hach HQ40d pH and D.O. meter and a HQ14d conductivity meter. These instruments were calibrated weekly. Ammonia levels were measured weekly using API ammonia test strips for freshwater and seawater aquariums. Daily water quality monitoring was carried out by randomly selecting two of the ten culture beakers. If the D.O. fell below 80%, water renewal was initiated for both species. Two basic air conditioners were set at 21ºC and adjusted as necessary to keep the room temperature stable, this was especially important for *D. pulex* cultures.

Digested clam tissue samples were also analyzed for Li by ICP with a detection limit of 0.005mg/L Li. QA/QC methods included the use of a blank sample and a DORM 5 standard, which is a certified reference material (CRM) for fish proteins from the National Research Council of Canada (NRC), with information on total trace element and species content (NRC 2024).

2.2.7 Statistical Analyses

All analyses and graphs were performed using the R language environment. Effects and lethal concentration were calculated using DRC (dose-response) package. The dose-response analysis identified the main relationships between administered dose and observed effects, including lethal concentrations affecting 50, 25 and 10% ($LC_{50,25,10}$) of the tested individuals and effective doses affecting 50, 25 and 10% ($EC_{50,25,10}$) of tested individuals. Analysis of variance (ANOVA), for alpha risks ≤ 0.05 was used to statistically determine the effect of Li concentrations on the different end-points for both species.

The Li bioconcentration factor (BCF L/Kg) was calculated in soft tissues and shells for all treatments using equation 2 (Arnot & Gobas, 2006).

$$\boldsymbol{BCF = \frac{Corgl}{Co}} \qquad \textbf{(4)}$$

Which is the ratio between Li concentrations in the organisms (C_{orgl}, µg/g) and the initial Li concentration in the treatment water (C_0, µg/L or µg/g, assuming the approximation 1 L = 1 kg).

2.3 Results

2.3.1 Lithium Partitioning and Solubility

The average net change in concentration of five 100 mg/L loadings of LiCl over 24 hours at pH 6 and 8.5 were 15.64 and 15.57 mg/L of Li, respectively. The theoretical (i.e., calculated) concentration for 100% LiCl dissolution is 16.37 mg/L Li, therefore >95% of the Li was in solution after 24 hours. This suggests that LiCl is readily soluble in the aquatic media at both pH 6 and 8.5 and agrees with Oliveira et al., 2011, who report a high solubility of LiCl in water of 83.5 g per 100 ml at 20°C. All blank samples, both at 0 and 24 hours, were reported as less than the limit of quantification (LOQ) of 1.2 µg/L demonstrating no contamination from glassware, filters or reagents.

The results of the 28-day partitioning test with a well characterized river sediment (Huntsman et al., 2019) show that in the case of Li, very little partitioning occurs with approximately 95% of the 1 mg/L of Li staying in solution after 28 days (on par with the test run under the same conditions but without sediment). In contrast, other metals measured (i.e., Co, Cu, Pb, Zn) demonstrate fairly rapid partitioning of the element of interest from the water into the sediment, in particular for Cu and Pb.

Concerning the Li partitioning, the ratios between unfiltered and filtered samples were generally very close to 1, irrespective of lithium concentration (0, 5, or 10 mg/L), treatment (water, sand, sand + food), or elapsed time (0, 24, 48 hours) (Table 6). Most measurements showed low variability, with generally small standard deviations. No significant change was observed over time for all treatments and concentrations. The different treatments did not appear to significantly affect the ratios, which remained stable whatever the initial lithium concentration. In sum, these results (Table 6) suggest that filtration has no significant impact on the measured lithium concentration, indicating that lithium probably remains in solution and is not adsorbed by particles that would be retained during filtration. This observation corroborates the work of Kszos et al. (2003), who reported high lithium solubility in aquatic systems. The presence of sand and food had no significant effect on lithium solubility, indicating that these materials neither significantly promoted nor inhibited its solubility under the tested conditions. The observed toxicity effects were therefore due to exposure to lithium in aqueous solution.

Table 6. Ratio of Unfiltered/Filtered lithium concentrations in different treatments (Water, Sand, Sand + Food) at 0, 5, and 10 mg/L Over 48 Hours.

Treatment [Li] (mg/L)	Treatment	Time (hours):		
		0	24	48
0 mg/L	Sand+Food	0.994±0.119	0.987±0.180	1.082±0.533
	Water	0.999±0.013	0.998±0.012	1.003±0.038
5 mg/L	Sand	1.005±0.0014	1.001±0.013	1.000±0.010
	Sand+Food	1.013±0.005	0.990±0.013	0.992±0.022
	Water	1.014±0.019	1.007±0.005	1.002±0.013
10 mg/L	Sand	1.015±0.021	1.009±0.0012	0.995±0.013
	Sand+Food	0.995±0.010	1.007±0.011	0.995±0.015

The results of the chemical analyses of the RSW (Table 7) used for toxicity testing showed that the concentrations measured corresponded to the nominal concentrations. Filtered (dissolved Li) and unfiltered (total Li) samples were measured for treatment-related Li in *Pisidium dubium* and *Daphnia pulex* species. Dissolved organic carbon (DOC) and Total organic carbon (TOC) concentrations measured in the samples averaged 1.245 mg/L for DOC and 1.184 mg/L for TOC, indicating an aquatic system with a low organic load. Although there was some variability between samples and replicates, overall, these values suggest good water quality in terms of organic load, typical of natural, unpolluted waters. The average sodium concentration was 12.48 mg/L *for D.*

pulex and 24.84 mg/L for *P. dubium*. The higher sodium level observed in *P. dubium* is likely attributable to the presence of sand.

Table 7. Measured lithium concentrations in exposure treatments to *Pisidium dubium.*

Comparison of nominal and measured Li concentrations (mg/L) in the treatment. The concentrations are the calculated averages of dissolved Li concentration from 3 single measurements for each treatment at the beginning of the test. Filtered or dissolved concentration was obtained by filtration through a 0.45 membrane filter.

Test Species	**Lithium (mg/L)**		
	Nominal	**Filtered**	**Unfiltered**
Daphnia pulex	0	< 0.005	< 0.005
	0.5	0.523	0.521
	1	1.138	1.172
	1.5	1.743	1.753
	2	1.741	1.675
	2.5	2.848	2.788
	3	3.449	3.407
Pisidium Dubium	0	< 0.005	< 0.005
	0.05	0.046	0.047
	0.1	0.0909	0.089
	0.5	0.469	0.453
	1	0.934	0.91
	5	4.59	4.39
	10	9.39	8.97

2.3.2 Toxicity results

The chronic toxicity values obtained when *P. dubium* and *D. pulex* were exposed to Li are presented in Table 8. These values represent the lethal or effective concentrations that caused adverse effects in 50, 20 and 10% test organisms ($LC_{50,20,10}$ or $EC_{50,20,10}$) over the experimental periods.

Table 8. Impact of Lithium Concentration on Biological Parameters in *Pisidium Dubium* and *Daphnia pulex* showing the ECx/LCx values.

Test Species	Indicator	Description	Endpoint	Endpoint Estimates			
				Mean	±SD	95% CI	
Pisidium dubium	Survival		LC_{50}	1.369	1.407	-1.598	4.338
			LC_{25}	1.025	0.433	0.112	1.937
			LC_{10}	0.766	0.445	-0.172	1.705
	Burrowing		EC_{50}	1.59	1.683	-1.962	5.141
			EC_{25}	1.256	0.799	-0.431	2.943
			EC_{10}	0.992	0.391	0.168	1.817
Daphnia pulex	Growth	Length (mm)	EC_{50}	3.013	0.772	1.465	4.56
			EC_{25}	2.434	0.365	1.703	3.167
			EC_{10}	1.968	0.201	1.564	2.372
	Reproduction	Mean neomates	EC_{50}	1.775	0.092	1.589	1.96
			EC_{25}	1.588	0.104	1.38	1.796
			EC_{10}	1.421	0.217	0.988	1.854

3.2.2.1 *Pisidium dubium*

After 28 days of exposure, clams exposed to 5 and 10 mg/L Li did not survive (Figure5). The lethal concentration (LC_{50}) was calculated at 1.37 mg/L, with a standard deviation (SD) of ±1.41 (Table 8). The 95% confidence intervals (CI: -1.60 to 4.34) indicate some variability in the endpoint estimate, and include both positive and negative values, suggesting uncertainty as to the true magnitude of the effect. The values for LC_{25} (1.03mg/L) and LC_{10} (0.77 mg/L) indicate concentrations at which milder effects on survival can be observed. However, the associated SDs (0.43 and 0.45 respectively) for LC_{25} and LC_{10} suggest lower variability in these estimates.

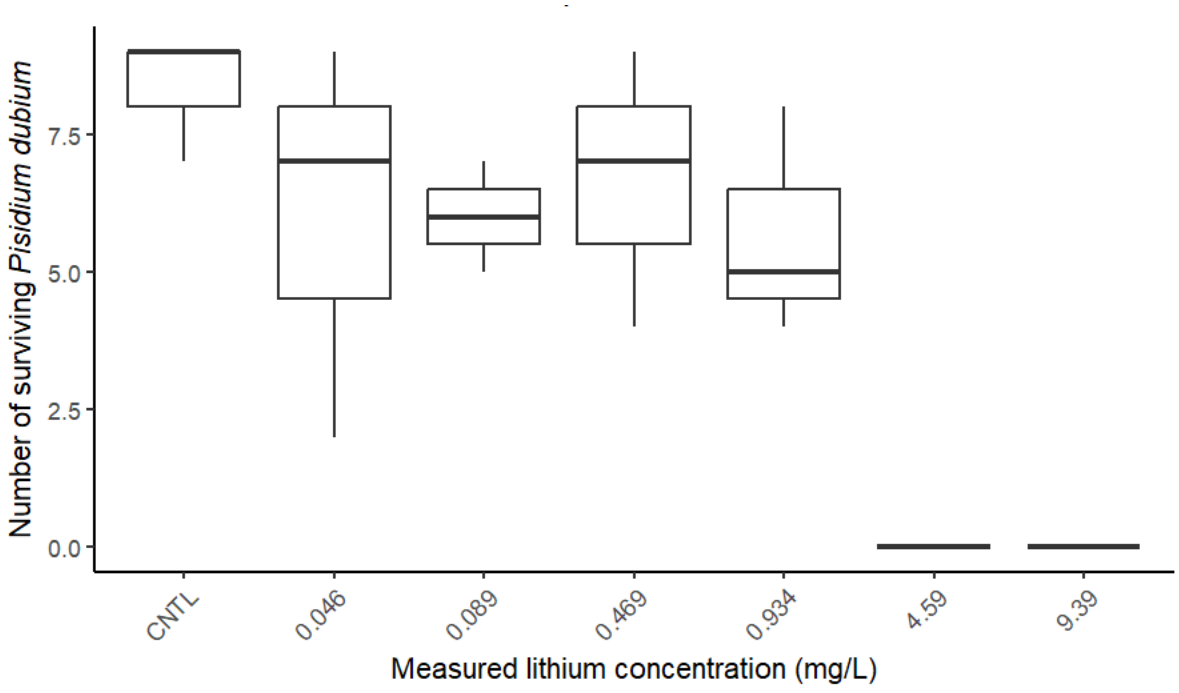

Figure 5. Effect of lithium on the survival of *Pisidium dubium* after 28 days of exposure. Box plot showing the number of surviving *Pisidium dubium* to measured lithium concentrations (mg/L). Each box represents the interquartile range (IQR), and the horizontal line inside the box indicates

the median number of surviving clams. Whiskers extend up to 1.5 times the IQR, and points beyond the whiskers represent outliers. n = 9 - 10 per treatment (n = 7).

Burrowing behavior in clams typically represents their ability to dig into the substrate (usually sand or mud) to protect themselves from predators, avoid harsh environmental conditions, or position themselves for optimal feeding. This behavior is an essential survival mechanism for many clam species (Ledoux et al., 2023). Figure 6 illustrates the burrowing behavior of Pisidium dubium under various lithium concentrations after 28 days of exposures. Increasing lithium concentration led to a significant decrease in this burrowing behavior. The threshold effect is marked: burrowing behavior remains relatively stable (between 75% and 90%) until the lithium concentration reaches around 0.089 mg/L, after which there is a sharp drop. At high lithium concentrations (4.59 and 9.39 mg/L), as clams did not survive at these levels, burrowing behavior was completely inhibited, reaching 0%, as there were no survivors at these concentrations. In terms of clam burrowing rates, the results (Table 8) suggest a relatively lower sensitivity to lithium compared to that of survival. The EC_{50}, EC_{25}, and EC_{10} for burial are estimated at 1.59, 1.26, and 0.99 mg/L, respectively. The SDs (1.68, 0.80 and 0.39, respectively) and the 95% CI (-1.96 to 5.14), (0.11 to 1.93) and (-0.17 to 1.70) reflect a high degree of variability in the estimated of the EC_{50} for burrowing behaviour.

