The Mechanism of Waterborne Lead Uptake and Toxicity in *Daphnia magna*

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By

SAYANTY ROY

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ABSTRACT

Lead is an omnipresent pollutant, and its contamination in natural waters is an issue of current regulatory concern throughout the world including Canada. The free divalent ion of lead (Pb^{2+}) is considered to be the most bioavailable and toxic form of lead. Pb^{2+} is known to be a calcium antagonist in vertebrates including fish. It is believed that lead causes toxicity to freshwater fish primarily by disrupting ionic homeostasis both during acute and chronic waterborne exposure. Lead can also potentially act as a respiratory toxicant since it is known to impair hemoglobin synthesis in both vertebrates. To date, the mechanistic underpinnings of lead accumulation and toxicity in aquatic invertebrates are not well understood, particularly during acute exposure. Therefore, the main objectives of the present study were in two folds: (i) to investigate the mechanisms of waterborne lead uptake, and (ii) to understand the physiological basis of lead toxicity during acute exposure. I used freshwater crustacean, *Daphnia magna*, as a model freshwater invertebrate species for my study. *Daphnia* are known to be quite sensitive to metals and widely used as a model species for toxicity assessments. The results of my study suggest that lead inhibits waterborne Ca^{2+} uptake in *Daphnia* in a concentration dependent manner, and this inhibition occurs predominantly through a direct competitive interaction. The entry of waterborne Pb^{2+} in *Daphnia* likely occurs *via* both lanthanum-sensitive and verapamil-sensitive epithelial calcium channels. Moreover, my results also indicate that acute waterborne lead exposure severely disrupts both Ca^{2+} and Na^{+} uptake from water, which are concomitant with the increase in the lead body burden in *Daphnia*. Interestingly however, acute exposure to lead does not affect the rate of oxygen

consumption in *Daphnia*, indicating no acute respiratory toxicity of lead. Overall, it appears that lead acts as an ionoregulatory toxicant to *Daphnia* during acute waterborne exposure.

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CHAPTER 1
INTRODUCTION

1.1. Lead contamination in natural waters

Lead is neither beneficial nor essential to living organisms. It is a group IV-A element in the periodic table and is considered a heavy metal (atomic weight: 207). It is a naturally occurring metal present in the earth's crust, rock, soil, and water. Lead enters aquatic environments by various different pathways. The Geological weathering and volcanic activity account for natural pathways, but most of the lead in natural waters derives from anthropogenic activities such as mining and smelting, burning of fossil fuels such as coal and gasoline, and industrial processes such as manufacturing of batteries, paints and cement (World Health Organization, 1995). Contamination of the aquatic systems by these processes is the primary cause of lead poisoning in aquatic organisms (Sorensen, 1991).

Lead is a ubiquitous and a common contaminant in natural waters throughout the world. This is because aquatic environments are the major recipients of lead inputs, and often act as the ultimate sink of lead in the environment. Lead concentration in natural waters usually varies within the range of 0.0006-0.12 mg/L (0.003- 0.6 μM) (Demayo et al., 1982). Though waterborne lead concentrations do not normally exceed 125 μg/L, levels as high as 890 μg/L have previously been reported in highly contaminated water bodies (Research Triangle Institute, 1999). The ambient water quality criteria (AWQC) developed by the United States Environmental Protection Agency (USEPA), for the protection of aquatic life against chronic and acute toxicity of lead are 3.2 and 82.0 μg/L, respectively (US EPA, 2002). In Canada, the general AWQC for the protection of aquatic life against lead toxicity in fresh water is 1-7 μg/L (depending on the hardness of the ambient water) (Canadian Council of Ministers of the Environment, 2007).

1.2. Influence of water chemistry on lead bioavailability and toxicity in freshwater animals

The bioavailability and toxicity of lead in the aquatic environment primarily depend on the speciation of lead, which is profoundly influenced by the chemistry of the ambient water. Lead can exist in various chemical forms in natural waters, however it is widely believed that the most

bioavailable and toxic species of lead is the divalent free ion of lead (Pb^{2+}) (Niyogi and Wood, 2004). The bioavailability and acute toxicity of lead in aquatic organisms decrease with increasing hardness of the water. This is because hard water contains more natural divalent cations ($Ca^{2+}$and Mg^{2+}) which compete with Pb^{2+