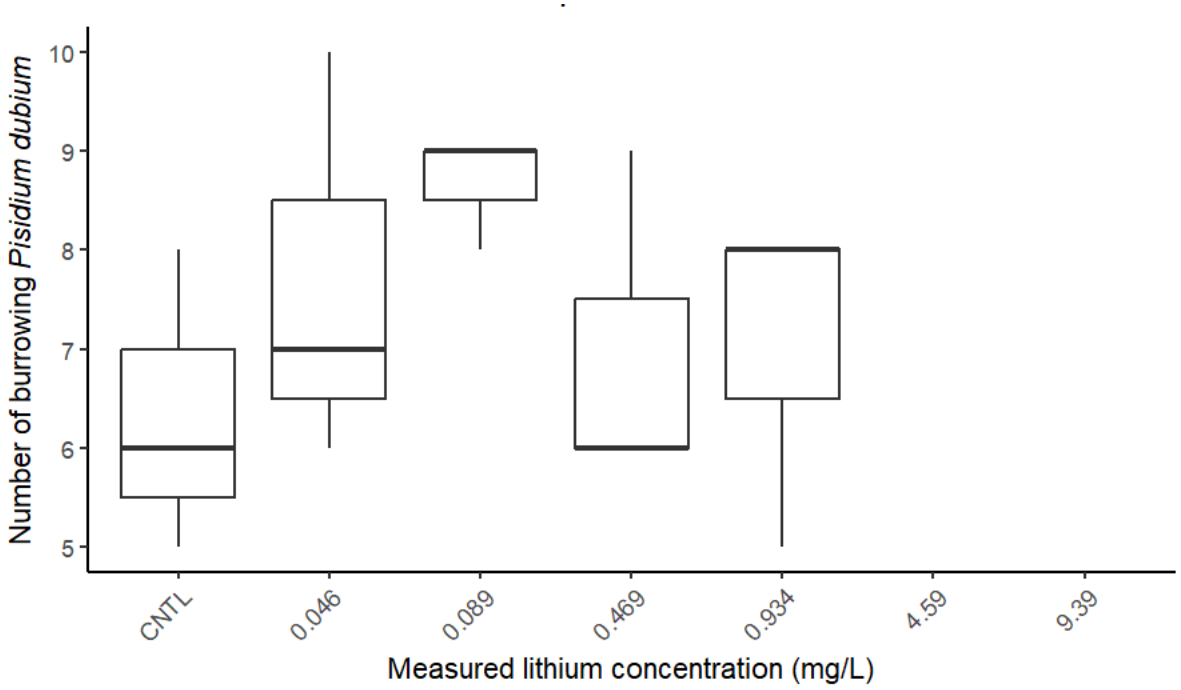

Figure 6 . Effect of lithium on the burrowing behavior of *Pisidium dubium* after 28 days of exposure. Box plot showing the number of *Pisidium dubium* buried after 28 days of exposure to measured lithium concentrations (mg/L). Each box represents the interquartile range (IQR), and the horizontal line in the box indicates the median number of buried clams. Whiskers extend to 1.5 times the IQR, and points beyond the whiskers represent outliers.

3.2.2.2 Bioconcentration

Lithium accumulation in clam shells and tissues were measured with data on surviving clams after 28 days of exposure, excluding dead clams exposed at 5 and 10 mg/L. Table 9 shows that lithium concentration in shells varied from 0.04 to 0.06 µg/g dry weight, while it varied from 3.62 to 5.01 µg/g dry weight in soft tissue. Total lithium in clams (shell + tissue) followed a similar trend to the soft tissue concentrations and ranged from 3.66 to 5.06 µg/g dry weight. The

bioconcentration factors (BCF) (Table 9) show a significant decrease with increasing lithium concentration in water, from 726 L/Kg at control (below the detection limit < 0.005 mg/L in water) to 4.89 L/Kg at 910 µg/L Li exposure.

Table 9. Lithium concentrations measured in *Psidium dubium* shell and clam tissue at different measured doses in water, with the corresponding bioconcentration factor (BCF).

Lithium Measurements					
Treatment (Nominal Concentration)	**Water**	**Shell**	**Clam Tissue**	**Total Li in Clam**	**BCF**
mg/L	**µg/L**	**µg/g d.w.**	**µg/g d.w.**	**µg/g d.w.**	**L/Kg**
Control	<DL[a]	0.039	3.622	3.661	726
0.01	47	0.05	4.547	4.597	96.766
0.05	89	0.05	5.013	5.058	56.326
0.5	453	0.05	4.08	4.126	9.011
1	910	0.06	4.447	4.507	4.888

[a]DL =5 ug/L

3.2.2.3 *Daphnia pulex*

Lithium had a significant dose-dependent effect on *D. pulex* reproduction (Figure 7). At the start of exposure, reproduction was similar between treatments, at around 10-20 neonates per day. However, significant differences appeared over time. In the 0 mg/L control group, production rose steadily, reaching around 160 neonates per day by the end of the experiment (21 days). At concentrations of 0.5 and 1 mg/L, production of neonates also increased, but moderately, reaching 100-120 neonates per day; in contrast, at concentrations of 1.5 mg/L and above, neonate production was increasingly limited, plateauing at around 60-80 neonates per day. At 3 mg/L, production reached only 40-50 neonates per day by the end of the experiment. The EC_{50} for the number of live offspring was 1.78 mg/L, with a standard deviation of 0.09 mg/L and a 95% confidence interval (95% CI) ranging from 1.58 to 1.96 (Table 8). The EC_{25} was 1.59 mg/L with a standard deviation of 0.10 and a 95%CI of 1.38 to 1.80, and the EC_{10} was 1.42 mg/L with a standard deviation of 0.22 and an 95% CI of 0.99 to 1.85 (Table 8). The narrowness of the confidence intervals suggests moderate variability in individual responses as showed the Figure 7.

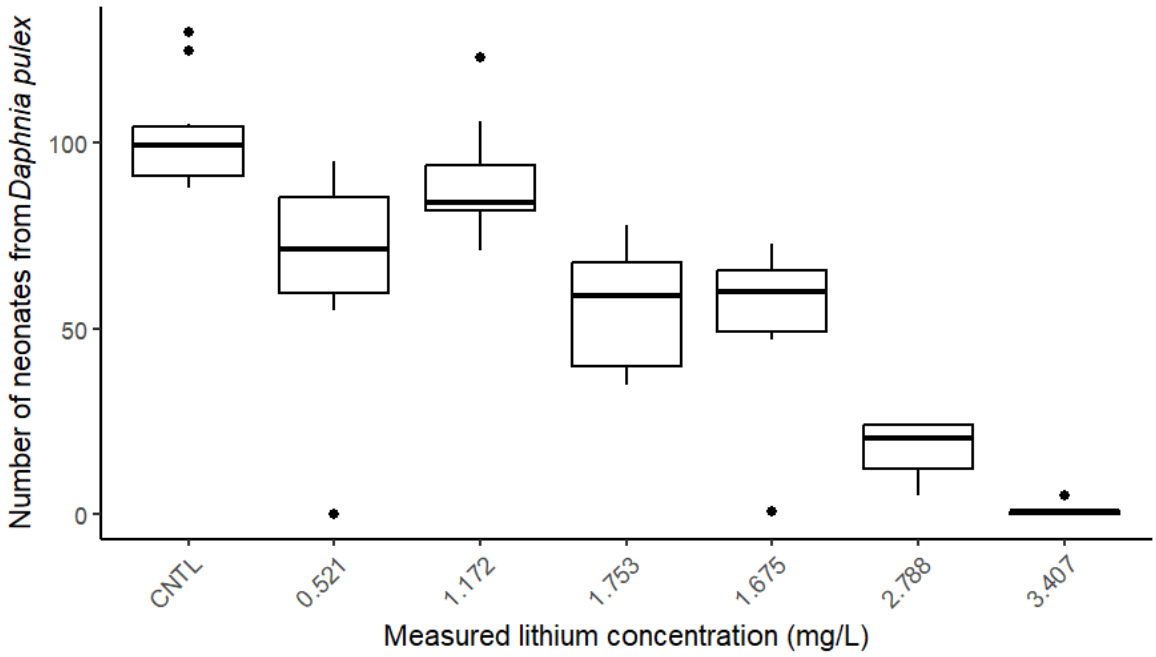

Figure 7. Effect of lithium on neonate production in *Daphnia pulex* after 21 days of exposure.

Box plot shows the total number of neonates produced by living *Daphnia pulex* after 21 days of exposure to different measured lithium concentrations. Each box represents the interquartile range (IQR), and the horizontal line within the box indicates the median number of neonates produced. Whiskers extend to 1.5 times the IQR, and points beyond the whiskers represent outliers.

Results concerning somatic growth in *Daphnia* after 21 days of exposure showed that the average length of 21 days neonates (newborn) varied inversely with the concentration of lithium in the medium (Figure 8). Neonates (less than 24 h old) measured on average 0.9mm; after 21 days, the average length of control individuals was around 2.93 mm. There was a linear decrease in individual size as lithium concentration increased. At 3 mg/L the average length was 2.21±0.15mm. These results indicate that lithium exposure had a significant negative impact on the growth and

development of young *Daphnia pulex*. The higher the concentrations, the smaller the size of the neonates at the end of the experiment. The EC_{50} was 3.01 mg/L with a standard deviation of 0.77 and a 95% CI of 1.46 to 4.56. Similarly, the EC_{25} was 2.43 mg/L with a standard deviation of 0.37 and, a 95 % CI of 1.70 to 3.16 while the EC_{10} was 1.97 mg/L with a standard deviation of 0.20 an 95 % CI of 1.56 to 2.37 (Table 8).

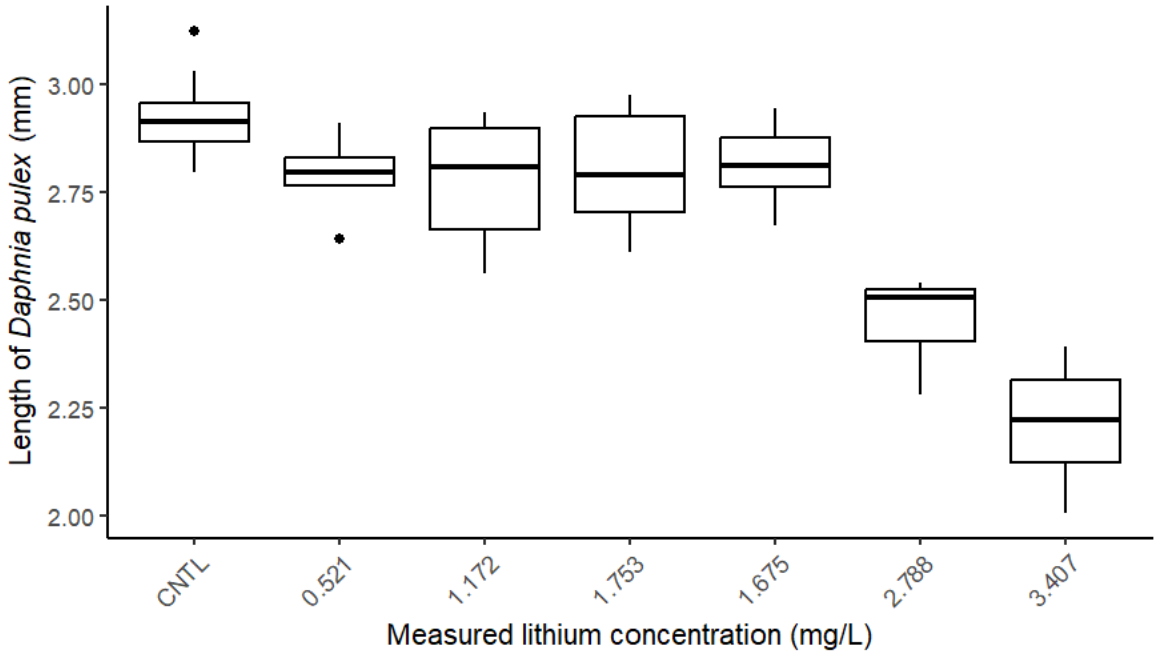

Figure 8. Effect of lithium on body length of *Daphnia pulex* after 21 days of exposure. Box plots of the mean body length of *Daphnia pulex* (in mm) after 21 days of exposure at different measured concentrations of lithium (mg/L). Each box represents the interquartile range (IQR), with the horizontal line indicating the median body length. Whiskers extend to 1.5 times the IQR, and points beyond the whiskers represent outliers.

2.4 Discussion

2.4.1 *Pisidium dubium*

For the fingernail clam *P. dubium*, the LC/EC_{50} values for survival (1.37 mg/L) and burrowing behavior (1.59 mg/L) were similar, suggesting that both endpoints are potentially equally sensitive to Li exposure. For the marine Mediterranean mussel *Mytilus galloprovincialis*, Fraga *et al.* (2022) reported an LC_{50} of 153.78 mg/L after 9 days of exposure to Li. The latter results are comparable to the LC_{50} of the freshwater and brackish (sometimes estuarine) water mussel *Dreissena polymorpha*, established at 185–232 mg/L Li after only 24 hours of exposure to LiCl (Fraga et al., 2022). These values are therefore significantly higher than the LC_{50} of 1.37 mg/L observed for the survival of *P. dubium*, suggesting a higher tolerance to lithium in marine mussels. This high tolerance could be explained by high Na levels in marine waters (Kszos *et al.*, 2003). It is important to note that in this study, no survivors were observed at concentrations of 5 and 10 mg/L, further emphasizing *P. dubium's* sensitivity to lithium.

The confidence intervals for *P. dubium* end-points were wide, which suggests considerable variability in responses, which is not uncommon in ecotoxicological studies (Kilgour et al., 2018). One explanation for this wide variability was the use of clams of difference size and age in each treatment at the start; a solution would be to collect clams in the spring and conduct toxicity tests on released new-born individuals(Nautilus Environmental Company Inc., 2019). Another solution would be to conduct toxicity tests within a narrower range of exposure.

2.4.2 Bioconcentration

Although lithium concentrations in soft tissues remained relatively low, the effects observed on the burrowing behavior of *Pisidium dubium* (EC_{10} of 0.99 mg/L) suggest that this metal affects physiological processes even at apparently low internal concentrations. This phenomenon illustrates the concept of "spill over", where the effects of the contaminant are manifested not only by direct accumulation in tissues, but also by physiological and behavioral disturbances (Campbell et al., 2003). This observation is in line with results reported for marine mussel species. In *Mytilus galloprovincialis*, at a concentration of 0.75 mg/L, significant negative effects were observed, including reduced metabolic activity, increased neurotoxicity and severe oxidative stress (Viana et al., 2020). Furthermore, studies on the same marine mussel species revealed sublethal effects progressing to histopathological alterations at lithium concentrations ranging from 0.1 mg/L to 10 mg/L over a 21-day period (Fraga et al., 2022).

To my knowledge, no Li bioaccumulation data exist for freshwater clams. However, studies on much larger marine bivalves, including *Venerupis corrugata*, *Mytilus galloprovincialis*, and *Mytilus edulis* (Barbosa et al., 2023; Fraga et al., 2022; Thibon et al., 2021; Viana et al., 2020), suggest that Li accumulation increases with higher exposure. Barbosa et al. (2023) found that Li concentrations in *V. corrugata* tissues ranged from 1.9 to 7.1 µg/g when exposed to Li concentrations of 0, 0.2, 0.4, and 0.8 mg/L. Similarly, *M. galloprovincialis* accumulated 0.7 to 1.4 µg/g when exposed to 0.1, 0.25,2and 0.75 mg/L (Viana et al., 2020), and *M. edulis* showed tissue Li concentrations of 0.5 to 2.2 µg/g after a four-day exposure to 0.18, 0.5, 1.0, and 1.5 mg/L (Thibon et al., 2021). These marine bivalves show higher bioaccumulation compared to *P. dubium*.

In contrast to marine mussels, which show proportional Li accumulation with increased water concentrations, Li levels in *P. dubium* remained constant despite increasing exposure. However, similar to trends observed in marine mussels, bioconcentration factor (BCF) in *P. dubium* decreased with rising lithium concentrations in the water (Barbosa et al., 2023; Fraga et al., 2022; Thibon et al., 2021; Viana et al., 2020). This pattern may indicate saturation or restricted lithium uptake pathways in *P. dubium*, suggesting that as external Li levels rise, the clams reduce uptake likely due to reduced filtration, respiration, or metabolic depression as avoidance mechanisms (Viana et al., 2020). Similar trends in decreasing BCF values have been observed with other pollutants, such as lanthanum, where reduced filtration and respiration or metabolic depression mitigate pollutant uptake in bivalves (Pinto et al., 2019).

While little information exists on Li toxicity mechanisms in freshwater clams, molecular studies in marine mussels provide some insights (Barbosa et al., 2023; Thibon et al., 2021; Viana et al., 2020). The digestive gland, essential for metabolism, detoxification, and pollutant response in mollusks, is a primary target of Li toxicity (Garmendia et al., 2010). Fraga et al. (2022) observed significant atrophy of the digestive alveoli epithelium in response to 1 and 10 mg/L of Li, likely due to reactive oxygen species (ROS) production induced by Li exposure (Regoli & Giuliani, 2014). In response to ROS, organisms activate antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), to neutralize ROS (Regoli and Giuliani, 2014). Barbosa et al. (2023) also observed an increase in enzymatic defenses in *V. corrugata* to protect cells from oxidative stress at lithium concentration of 0.5 mg/L in seawater. Additionally, enzymes like carboxylesterases (CbEs) and glutathione S-transferases (GSTs) metabolize contaminants, facilitating their excretion (Nebbia, 2001). Increased CbEs and GSTs activities with Li exposure concentrations may help limit cellular damage through Li elimination (Marín

Rodríguez et al., 2022). However, if defense mechanisms fail, excess ROS may cause oxidative damage to cell membranes through lipid peroxidation (LPO) or protein oxidation (protein carbonylation) (Regoli and Giuliani, 2014), potentially contributing to the epithelium atrophy observed by Fraga et al. (2022).

Li exposure can also lead to neurotoxicity. Acetylcholinesterase (AChE), an enzyme crucial for nerve impulse regulation, is inhibited by Li, causing acetylcholine buildup and negative neurological effects (Lionetto et al., 2013). Viana et al. (2020) reported neurotoxic effects in bivalves with increased Li exposure, likely due to Li's interaction with sodium (Na) and magnesium (Mg) macromolecular sites, which disrupts ion transport and cell membrane potential (Jakobsson et al., 2017).

2.4.3 *Daphnia pulex*

There are few data on lithium toxicity in *Daphnia pulex*, and the available information is limited. For acute exposures, Lilius *et al.* (1995) reported a 24-hour EC_{50} (immobilization) of 17.1 mg/L for lithium in *D. pulex* and *24.85* mg/L for *D. magna* with 55 mg/L of Na (sodium). A recent study by Chen et al. (2024) provided more detailed insights into the acute toxicity of lithium to *D. magna*. The reported LC_{50} values for *D. magna* at 24, 48, and 64 hours were 10.2 mg/L, 4.1 mg/L, and 1.2 mg/L, respectively, with 8.36 mg/L of Na. From these results it would appear that *Daphnia magna* seems more sensitive to lithium. However, the study by Lilius *et al.* (1995) noted no significant difference in the overall sensitivity of *D. magna* and *D. pulex* when exposed for 24 hours to 30 reference chemicals including Li. This could be the result of different genetic strains or culture media.

From a chronic exposure perspective, a 21-day study on *D. magna* reported an EC_{50} of 0.039 mg/L and an EC_{10} of 0.023 mg/L (Martins et al., 2022) for reproduction with a moderate concentration of Na of ~ 52.5 mg/L. This is in stark contrast to the present study, which found an EC_{50} of 1.78 mg/L and EC_{10} 1.42 mg/L with 12.48 mg/L of Na for *D. pulex* reproduction over the same period. These findings suggest that *D. magna* is likely more sensitive to lithium compared to *D. pulex*, especially over long exposure periods.

The above comparison of lithium sensitivity between the two *Daphnia* species reveals significant differences, underlining the need to consider interspecific variability when assessing ecotoxicological risks. The significant difference in sensitivity to lithium observed between *D. pulex* and *D. magna* shows that comparative studies on different species are essential to better characterize the effects of pollutants in aquatic ecosystems.

An integrated biomarker (IBR) analysis by Chen *et al.* (2024) has recently shed light on the mechanism of action of lithium in *D. magna*. They demonstrated that lithium exposure leads to a concentration-dependent reduction in energy reserves, with significant effects on protein and glycogen contents. This reduction in energy reserves indicates a disturbance in energy metabolism, which is primarily oriented towards detoxification or homeostatic regulation processes, to the detriment of growth and reproduction. Furthermore, the metabolomic study of Nagato *et al.* (2013) showed that copper (0.012 mg/L) and lithium (1.15 mg/L) induced similar metabolic changes in *D. magna*, suggesting a comparable mode of action for these two metals. These mechanisms may explain the toxicity effects observed in *Daphnia pulex*.

2.4.4 Relevance to Li sources management

In urban environments, lithium levels at present occur below toxic thresholds for aquatic organisms as Canadian surface waters and urban stormwater ponds exhibit concentrations ranging from 0.001 to 0.200 mg/L (Ouedraogo & Pick unpub.; Paquet & Mueller, 2022), well below the lowest observed EC10 of 0.77 mg/L for *Pisidium dubium* survival. Even in more impacted urban areas like the Han River in South Korea, where concentrations reach 1.57 mg/L (Choi et al., 2019), levels remain close to, but do not consistently exceed, toxicity thresholds for *P. dubium* and *Daphnia pulex.*

In contrast, mining areas can approach or exceed toxicity thresholds for aquatic organisms. Near active mines in Canada, lithium surface water for unaffected area concentrations, range from 0.735 to 1.0 mg/L(GENIVAR, 2013), while tailing pore waters in Quebec reach 1-2 mg/L (Roy, 2023). These levels overlap with or are similar to multiple toxicity endpoints for both *P. dubium and D. pulex*. More extreme cases, such as the Williston basin in the USA with 3.5 mg/L (Lauer et al., 2016) and the Donbass River in Ukraine with 1.18-1.37 mg/L (Sobolev et al., 2019) surpass most of the observed toxicity thresholds determined in the present study.

These findings highlight the potential ecological risks in mining areas and heavily polluted water bodies, where lithium concentrations frequently exceed levels now known to cause adverse effects in aquatic organisms. While urban environments currently maintain relatively safe lithium levels, the increasing use of lithium in modern technologies may lead to rising urban concentrations in the future. This underscores the need for continued monitoring and rigorous management of

lithium, particularly in high-risk areas, to protect aquatic ecosystems from potential long-term impacts.

2.4.5 Antagonist effect of sodium

While lithium levels can potentially affect aquatic environments in the vicinity of anthropogenic sources, it is important to consider the antagonistic effect of sodium on lithium toxicity, as previously reported by Kszos et al. (2003). These authors observed that higher sodium concentrations enabled organisms to tolerate higher levels of lithium. In their toxicity experiment with the fish *Pimephales promelas*, the concentration of lithium inhibiting 25% of growth (IC_{25}) increased from 0.38 mg/L to 1.99 mg/L when sodium levels were increased from 2.8 to 17 mg/L. Similarly, for the crustacean *Ceriodaphnia dubia*, the IC_{25} for reproduction increased from 0.32 to 3.33 mg/L Li for the same increase in sodium concentrations (Kszos et al., 2003). Na plays an important role in Li uptake by living organisms, as it conditions pH, which in turn influences the Na-K equilibrium and, consequently, lithium uptake (Paquet & Mueller, 2022). Due to its physical similarity to Na, Li is absorbed through processes analogous to those used for sodium. Indeed, as a result of its atomic radius, lithium can replace other cations, notably sodium (Na^+), in living organisms (Paquet & Mueller, 2022). This ability is enhanced by the fact that Li enters cells via Na channels, facilitating its uptake.

In the present study, toxicity tests were carried out with mean sodium concentrations of 12.5 mg/L for *D. pulex* and 24.8 mg/L for *P. dubium*. These level of Na are above mean Na concentrations of 2.0 mg/L encountered in unaffected lakes near Uranium mines and Mills in Northern Saskatchewan (Areva resources, 2012; Cameco, 2009, 2015, 2018; CanNorth, 2011) and

1.5 mg/L in unaffected lakes near proposed lithium mining areas in Northern Quebec (Galaxy lithium inc. & WSP Canada inc., 2018; Nemaska Lithium Inc., 2013; WSP Canada Inc., 2019). Therefore, the lithium chronic toxicity data reported in the present study may not be sufficiently protective. In other words, if the Li toxicity experiments were conducted at lower Na concentrations of 1-2 mg/L per liter, lower toxicity values than the one reported in this thesis would likely be identified (provided Na alleviates Li toxicity). However, considering that Li mining effluent could contain an appreciable amount of Na, it is possible that the toxicity results conducted at 12.5 and 24.8 mg/L of Na are sufficiently protective for species living downstream of lithium mining. While ore processing is different, data downstream of Uranium mines and mills in Northern Saskatchewan report mean concentrations of Na of 26 mg/L, higher than levels tested in the present study. Therefore, it is possible that Li mining effluent containing Na could moderate the effects of lithium exposure on aquatic organisms.

Long-term accumulation of Li and Na in sediment should also be considered when predicting effects on the aquatic environment from long-term mining operations. Such research would help in planning adequate measures for the protection of aquatic ecosystems downstream of Li sources. It would also be pertinent to carry out more toxicity studies to investigate this antagonistic relationship on aquatic species. Furthermore, just as guidelines for metals like copper are adjusted based on water hardness, future environmental guidelines for Li could potentially be tailored to specific water chemistry parameters. This approach would lead to more nuanced and effective environmental protection strategies, highlighting the practical importance of studying these antagonistic relationships in depth.

Chapter 3: Conclusion

The increasing use of lithium (Li) worldwide will, without adequate guidelines, lead to an increase in Li-containing waste and effluents, resulting in higher concentrations of Li in the environment. The choice of adequate controls needs to be based on in-depth knowledge of the chronic toxicity of Li to the environment. However, the ecotoxicological implications of Li remain poorly understood, and data on its chronic toxicity is limited. It is surprising that in this context of growing demand for Li, in most countries (including Canada), there are currently no discharge limits for municipal or mining effluents, and no directives on water quality, whether acute or chronic, for the protection of aquatic life.

3.1 Chronic effects of lithium on *Daphnia pulex* and *Pisidium dubium*

The present thesis provided insight into the chronic toxicity of lithium on two common freshwater invertebrates with different life history strategies, *Pisidium dubium* and *Daphnia pulex*. Overall, the results led to rejection of the null hypothesis (H0) that lithium does not affect these species at concentrations likely to be encountered near Li non-point and point sources. The observations supported the alternative hypothesis, and showed effects of Li during chronic exposure on reproduction (EC_{10} = 1.42 mg/L) and growth in *D. pulex* (EC_{10} = 3.01 mg/L), as well as on burrowing (EC_{10} = 0.99 mg/L) and survival in *P.dubium* (EC_{10} = 0.77 mg/L). The results corroborated the hypothesis demonstrating the sensitivity of *Daphnia pulex* and *Pisidium dubium* to Li varies with Li concentration.

Lithium bioaccumulation mechanisms in bivalves present a complex dynamic. Although in the present study tissue lithium concentration remains constant with exposure, the bioconcentration factor (BCF) follows an inverse trend, reaching its minimum values at the highest exposure concentrations. This inverse relationship suggests the activation of specific defense mechanisms. Bivalves appear to implement detoxification systems involving glutathione S-transferases (GST) and carboxylesterases (CbE) (Barbosa et al., 2023; Viana et al., 2020). These enzymatic responses represent an adaptive strategy aimed at limiting bioaccumulation and preventing toxicity. This phenomenon is often accompanied by metabolic depression and reduced respiratory capacity, as demonstrated in several studies on marine bivalves (Barbosa et al., 2023; Thibon et al., 2021; Viana et al., 2020). The regulation of lithium accumulation therefore appears to be an active process involving coordinated biochemical and physiological mechanisms to maintain homeostasis in the face of increasing exposure.

Concerning Li mechanism toxicity, it operates through multiple pathways. Studies have shown that lithium can induce oxidative stress in organisms, leading to increased activity of antioxidant enzymes such as peroxide dismutase and catalase (Barbosa, et al., 2023; Thibon et al., 2021; Viana et al., 2020). Li's detoxification mechanism involves a reallocation of cellular energy, where lithium, on entering the body, disrupts normal physiological functions. This disruption leads to a depletion of energy reserves (proteins and glycogen), towards detoxification processes and homeostatic regulation to maintain internal equilibrium (Chen et al., 2024). These energy defense mechanisms prioritize the elimination or management of toxic lithium to the detriment of other vital functions (growth, reproduction, burial).

3.2 Proposed Li chronic guideline value

Comparative studies of lithium toxicity reveal significant differences in sensitivity between aquatic species, with important implications for setting water quality standards. The LC/EC values obtained for *Daphnia magna* in previous studies were lower than those found here for *Daphnia pulex*, indicating that *D. magna* may be more sensitive to lithium than *D. pulex*. This is supported by the species sensitivity distribution curve (Figure 9) based on the chronic data available to date from literature and the present study. *Daphnia magna* appears to be the most sensitive aquatic species to lithium among those tested, with effects observed at very low concentrations (below 0.1 mg/L). In contrast, *Daphnia pulex* and *Pisidium dubium* are moderately sensitive freshwater invertebrates.

Using the species sensitivity distributions (Figure 9), the estimated water quality guideline for the protection of freshwater aquatic life is approximately 0.0579 mg/L (57.9 μg/L) (standard error: 0.110 mg/L, with a 95% confidence interval from 0.0024 to 0.416 mg/L). This value corresponds to the hazardous concentration threshold for 5% or HC5 (Posthuma et al., 2001). The graph was produced using the R package ssdtools, which includes statistical models for fitting species sensitivity distributions (SSDs). Although a precautionary approach using a lower effect concentration as a conservative reference is advised, additional data would significantly improve the precision of the HC_5 estimate, reduce uncertainty and enhance the reliability of environmental management decisions concerning lithium in freshwater ecosystem.

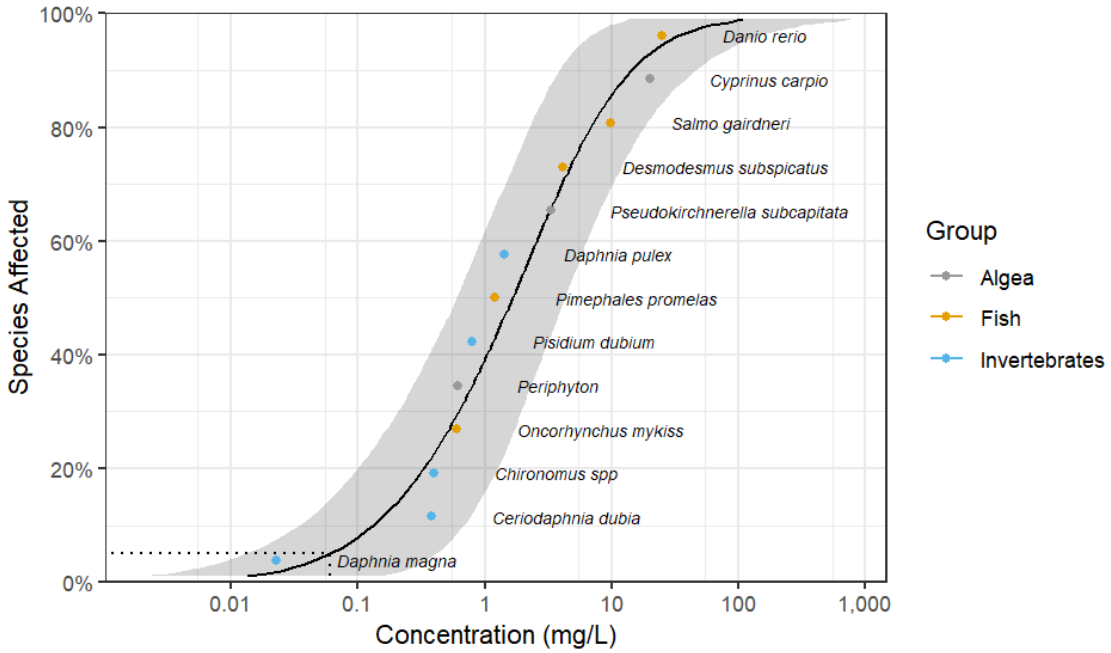

Figure 9. Species Sensitivity Distribution (SSD) for Long-term Lithium Toxicity in Freshwater Ecosystems**:** illustration of the cumulative distribution of chronic lithium toxicity across various species **(**n = 13**)**, with concentration (mg/L) on a logarithmic x-axis and percentage of affected species on the y-axis. Data points are color-coded by group (algae, fish, invertebrates), with the black curve showing the fitted distribution and the gray area representing the 95% confidence interval. The hazardous concentration affecting 5% of species (HC5) is estimated at 0.0579 mg/L (standard error: 0.110 mg/L), with a 95% confidence interval from 0.0024 to 0.416 mg/L.

Although natural levels of lithium in most surface waters are generally below the chronic toxicity thresholds for both species and the estimated water quality guideline, the elevated levels observed in some municipal and mining areas (0.003-3.5 mg/L; see Table 1) could potentially have negative impacts on sublethal parameters such as reproduction, growth or behavior. It is therefore recommended that mining companies and municipalities consider adopting appropriate controls on Li discharges and implementing adequate monitoring programs to confirm the effectiveness of these controls.

While this derivation of a water quality guideline for lithium is necessary, further studies are recommended to elucidate the physiological and biochemical mechanisms underlying lithium sensitivity in aquatic species. This deeper understanding would not only improve knowledge of the effects of lithium but would also contribute to the development of more sophisticated risk assessment models. Furthermore, the interaction between lithium and sodium, as well as the influence of other factors such as water hardness, underline the need to develop guidelines based on these parameters in the future. This improved knowledge would enable more informed decision-making when implementing mitigation strategies, thus ensuring better protection of aquatic ecosystems from the potential impacts of lithium.

3.3 Limitations and recommendations

While *Daphnia* species are typical test organisms in ecotoxicology, *Pisidium dubium* is a relatively new species for toxicity testing. The use of this species presented some challenges, and some limitations. Specimens were collected exclusively during the summer and are not

cultivated in a laboratory setting. Furthermore, it was challenging to ascertain the age of individuals, which prompted the implementation of experiments utilizing a diverse pool of individuals. It is therefore recommended that improvements be made to the methods and protocols used in future tests. For instance, Nautilus Environmental Company Inc. (2019)collect clams in the spring and conduct tests on offsprings. Moreover, reproduction was not examined in the *P. dubium* experiments; it would be of interest to open the individuals at the conclusion of the tests to determine whether reproduction has occurred. However, their small size is a constraint. Using larger species like *Sphaerium s*pp. could be an alternative. Finally, the 95% confidence intervals for the *P.dubium* tests yielded negative values, which is likely due to a significant difference between the exposure concentrations of 5 and 10 mg/L.

These tests should be repeated with intermediate values to obtain more accurate results. To enhance the precision of lithium effect tests, ideally it may be preferable to study different endpoints (survival, reproduction, and growth) separately, increase the number of treatments from 7 to 10, and use individuals of the same age. These changes, while increasing the cost and complexity of experiments, would yield more reliable results with narrower confidence intervals, significantly improving understanding of lithium's effects on various aspects of aquatic organisms' biology. Initially, clam burrowing levels were observed without disturbing the aquarium, which could lead to inaccurate assessments as dead clams might remain buried for extended periods unnoticed. To address this issue and obtain more accurate data on the burial endpoint, one recommendation would be to gently shake the mini aquariums housing the clams and observe their reactions. This method would help to distinguish between living clams that actively rebury themselves and those that remain unresponsive, potentially indicating mortality.

Finally, Lithium bioconcentration data in this thesis was measured by pooling all surviving individual per treatment. This reduced the estimation of variability in bioconcentration estimates. While all 25 individuals survived in the control, an average of 18 individuals survived in each treatment, leading to Li concentration in acid extract ranging between 0.062 and 0.080 mg/L. With a typical detection limit for an ICP-MS of 0.005 mg/L, we can conservatively recommend that at these range of lithium exposure (i.e. 0.05 to 1 mg/L) the tissue of three individuals could have been pooled together and obtain detectable level of Li. Pooling three individuals together would have provided 6 replicates per treatment in the assessment of bioaccumulation.

3.4 The antagonistic effect of sodium on lithium toxicity

An important aspect of lithium toxicity is the reported antagonistic effect of sodium (Kszos et al., 2003). Indeed, sodium plays a protective role, reducing lithium toxicity to freshwater organisms. In the presence of sufficient sodium levels, species can likely tolerate much higher concentrations of lithium than in its absence. This antagonistic relationship follows an exponential model, suggesting that a certain sodium/lithium ratio can prevent harmful effects on the reproduction and growth of organisms (Kszos et al., 2003b). These findings have important implications for the management of lithium releases to the environment and highlight the need to consider sodium concentrations when assessing the risks associated with lithium in aquatic ecosystems.

However, it is important to note that despite high sodium levels in marine environments, toxic effects of lithium have still been observed in certain marine species (Barbosa et al., 2023;

Fraga et al., 2022; Thibon et al., 2021; Viana et al., 2020). The sodium/lithium ratio needed to prevent toxicity requires further study.

3.5 Future research directions and implications

For future research, it is also recommended that more aquatic species be tested with environmentally relevant lithium concentrations, considering the alleviating effects of sodium, and examining the short- and long-term effects. Depending on the results of these investigations, the Li water quality guideline for the protection of aquatic life may need to consider the protective effect of sodium. Additionally, further research will also be essential to develop more accurate predictive models for assessing the ecological risks associated with lithium and improve decision making on the implementation of mitigation measures.

Finally, the results of this thesis have significant implications for the mining, pharmaceutical, and municipal sectors. Developing water quality criteria for aquatic life protection will enhance risk assessment models, facilitating the refinement of wastewater treatment protocols to reduce lithium discharges and their environmental impacts. The identification of aquatic invertebrates as early indicators of disturbance offers a valuable opportunity for establishing effective biomonitoring programs, enabling early detection and prevention of contamination before significant ecosystem disruption occurs. This research advances freshwater ecosystem protection, emphasizing the need for vigilant monitoring and management of lithium levels, particularly in high-risk areas. By translating scientific insights into actionable strategies, we can better protect and sustain aquatic environments for future generations.

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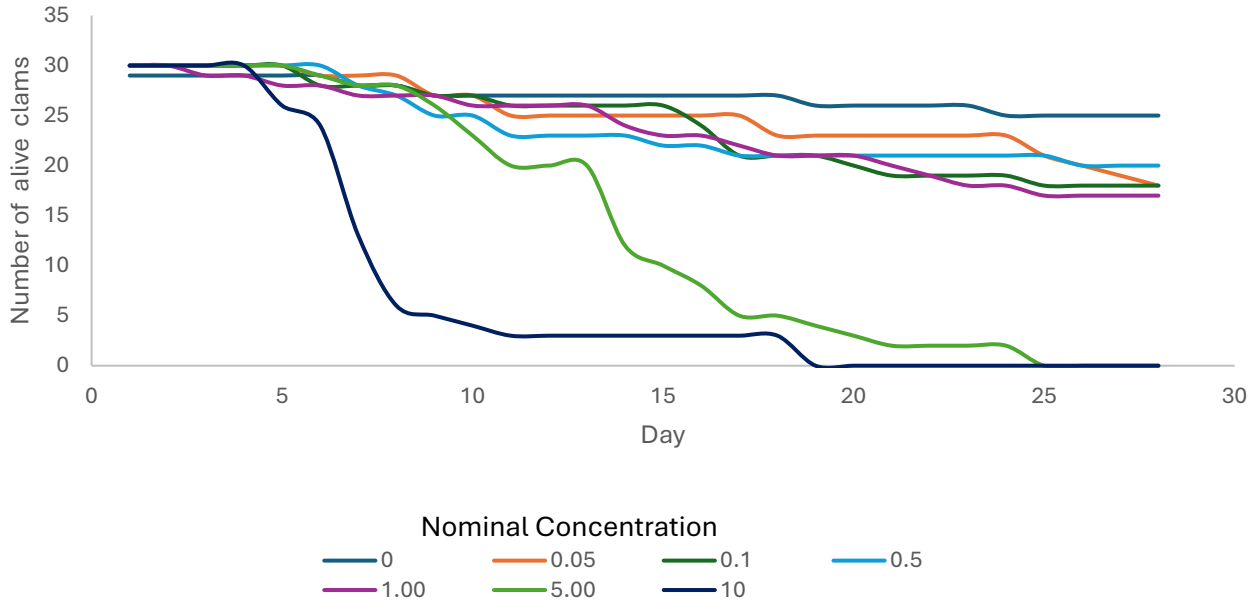
Appendix

Appendix1: *Pisidium dubium*

FigureA1. *Pisidium dubium,* fingernail clam species.

FigureA2. Photograph of the initial fingernail clam (*Pisidium dubium)* culture setup. A total of 10 culture beakers were setup and maintained simultaneously.



FigureA3. Survival trend curves for Pisidium dubium. (n = 9-10 per treatment group) exposed to lithium treatment (n=7) over a 28-day period. Points represent the percentage of organisms surviving over time for different lithium concentrations.

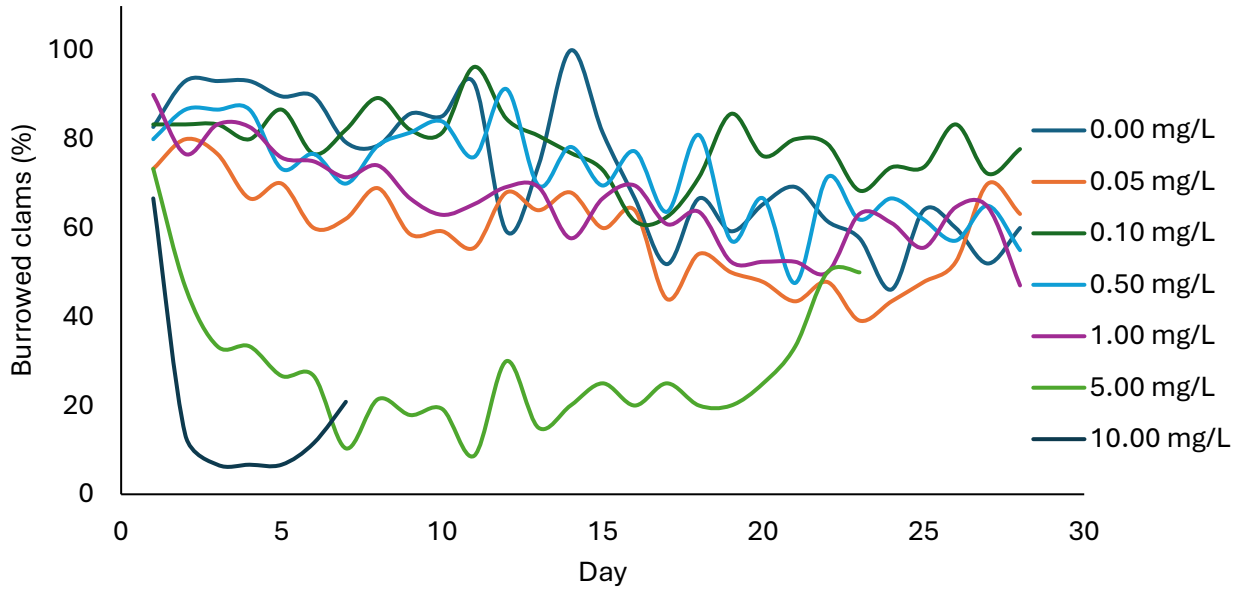


Figure A4. Burrowing trend curves for Pisidium dubium (n = 9-10 per treatment group) exposed to lithium treatment (n = 7) over a 28-day period. Curves represent the percentage of burrowed bivalves over time for each tested concentration (mg/L). A significant decrease in burrowing rate is observed at higher concentrations (5.00 and 10.00 mg/L), suggesting a dose-dependent effect of lithium on burrowing behavior.

Appendix 2: Bioconcentration

Table1A. Raw data of lithium and sodium (ppb) concentrations samples for clam (*Pisidium dubium*) soft tissue (CT) and shell (SH) after 28 days of exposures. DORM5 is the standar.

SAMPLE DESCRIPTION	Li(ppb)	Na(ppb)
CT-0	79.7	998.4
CT-0.05	71.21	833.1
CT-0.10	64.97	723.5
CT-0.50	62.04	684.5
CT-1.00	63.51	837.2
DORM5-CT-1	59.12	11640
DORM5-CT-2	55.02	10810
DORM5-CT-3	48.58	10322
SH-0	51.7	56201
SH-0.05	48.39	60768
SH-0.10	35.7	50013
SH-0.50	40.48	52382
SH-1.00	41.36	48650
DORM5-SH-1	48.09	401174
DORM5-SH-2	59.51	389854
DORM5-SH-3	58.04	415676

TableA2. Raw data of Pisidium dubium weigth and numbers of individuals for soft tissue (CT) and shell (SH) after 28 days of exposures. DORM5 is the standar.

Name	Weight (g)	Number of Individuals
CT-0	0.0088	25
CT-0.05	0.0087	18
CT-0.10	0.0072	18
CT-0.50	0.0080	19
CT-1.00	0.0084	17
DORM5-CT-1	0.0122	--
DORM5-CT-2	0.0113	--
DORM5-CT-3	0.0107	--
SH-0	0.5213	25
SH-0.05	0.5371	18
SH-0.10	0.4359	18
SH-0.50	0.4550	19
SH-1.00	0.4054	17
DORM5-SH-1	0.4999	--
DORM5-SH-2	0.5046	--
DORM5-SH-3	0.5049	--

Appendix 3: *Daphnia pulex*

Figure A5. *Daphnia pulex*

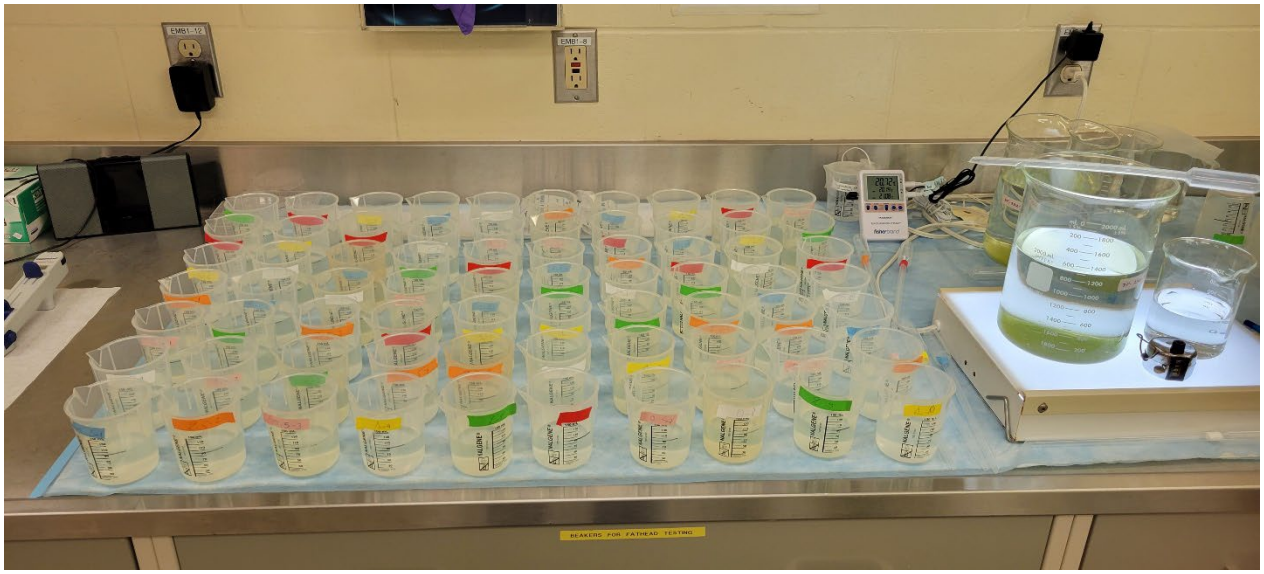
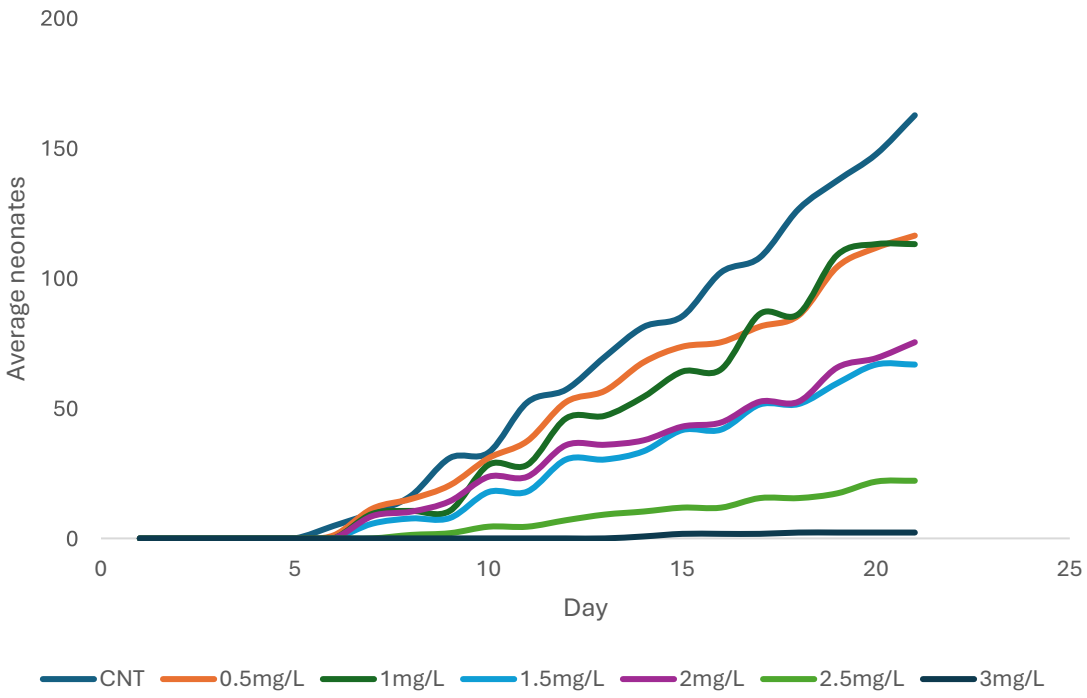


Figure A6. Photograph of the set-up for the toxicity test on Daphnia pulex (water flea). A total of 10 breakers for each lithium treatment (n=7), with one daphnia per beaker.



FigureA6. Average neonate production per day from alive *Daphnia pulex* during 21 days of exposure to lithium concentrations (n=7).

TableA.3: Raw data (survival and reproduction) of Daphnia pulex exposure to copper as quality test control for 21 days. The data include , pH, conductivity , temperature and dissolved oxygen.

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

SET 1: 0 - 80µg/L Cu — Feeding: 700µl YCT + 700 µl Algae per 100ml daily — Dilution media: Reconstituted Soft water

Start Date: August 24, 2023 — one (1) starter (neonate) *daphnia* per test vessel

Replicate I.d. = number of live neonates produced + deaths

Age	Date	Time	1.25	5.0	0	10.0	2.5	80.0	48.8	20.0	Comments	Initials
Day 0	24-Aug-23	13:00	—	—	—	—	—	—	—	—		GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		ALL ALIVE	GP
Day 2	26-Aug-23	14:20	—	—	—	—	—	X	X	—		GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER			
Day 4	28-Aug-23	10:37	—	—	—	—	—			—		GP
Day 5	29-Aug-23	11:48	—	—	—	—	—			—		GP
Day 6	30-Aug-23	10:15	8	Ø	12	Ø	Ø			Ø		GP
Day 7	31-Aug-23	10:13	Ø	11	Ø	14	11			12		GP
Day 8	01-Sep-23	10:38	Ø	Ø	18	Ø	Ø			Ø		GP
Day 9	02-Sep-23	12:54	19	26	Ø	24	22			21		GP
Day 10	03-Sep-23	12:03	Ø	Ø	25	Ø	Ø			Ø		ES
Day 11	04-Sep-23	10:56	23	32	Ø	25	25			25		MO
Day 12	05-Sep-23	10:25	Ø	Ø	Ø	Ø	Ø			Ø		GP
Day 13	06-Sep-23	8:48	26	37	32	X 7	Ø			35		ES
Day 14	07-Sep-23	8:20	Ø	Ø	Ø		30			Ø		MO
Day 15	08-Sep-23	10:33	27	30	32		Ø			Ø		MO
Day 16	09-Sep-23	12:00	Ø	Ø	Ø		38			10		GP
Day 17	10-Sep-23	9:27	Ø	Ø	31		Ø			Ø		MO
Day 18	11-Sep-23	10:21	Ø	37	Ø		35			20		GP
Day 19	12-Sep-23	13:15	Ø	Ø	34		Ø			Ø		GP
Day 20	13-Sep-23	10:31	40	30	Ø		33			16 X		GP
Day 21	14-Sep-23	10:20	Ø	ØX	Ø		Ø			↓		GP
			1.25	5.0	0	10.0	2.5	80.0	48.8	20.0		

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

SET 2: 0 - 80µg/L Cu | Feeding: 700µl YCT + 700 µl Algae per 100ml daily | Dilution media: Reconstituted Soft water

Start Date: August 24, 2023 | one (1) starter (neonate) *daphnia* per test vessel

Replicate l.d. = number of live neonates produced + deaths

Age	Date	Time	5.0	20.0	10.0	40.0	0	1.25	2.5	80.0	Comments	Initials
Day 0	24-Aug-23	13:00	—	—	—	—	—	—	—	—		GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		ALL ALIVE	GP
Day 2	26-Aug-23	14:25	—	—	—	—	—	—	—	—		GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER			
Day 4	28-Aug-23	10:42	—	—	—	—	—	—	—	X		GP
Day 5	29-Aug-23	11:51	—	—	—	—	—	—	—	↓		GP
Day 6	30-Aug-23	10:27	5	6	Ø	Ø X	Ø	Ø	Ø	↓		GP
Day 7	31-Aug-23	10:24	Ø X	Ø	13	↓	10	8	11	↓		GP
Day 8	01-Sep-23	10:49	↓	20	Ø	↓	Ø	Ø	Ø	↓		GP
Day 9	02-Sep-23	13:03	↓	Ø	20	↓	23	23	23	↓		GP
Day 10	03-Sep-23	12:37	↓	27	Ø	↓	Ø	Ø	Ø	↓		ES
Day 11	04-Sep-23	11:00	↓	Ø	23	↓	23	28	23	↓		MO
Day 12	05-Sep-23	10:32	↓	Ø	Ø	↓	Ø	Ø	Ø	↓		GP
Day 13	06-Sep-23	9:07	↓	29	27	↓	31	25	25	↓		ES
Day 14	07-Sep-23	8:23	↓	Ø	Ø	↓	Ø	9	3	↓		MO
Day 15	08-Sep-23	10:36	↓	41	80	↓	20	7	Ø	↓		MO
Day 16	09-Sep-23	12:10	↓	Ø X	Ø	↓	12	24	31	↓		GP
Day 17	10-Sep-23	8:30	↓	↓	Ø	↓	Ø	Ø	Ø	↓		MO
Day 18	11-Sep-23	10:20	↓	↓	37	↓	32	15	36	↓		GP
Day 19	12-Sep-23	13:22	↓	↓	Ø	↓	Ø	Ø	Ø	↓		GP
Day 20	13-Sep-23	10:41	↓	↓	40	↓	39	Ø	30	↓		GP
Day 21	14-Sep-23	10:26	↓	↓	Ø	↓	Ø	15	Ø	↓		GP
			5.0	20.0	10.0	40.0	0	1.25	2.5	80.0		

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

SET 3: 0 - 80µg/L Cu | Feeding: 700µl YCT + 700 µl Algae per 100ml daily | Dilution media: Reconstituted Soft water

Start Date: August 24, 2023 | one (1) starter (neonate) *daphnia* per test vessel

Replicate l.d. = number of live neonates produced + deaths

Age	Date	Time	10.0	40.0	5.0	0	80.0	2.5	1.25	20.0	Comments	Initials
Day 0	24-Aug-23	13:00	—	—	—	—	—	—	—	—		GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		ALL ALIVE	GP
Day 2	26-Aug-23	14:30	—	—	—	—	X	—	—	—		GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER			
Day 4	28-Aug-23	10:46	—	X	—	—		—	—	—		GP
Day 5	29-Aug-23	11:54	—		—	—		—	—	—		GP
Day 6	30-Aug-23	10:37	Ø		11	Ø		8	Ø	Ø		GP
Day 7	31-Aug-23	10:33	13		Ø	10		Ø	17	12		GP
Day 8	01-Sep-23	10:56	Ø		19	Ø		15	ØX	Ø		GP
Day 9	02-Sep-23	13:11	21		Ø	20		10		24		GP
Day 10	03-Sep-23	12:47	Ø		Ø	Ø		Ø		Ø		ES
Day 11	04-Sep-23	11:09	25		25	~~19~~ 20		26		~~X~~ 30		MO
Day 12	05-Sep-23	10:39	Ø		Ø	Ø		Ø				GP
Day 13	06-Sep-23	9:20	Ø		31	37		31				ES
Day 14	07-Sep-23	8:26	23		Ø	Ø		Ø				MO
Day 15	08-Sep-23	10:39	Ø		24	Ø		28				MO
Day 16	09-Sep-23	12:18	29		Ø	24		Ø				GP
Day 17	10-Sep-23	9:33	Ø		34	Ø		Ø				MO
Day 18	11-Sep-23	10:37	36		ØX	31		37				GP
Day 19	12-Sep-23	13:26	Ø			Ø		Ø				GP
Day 20	13-Sep-23	10:48	30			38		48				GP
Day 21	14-Sep-23	10:33	Ø	✓	✓	Ø		Ø	✓	✓		GP
			10.0	40.0	5.0	0	80.0	2.5	1.25	20.0		

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

SET 4: 0 - 80µg/L Cu — Feeding: 700µl YCT + 700 µl Algae per 100ml daily — Dilution media: Reconstituted Soft water

Start Date: August 24, 2023 — one (1) starter (neonate) *daphnia* per test vessel

Replicate I.d. = number of live neonates produced + deaths

Age	Date	Time	2.5	5.0	80.0	20.0	40.0	1.25	10.0	0	Comments	Initials
Day 0	24-Aug-23	13:00	—	—	—	—	—	—	—	—		GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		ALL ALIVE	GP
Day 2	26-Aug-23	14:36	—	—	—	—	—	—	—	—		GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER			
Day 4	28-Aug-23	10:50	—	—	X	—	—	—	—	—		GP
Day 5	29-Aug-23	11:57	—	—	|	—	ØX	—	—	—		GP
Day 6	30-Aug-23	10:46	Ø	Ø	|	Ø	|	Ø	Ø	14		GP
Day 7	31-Aug-23	10:40	8	13	|	13	|	9	12	Ø		GP
Day 8	01-Sep-23	11:04	Ø	Ø	|	Ø	|	Ø	Ø	18		GP
Day 9	02-Sep-23	13:18	25	22	|	22	|	19	25	Ø		GP
Day 10	03-Sep-23	12:52	Ø	Ø	|	Ø	|	Ø	Ø	Ø	1.25 - Air bubble	ES
Day 11	04-Sep-23	11:05	28	26	|	22	|	19	25	19		MO
Day 12	05-Sep-23	10:43	Ø	Ø	|	Ø	|	Ø	Ø	Ø		GP
Day 13	06-Sep-23	10:03	32	30	|	26	|	32	34	30		ES
Day 14	07-Sep-23	8:29	Ø	Ø	|	Ø	|	Ø	Ø	Ø		MO
Day 15	08-Sep-23	10:41	23	22	|	X	|	Ø	Ø	25		MO
Day 16	09-Sep-23	~~10~~ 12:23	12	Ø	|	|	|	28	39	Ø		GP
Day 17	10-Sep-23	9:36	Ø	Ø	|	|	|	Ø	Ø	Ø		MO
Day 18	11-Sep-23	10:46	35	38	|	|	|	37	35	27		GP
Day 19	12-Sep-23	13:29	Ø	Ø	|	|	|	Ø	Ø	Ø		GP
Day 20	13-Sep-23	10:54	36	42	✓	|	✓	33	33	36		GP
Day 21	14-Sep-23	10:38	ØX	Ø	|	↓	|	Ø	Ø	Ø		GP
			2.5	5.0	80.0	20.0	40.0	1.25	10.0	0		

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

SET 5: 0 - 80µg/L Cu — Feeding: 700µl YCT + 700 µl Algae per 100ml daily — Dilution media: Reconstituted Soft water

Start Date: August 24, 2023 — one (1) starter (neonate) *daphnia* per test vessel

Replicate l.d. = number of live neonates produced + deaths

Age	Date	Time	40.0	0	10.0	2.5	1.25	20.0	80.0	5.0	Comments	Initials
Day 0	24-Aug-23	13:00	—	—	—	—	—	—	—	—		GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		ALL ALIVE	GP
Day 2	26-Aug-23	14:42	—	—	—	—	—	—	—	—		GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER			
Day 4	28-Aug-23	10:55	X	—	—	—	—	—	X	—		GP
Day 5	29-Aug-23	12:00		—	—	—	—	—		—		GP
Day 6	30-Aug-23	10:53		8	10	Ø	Ø	16		Ø		GP
Day 7	31-Aug-23	10:49		Ø	Ø	15	10	Ø		12		GP
Day 8	01-Sep-23	11:10		25	19	Ø	Ø	22		Ø		GP
Day 9	02-Sep-23	13:27		Ø	Ø	17	23	Ø		24		GP
Day 10	03-Sep-23	12:59		28	67	Ø	Ø	31		Ø		ES
Day 11	04-Sep-23	11:08		Ø	17	21	22	Ø		24		MO
Day 12	05-Sep-23	10:49		~~Ø~~	Ø	Ø	Ø	25		Ø		GP
Day 13	06-Sep-23	10:19		32	28	30	27	Ø		35		ES
Day 14	07-Sep-23	8:39		Ø	Ø	Ø	Ø	Ø		Ø		MO
Day 15	08-Sep-23	10:45		19	~~Ø~~22	5	Ø	~~33~~		Ø		MO
Day 16	09-Sep-23	12:31		Ø	ØX	25	23			39		GP
Day 17	10-Sep-23	9:38		27		Ø	Ø			Ø		MO
Day 18	11-Sep-23	10:57		Ø		36	35			37		GP
Day 19	12-Sep-23	13:32		41		Ø	Ø			Ø		GP
Day 20	13-Sep-23	11:02		Ø		36	35			34		GP
Day 21	14-Sep-23	10:43		Ø		Ø	Ø			Ø		GP
			40.0	0	10.0	2.5	1.25	20.0	80.0	5.0		

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

SET 6: 0 - 80µg/L Cu			Feeding: 700µl YCT + 700 µl Algae per 100ml daily							Dilution media: Reconstituted Soft water	
Start Date: August 24, 2023			one (1) starter (neonate) *daphnia* per test vessel								
			Replicate i.d. = number of live neonates produced + deaths								
Age	Date	Time	20.0	0	40.0	80.0	10.0	1.25	5.0	2.5	Comments	Initials
Day 0	24-Aug-23	13:00	—	—	—	—	—	—	—	—		GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		ALL ALIVE	GP
Day 2	26-Aug-23	14:52	—	—	—	—	—	—	—	—		GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER			
Day 4	28-Aug-23	11:00	—	—	—	X	—	—	—	—		GP
Day 5	29-Aug-23	12:12	—	—	ØX		—	—	—	—		GP
Day 6	30-Aug-23	11:11	Ø	Ø			Ø	Ø	9	Ø		GP
Day 7	31-Aug-23	11:20	11	16			12	13	Ø	10		GP
Day 8	01-Sep-23	11:40	Ø	Ø			Ø	Ø	19	Ø		GP
Day 9	02-Sep-23	13:43	27	21			19	23	Ø	18		GP
Day 10	03-Sep-23	13:14	Ø	Ø			Ø	Ø	Ø	Ø		ES
Day 11	04-Sep-23	11:11	24	23			~~19~~ 21	25	24	25		MO
Day 12	05-Sep-23	11:04	Ø	Ø			Ø	Ø	Ø	Ø		GP
Day 13	06-Sep-23	10:35	31	35			Ø	10	30	15	2.5- something attached to body	ES
Day 14	07-Sep-23	8:35	Ø	Ø			28	Ø	Ø	X 13		MO
Day 15	08-Sep-23	10:50	Ø	~~28~~			Ø	Ø	25			MO
Day 16	09-Sep-23	12:38	10	16			35	28	Ø			GP
Day 17	10-Sep-23	9:44	Ø	Ø			Ø	Ø	Ø			MO
Day 18	11-Sep-23	11:06	13	38			31	18 X*	32		*1.25+7 dead neonates	GP
Day 19	12-Sep-23	13:39	Ø	Ø			Ø		Ø			GP
Day 20	13-Sep-23	11:08	Ø	36			29		31			GP
Day 21	14-Sep-23	10:48	12	Ø			Ø		Ø			GP
			20.0	0	40.0	80.0	10.0	1.25	5.0	2.5		

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

SET 7: 0 - 80µg/L Cu — Feeding: 700µl YCT + 700 µl Algae per 100ml daily — Dilution media: Reconstituted Soft water

Start Date: August 24, 2023 — one (1) starter (neonate) *daphnia* per test vessel

Replicate I.d. = number of live neonates produced + deaths

Age	Date	Time	0	80.0	20.0	10.0	2.5	5.0	1.25	40.0	Comments	Initials
Day 0	24-Aug-23	13:00	—	—	—	—	—	—	—	—		GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		ALL ALIVE	GP
Day 2	26-Aug-23	14:57	—	—	—	—	—	—	—	—		GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER			
Day 4	28-Aug-23	11:10	—	X	—	—	—	—	—	X		GP
Day 5	29-Aug-23	12:16	—		—	—	—	—	—			GP
Day 6	30-Aug-23	11:20	Ø		Ø	Ø	10	Ø	12			GP
Day 7	31-Aug-23	11:28	13		12	12	Ø	14	Ø			GP
Day 8	01-Sep-23	11:48	Ø		Ø	Ø	24	Ø	Ø			GP
Day 9	02-Sep-23	13:52	25		18	22	Ø	23	26			GP
Day 10	03-Sep-23	13:23	Ø		Ø	Ø	Ø	Ø	Ø			ES
Day 11	04-Sep-23	11:14	29		34	26	22	22	27			MO
Day 12	05-Sep-23	11:10	Ø		Ø	Ø	Ø	Ø	Ø			GP
Day 13	06-Sep-23	10:49	30		33	Ø	X 33	Ø	34			ES
Day 14	07-Sep-23	8:38	Ø		Ø	29		28	Ø			MO
Day 15	08-Sep-23	10:53	Ø		Ø	Ø		Ø	Ø			MO
Day 16	09-Sep-23	12:45	33		28 X	29		29	24			~~GP~~
Day 17	10-Sep-23	9:44	Ø			Ø		Ø	Ø			MO
Day 18	11-Sep-23	11:17	33			Ø		27	34			GP
Day 19	12-Sep-23	13:43	Ø			22		Ø	Ø			GP
Day 20	13-Sep-23	11:15	37			Ø		Ø	38			GP
Day 21	14-Sep-23	10:53	Ø			17		37	Ø			GP
			0	80.0	20.0	10.0	2.5	5.0	1.25	40.0		

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

SET 8: 0 - 80µg/L Cu | Feeding: 700µl YCT + 700 µl Algae per 100ml daily | Dilution media: Reconstituted Soft water

Start Date: August 24, 2023 | one (1) starter (neonate) *daphnia* per test vessel

Replicate I.d. = number of live neonates produced + deaths

Age	Date	Time	5.0	10.0	2.5	0	20.0	40.0	1.25	80.0	Comments	Initials
Day 0	24-Aug-23	13:00	—	—	—	—	—	—	—	—		GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		ALL ALIVE	GP
Day 2	26-Aug-23	15:03	—	—	—	—	—	X	—	—		GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER			
Day 4	28-Aug-23	11:20	—	—	—	—	—		—	X		GP
Day 5	29-Aug-23	12:19	—	—	—	—	—		—			GP
Day 6	30-Aug-23	11:28	Ø	Ø	Ø	Ø	11		Ø			GP
Day 7	31-Aug-23	11:37	11	12	15	13	Ø		7			GP
Day 8	01-Sep-23	11:55	Ø	Ø	Ø	Ø	Ø		Ø			GP
Day 9	02-Sep-23	14:02	22	24	20	24	26		18			GP
Day 10	03-Sep-23	13:30	Ø	Ø	Ø	Ø	Ø		Ø			ES
Day 11	04-Sep-23	11:17	Ø	24	28	23	30		Ø			MO
Day 12	05-Sep-23	11:15	28	Ø	7	Ø	Ø		28			GP
Day 13	06-Sep-23	11:00	X Ø	Ø	Ø	33	30		Ø			ES
Day 14	07-Sep-23	8:41		32	36	Ø	Ø		34			MO
Day 15	08-Sep-23	10:56		Ø	Ø	Ø	Ø		Ø			MO
Day 16	09-Sep-23	13:03		35	36	41	27		26*		*1.25 stuck to carapace.	GP
Day 17	10-Sep-23	9:46		Ø	Ø	Ø	Ø		X			MO
Day 18	11-Sep-23	11:34		36	3	45	17					GP
Day 19	12-Sep-23	13:48		Ø	31	Ø	Ø					GP
Day 20	13-Sep-23	11:22		28	Ø	36	Ø					GP
Day 21	14-Sep-23	10:59		9	39	Ø	4					GP
			5.0	10.0	2.5	0	20.0	40.0	1.25	80.0		

Test: 21 day Daphnia pulex test in RSW with Cu ($CuSO_4*5H_2O$)

SET 9: 0 - 80µg/L Cu — Feeding: 700µl YCT + 700 µl Algae per 100ml daily — Dilution media: Reconstituted Soft water

Start Date: August 24, 2023 — one (1) starter (neonate) *daphnia* per test vessel

Replicate I.d. = number of live neonates produced + deaths

Age	Date	Time	1.25	2.5	40.0	5.0	0	20.0	80.0	10.0	Comments	Initials
Day 0	24-Aug-23	13:00	—	—	—	—	—	—	—	—		GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		ALL ALIVE	GP
Day 2	26-Aug-23	15:10	—	—	—	—	—	—	X	—		GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER			
Day 4	28-Aug-23	11:25	—	—	X	—	—	—		—		GP
Day 5	29-Aug-23	12:22	—	—		—	—	—		—		GP
Day 6	30-Aug-23	11:36	Ø	Ø		Ø	Ø	Ø		Ø		GP
Day 7	31-Aug-23	11:46	8	10		11	14	14		11		GP
Day 8	01-Sep-23	12:02	Ø	Ø		Ø	Ø	Ø		Ø		GP
Day 9	02-Sep-23	14:11	15	27		25	23	25		23		GP
Day 10	03-Sep-23	13:36	Ø	Ø		Ø	Ø	Ø		Ø		ES
Day 11	04-Sep-23	11:20	Ø	~~27~~ 30		24	Ø	27		27		MO
Day 12	05-Sep-23	11:25	28	Ø		Ø	29	Ø X		Ø		GP
Day 13	06-Sep-23	11:08	Ø	Ø		21	Ø			Ø		ES
Day 14	07-Sep-23	8:44	~~28~~	28		8	32			28		MO
Day 15	08-Sep-23	10:59	~~Ø~~	Ø		Ø	1			Ø		MO
Day 16	09-Sep-23	13:14	29	33		35	34			29		GP
Day 17	10-Sep-23	9:48	Ø	Ø		Ø	Ø			Ø		MO
Day 18	11-Sep-23	11:42	14	Ø		Ø	Ø			37		GP
Day 19	12-Sep-23	14:00	22	37		8	46			Ø		GP
Day 20	13-Sep-23	11:31	Ø	Ø		Ø	Ø			Ø		GP
Day 21	14-Sep-23	11:08	~~39~~	33	✓	19	41	✓	✓	37		GP
			1.25	2.5	40.0	5.0	0	20.0	80.0	10.0		

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

SET 10: 0 - 80µg/L Cu — Feeding: 700µl YCT + 700 µl Algae per 100ml daily — Dilution media: Reconstituted Soft water

Start Date: August 24, 2023 — one (1) starter (neonate) *daphnia* per test vessel

Replicate I.d. = number of live neonates produced + deaths

Age	Date	Time	80.0	1.25	5.0	2.5	20.0	0	10.0	40.0	Comments	Initials
Day 0	24-Aug-23	13:00	—	—	—	—	—	—	—	—		GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		ALL ALIVE	GP
Day 2	26-Aug-23	15:16	X	–	–	–	–	–	–	–		GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER			
Day 4	28-Aug-23	11:29		–	–	–	–	–	–	–		GP
Day 5	29-Aug-23	12:26		–	–	–	–	–	–	–		GP
Day 6	30-Aug-23	11:44		Ø	Ø	Ø	Ø	Ø	Ø	Ø X		GP
Day 7	31-Aug-23	11:54		5	12	10	11	8	10			GP
Day 8	01-Sep-23	12:08		Ø	Ø	Ø	Ø	Ø	Ø			GP
Day 9	02-Sep-23	14:21		16	18	20	25	21	24			GP
Day 10	03-Sep-23	13:45		Ø	Ø	Ø	Ø	Ø	Ø			ES
Day 11	04-Sep-23	11:23		Ø	Ø	Ø	Ø	Ø	Ø			MO
Day 12	05-Sep-23	11:36		29	28	26	30	26	27			GP
Day 13	06-Sep-23	11:13		Ø	Ø	Ø	Ø	Ø	Ø			ES
Day 14	07-Sep-23	8:47		31	29	27	33	28	31			MO
Day 15	08-Sep-23	11:04		Ø	Ø	2	Ø	Ø	Ø			MO
Day 16	09-Sep-23	13:22		31	27	31	24	28	28			GP
Day 17	10-Sep-23	9:50		Ø	Ø	Ø	Ø	Ø	Ø			MO
Day 18	11-Sep-23	11:50		Ø	Ø	Ø	Ø	Ø	Ø			GP
Day 19	12-Sep-23	14:08		41	32	26	11	35	34			GP
Day 20	13-Sep-23	11:37		Ø	Ø	Ø	Ø	Ø	Ø			GP
Day 21	14-Sep-23	11:14	↓	31	37	24	Ø	31	Ø	↓		GP
			80.0	1.25	5.0	2.5	20.0	0	10.0	40.0		

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

0 µg/L Cu

Start Date: August 24, 2023

Age	Date	Time	pH after	pH before	Temp °C after	Temp °C before	D.O. % after	D.O. % before	Conductivity uohms/cm after	Conductivity uohms/cm before	Initials
Day 0	24-Aug-23	13:00	x	7.26	x	21.5	x	105.5	x	163.1	GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		
Day 2	26-Aug-23		7.55	7.30	21.3	21.4	98.0	108.4	184.3	162.1	GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER		
Day 4	28-Aug-23		7.46	7.27	21.5	21.4	97.1	106.0	172.2	162.9	GP
Day 5	29-Aug-23		7.56	7.37	21.3	21.2	94.9	107.1	171.9	163.7	GP
Day 6	30-Aug-23		7.42	7.33	21.0	21.1	96.5	104.0	171.6	167.2	GP
Day 7	31-Aug-23		7.50	7.34	20.8	21.8	96.9	103.9	172.9	165.9	GP
Day 8	01-Sep-23		7.23	7.52	21.0	21.3	96.4	104.5	172.1	163.9	GP
Day 9	02-Sep-23		7.32	7.27	21.0	21.4	95.1	108.6	169.2	162.6	GP
Day 10	03-Sep-23		7.39	7.36	21.7	20.7	95.7	108.2	166.5	167.7	ES
Day 11	04-Sep-23		7.18	7.46	21.0	21.5	95.0	110.6	163.7	165.4	MO
Day 12	05-Sep-23		7.44	7.38	21.4	21.4	97.0	107.8	167.4	164.5	GP
Day 13	06-Sep-23		7.33	7.15	20.5	21.3	93.4	105.2	167.8	197.7	ES
Day 14	07-Sep-23		7.02	7.32	21.6	21.7	90.9	112.0	167.1	166.9	MO
Day 15	08-Sep-23		7.18	7.41	21.6	21.5	93.1	106.0	172.0	163.1	MO
Day 16	09-Sep-23		7.46	7.26	21.2	20.3	97.6	110.1	171.5	161.7	GP
Day 17	10-Sep-23		7.07	7.32	21.2	21.0	89.0	111.4	174.6	162.6	MO
Day 18	11-Sep-23		7.34	7.28	21.4	21.5	92.6	110.3	171.3	170.5	GP
Day 19	12-Sep-23		7.34	7.51	21.3	21.9	93.6	104.8	173.9	165.2	GP
Day 20	13-Sep-23		7.32	7.37	21.2	20.6	94.6	111.6	173.0	163.3	GP
Day 21	14-Sep-23		7.29	x	20.8	x	94.4	x	172.7	x	GP

1.25 µg/L Cu

Start Date: August 24, 2023

Age	Date	Time	pH after	pH before	Temp °C after	Temp °C before	D.O. % after	D.O. % before	Conductivity uohms/cm after	Conductivity uohms/cm before	Initials
Day 0	24-Aug-23	13:00	x	7.44	x	21.4	x	105.4	x	163.9	GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		
Day 2	26-Aug-23		7.58	7.35	21.4	21.7	97.9	109.2	173.5	164.6	GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER		
Day 4	28-Aug-23		7.64	7.37	21.4	21.5	98.8	105.8	176.2	163.4	GP
Day 5	29-Aug-23		7.41	7.39	21.5	21.6	96.0	107.8	175.0	164.2	GP
Day 6	30-Aug-23		7.39	7.36	20.7	20.8	95.2	105.0	173.5	163.9	GP
Day 7	31-Aug-23		7.52	7.41	20.7	21.6	96.9	106.1	175.0	163.7	GP
Day 8	01-Sep-23		7.38	7.42	20.8	21.6	96.9	103.9	173.6	164.7	GP
Day 9	02-Sep-23		7.39	7.33	21.3	21.3	96.5	109.2	170.3	162.6	GP
Day 10	03-Sep-23		7.32	7.37	21.4	21.6	95.0	107.7	168.1	164.4	ES
Day 11	04-Sep-23		7.32	7.44	20.9	21.5	95.3	110.0	169.6	165.4	MO
Day 12	05-Sep-23		7.45	7.35	21.2	21.4	95.7	107.5	168.7	165.2	GP
Day 13	06-Sep-23		7.36	7.38	20.8	21.3	95.5	103.5	168.5	165.7	ES
Day 14	07-Sep-23		7.22	7.32	21.3	21.7	93.3	109.2	172.2	161.7	MO
Day 15	08-Sep-23		7.21	7.43	21.3	21.4	91.2	106.4	169.6	162.3	MO
Day 16	09-Sep-23		7.42	7.32	21.2	20.4	97.1	110.5	175.9	165.1	GP
Day 17	10-Sep-23		7.21	7.34	21.2	21.1	88.2	110.7	172.6	161.7	MO
Day 18	11-Sep-23		7.31	7.35	21.3	21.0	91.6	110.8	174.5	162.9	GP
Day 19	12-Sep-23		7.37	7.52	21.0	21.3	95.7	104.7	176.2	165.8	GP
Day 20	13-Sep-23		7.30	7.38	21.2	20.2	92.5	109.5	174.1	164.0	GP
Day 21	14-Sep-23		7.32	x	20.8	x	93.9	x	175.9	x	GP

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

2.5 µg/L Cu

Start Date: August 24, 2023

Age	Date	Time	pH after	pH before	Temp °C after	Temp °C before	D.O. % after	D.O. % before	Conductivity uohms/cm after	Conductivity uohms/cm before	Initials
Day 0	24-Aug-23	13:00	x	7.48	x	21.5	x	103.9	x	165.0	GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		
Day 2	26-Aug-23		7.58	7.27	21.4	21.0	98.7	110.9	172.5	162.6	GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER		
Day 4	28-Aug-23		7.51	7.41	21.4	21.4	96.9	106.2	172.3	163.9	GP
Day 5	29-Aug-23		7.42	7.36	21.4	21.5	95.6	108.6	175.7	163.9	GP
Day 6	30-Aug-23		7.39	7.38	20.7	21.0	95.2	106.2	173.8	163.8	GP
Day 7	31-Aug-23		7.49	7.46	20.8	21.8	97.1	107.0	174.7	165.3	GP
Day 8	01-Sep-23		7.33	7.44	21.1	21.8	95.8	105.2	175.5	164.6	GP
Day 9	02-Sep-23		7.38	7.37	21.4	21.3	97.1	109.3	171.9	163.8	GP
Day 10	03-Sep-23		7.40	7.45	21.3	21.2	93.2	107.4	167.6	164.8	ES
Day 11	04-Sep-23		7.36	7.33	21.0	21.4	86.3	108	167.3	163.6	MO
Day 12	05-Sep-23		7.39	7.37	21.2	21.5	96.7	107.9	169.5	165.7	GP
Day 13	06-Sep-23		7.29	7.35	21.1	21.6	94.3	103.5	169.2	166.2	ES
Day 14	07-Sep-23		7.27	7.27	21.6	21.6	99.8	110.4	169.9	166.1	MO
Day 15	08-Sep-23		7.30	7.36	20.7	21.3	93.3	107.4	167.2	161.0	MO
Day 16	09-Sep-23		7.33	7.29	21.2	20.7	95.1	109.7	171.9	163.0	GP
Day 17	10-Sep-23		7.19	7.25	21.0	21.2	91.3	111.2	171.3	168.1	MO
Day 18	11-Sep-23		7.32	7.33	21.1	21.3	93.3	110.6	172.8	164.2	GP
Day 19	12-Sep-23		7.32	7.49	21.1	21.5	94.1	103.4	172.0	166.2	GP
Day 20	13-Sep-23		7.29	7.35	21.3	21.2	92.3	110.9	174.4	167.0	GP
Day 21	14-Sep-23		7.37	x	20.7	x	96.2	x	174.0	x	GP

5.0 µg/L Cu

Start Date: August 24, 2023

Age	Date	Time	pH after	pH before	Temp °C after	Temp °C before	D.O. % after	D.O. % before	Conductivity uohms/cm after	Conductivity uohms/cm before	Initials
Day 0	24-Aug-23	13:00	x	7.50	x	21.5	x	106.6	x	164.4	GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		
Day 2	26-Aug-23		7.58	7.44	21.5	21.7	98.5	113.9	172.4	164.1	GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER		
Day 4	28-Aug-23		7.52	7.40	21.4	21.0	96.6	107.0	172.5	163.9	GP
Day 5	29-Aug-23		7.34	7.41	21.4	21.1	94.8	107.9	173.8	163.3	GP
Day 6	30-Aug-23		7.33	7.39	20.7	21.1	94.1	105.0	174.2	164.1	GP
Day 7	31-Aug-23		7.46	7.47	20.8	21.9	97.0	107.4	176.6	165.1	GP
Day 8	01-Sep-23		7.37	7.47	21.0	21.7	96.8	104.0	174.5	166.3	GP
Day 9	02-Sep-23		7.40	7.39	21.4	21.0	97.8	100.3	171.6	164.0	GP
Day 10	03-Sep-23		7.42	7.44	21.3	21.3	96.7	108.8	169.0	165.4	ES
Day 11	04-Sep-23		7.35	7.40	21.0	21.6	96.0	111.2	168.2	163.8	MO
Day 12	05-Sep-23		7.33	7.37	21.3	21.5	96.6	107.2	168.8	165.9	GP
Day 13	06-Sep-23		7.27	7.33	21.4	21.7	95.2	103.6	170.6	166.1	ES
Day 14	07-Sep-23		7.24	7.37	21.6	21.8	92.8	111.4	169.7	165.2	MO
Day 15	08-Sep-23		7.16	7.30	20.8	21.4	85.3	107.5	169.4	166.4	MO
Day 16	09-Sep-23		7.36	7.34	21.2	20.2	96.4	110.0	172.7	164.5	GP
Day 17	10-Sep-23		7.18	7.32	21.0	20.9	86.8	109.6	172.3	168.0	MO
Day 18	11-Sep-23		7.34	7.38	21.4	21.6	94.5	111.2	173.7	165.7	GP
Day 19	12-Sep-23		7.32	7.48	21.2	21.5	93.2	105.2	174.4	166.8	GP
Day 20	13-Sep-23		7.24	7.38	21.4	20.8	92.1	111.2	174.2	166.0	GP
Day 21	14-Sep-23		7.28	x	20.7	x	92.4	x	174.8	x	GP

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

10.0 µg/L Cu

Start Date: August 24, 2023

Age	Date	Time	pH after	pH before	Temp °C after	Temp °C before	D.O. % after	D.O. % before	Conductivity uohms/cm after	Conductivity uohms/cm before	Initials
Day 0	24-Aug-23	15:00	x	7.47	x	21.6	x	105.9	x	165.1	GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		
Day 2	26-Aug-23		7.63	7.49	21.2	21.7	98.1	112.8	174.5	163.9	GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER		
Day 4	28-Aug-23		7.48	7.43	21.4	21.3	96.6	104.5	172.2	164.0	GP
Day 5	29-Aug-23		7.31	7.43	21.4	21.2	93.3	108.8	172.6	163.8	GP
Day 6	30-Aug-23		7.44	7.40	20.6	21.1	95.5	107.3	173.8	164.6	GP
Day 7	31-Aug-23		7.40	7.49	20.8	21.7	97.2	106.5	172.7	165.8	GP
Day 8	01-Sep-23		7.35	7.46	21.0	21.8	95.0	103.8	174.4	166.1	GP
Day 9	02-Sep-23		7.40	7.36	21.0	21.3	96.0	108.6	173.8	164.8	GP
Day 10	03-Sep-23		7.38	7.45	21.4	21.1	96.8	107.3	168.8	165.8	ES
Day 11	04-Sep-23		7.36	7.35	21.0	21.3	96.7	110.5	170.0	168.5	MO
Day 12	05-Sep-23		7.37	7.38	21.2	21.6	97.1	107.3	168.7	165.8	GP
Day 13	06-Sep-23		7.33	7.31	21.2	21.7	96.7	104.8	169.5	167.5	ES
Day 14	07-Sep-23		7.18	7.27	21.6	21.8	91.5	112.2	169.4	163.1	MO
Day 15	08-Sep-23		7.29	7.35	20.9	21.4	93.3	105.7	170.6	163.0	MO
Day 16	09-Sep-23		7.38	7.33	21.2	20.5	95.0	111.9	172.3	164.5	GP
Day 17	10-Sep-23		7.25	7.30	21.1	20.7	89.9	111.6	173.3	162.7	MO
Day 18	11-Sep-23		7.29	7.39	21.3	21.7	94.8	110.0	172.6	165.9	GP
Day 19	12-Sep-23		7.40	7.47	21.2	21.5	96.6	106.2	175.3	166.6	GP
Day 20	13-Sep-23		7.35	7.42	21.4	21.5	96.1	109.7	174.5	166.1	GP
Day 21	14-Sep-23		7.36	x	20.5	x	94.3	x	174.4	x	

20.0 µg/L Cu

Start Date: August 24, 2023

Age	Date	Time	pH after	pH before	Temp °C after	Temp °C before	D.O. % after	D.O. % before	Conductivity uohms/cm after	Conductivity uohms/cm before	Initials
Day 0	24-Aug-23	13:00	x	7.48	x	21.6	x	103.5	x	164.7	GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		
Day 2	26-Aug-23		7.62	7.52	21.1	21.3	95.4	112.7	171.2	163.6	GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER		
Day 4	28-Aug-23		7.47	7.42	21.5	21.5	97.5	104.9	172.6	165.1	GP
Day 5	29-Aug-23		7.44	7.43	21.2	21.6	95.9	107.3	175.0	165.0	GP
Day 6	30-Aug-23		7.41	7.42	20.8	21.3	95.2	106.4	174.5	165.5	GP
Day 7	31-Aug-23		7.48	7.42	20.8	21.9	97.3	107.9	175.5	166.5	GP
Day 8	01-Sep-23		7.31	7.41	21.1	21.5	95.6	107.1	175.6	165.3	GP
Day 9	02-Sep-23		7.38	7.35	21.2	21.5	96.3	~~113~~	170.1	165.5	GP
Day 10	03-Sep-23		7.41	7.39	21.3	21.2	96.8	104.8	168.5	165.7	ES
Day 11	04-Sep-23		7.29	7.41	21.1	21.3	97.2	109.5	168.3	163.1	MO
Day 12	05-Sep-23		7.30	7.35	21.5	21.6	97.1	107.4	169.6	165.1	GP
Day 13	06-Sep-23		7.36	7.33	21.6	21.8	96.9	104.4	168.9	166.0	ES
Day 14	07-Sep-23		7.23	7.30	21.5	21.5	93.2	113.9	168.3	164.7	MO
Day 15	08-Sep-23		7.27	7.35	20.9	21.3	91.7	107.3	169.7	162.6	MO
Day 16	09-Sep-23		7.45	7.29	21.0	20.4	97.4	110.0	174.8	165.3	GP
Day 17	10-Sep-23		7.21	7.34	21.0	21.0	90.2	110.3	172.6	162.1	MO
Day 18	11-Sep-23		7.47	7.34	20.9	21.5	96.6	110.9	175.4	167.2	GP
Day 19	12-Sep-23		7.40	7.51	21.0	21.6	93.8	102.6	175.2	167.5	GP
Day 20	13-Sep-23		7.36	7.42	21.4	21.4	93.9	106.5	176.0	168.1	GP
Day 21	14-Sep-23		7.37	x	20.2	x	94.3	x	175.6	x	GP

111.3

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

40.0 µg/L Cu

Start Date: August 24, 2023

Age	Date	Time	pH after	pH before	Temp °C after	Temp °C before	D.O. % after	D.O. % before	Conductivity uohms/cm after	Conductivity uohms/cm before	Initials
Day 0	24-Aug-23	13:00	x	7.47	x	21.5	x	105.1	x	164.3	GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		
Day 2	26-Aug-23		7.60	7.51	21.0	21.9	95.3	112.8	171.5	164.8	GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER		
Day 4	28-Aug-23		7.49	7.51	21.5	21.3	97.1	106.1	171.6	165.7	GP
Day 5	29-Aug-23		7.35	7.35	21.2	21.3	95.9	108.4	181.2	165.0	GP
Day 6	30-Aug-23		7.47	7.46	21.1	21.8	96.4	97.0	181.0	169.7	GP
Day 7	31-Aug-23										
Day 8	01-Sep-23										
Day 9	02-Sep-23										
Day 10	03-Sep-23										
Day 11	04-Sep-23										
Day 12	05-Sep-23										
Day 13	06-Sep-23										
Day 14	07-Sep-23										
Day 15	08-Sep-23										
Day 16	09-Sep-23										
Day 17	10-Sep-23										
Day 18	11-Sep-23										
Day 19	12-Sep-23										
Day 20	13-Sep-23										
Day 21	14-Sep-23			x		x		x		x	

80.0 µg/L Cu

Start Date: August 24, 2023

Age	Date	Time	pH after	pH before	Temp °C after	Temp °C before	D.O. % after	D.O. % before	Conductivity uohms/cm after	Conductivity uohms/cm before	Initials
Day 0	24-Aug-23	13:00	x	7.46	x	21.6	x	102.3	x	164.9	GP
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		
Day 2	26-Aug-23		7.57	7.53	21.0	22.0	96.3	110.7	172.1	164.9	GP
Day 3	27-Aug-23		N	O		TR	AN	SF	ER		
Day 4	28-Aug-23		7.51	✓	21.6	✓	97.0	✓	171.6	✓	BP
Day 5	29-Aug-23										
Day 6	30-Aug-23										
Day 7	31-Aug-23										
Day 8	01-Sep-23										
Day 9	02-Sep-23										
Day 10	03-Sep-23										
Day 11	04-Sep-23										
Day 12	05-Sep-23										
Day 13	06-Sep-23										
Day 14	07-Sep-23										
Day 15	08-Sep-23										
Day 16	09-Sep-23										
Day 17	10-Sep-23										
Day 18	11-Sep-23										
Day 19	12-Sep-23										
Day 20	13-Sep-23										
Day 21	14-Sep-23			x		x		x		x	

Test: 21 day Daphnia pulex test in RSW with Cu (CuSO4*5H2O)

10.0 µg/L Cu

Start Date: August 24, 2023

Age	Date	Time	pH after	pH before	Temp °C after	Temp °C before	D.O. % after	D.O. % before	Conductivity uohms/cm after	Conductivity uohms/cm before	Initials
Day 0	24-Aug-23		x		x		x		x		
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		
Day 2	26-Aug-23										
Day 3	27-Aug-23		N	O		TR	AN	SF	ER		
Day 4	28-Aug-23										
Day 5	29-Aug-23										
Day 6	30-Aug-23										
Day 7	31-Aug-23										
Day 8	01-Sep-23										
Day 9	02-Sep-23										
Day 10	03-Sep-23										
Day 11	04-Sep-23										
Day 12	05-Sep-23										
Day 13	06-Sep-23										
Day 14	07-Sep-23										
Day 15	08-Sep-23										
Day 16	09-Sep-23										
Day 17	10-Sep-23										
Day 18	11-Sep-23										
Day 19	12-Sep-23										
Day 20	13-Sep-23										
Day 21	14-Sep-23			x		x		x		x	

20.0 µg/L Cu

Start Date: August 24, 2023

Age	Date	Time	pH after	pH before	Temp °C after	Temp °C before	D.O. % after	D.O. % before	Conductivity uohms/cm after	Conductivity uohms/cm before	Initials
Day 0	24-Aug-23		x		x		x		x		
Day 1	25-Aug-23		N	O		TR	AN	SF	ER		
Day 2	26-Aug-23										
Day 3	27-Aug-23		N	O		TR	AN	SF	ER		
Day 4	28-Aug-23										
Day 5	29-Aug-23										
Day 6	30-Aug-23										
Day 7	31-Aug-23										
Day 8	01-Sep-23										
Day 9	02-Sep-23										
Day 10	03-Sep-23										
Day 11	04-Sep-23										
Day 12	05-Sep-23										
Day 13	06-Sep-23										
Day 14	07-Sep-23										
Day 15	08-Sep-23										
Day 16	09-Sep-23										
Day 17	10-Sep-23										
Day 18	11-Sep-23										
Day 19	12-Sep-23										
Day 20	13-Sep-23										
Day 21	14-Sep-23			x		x		x		x	

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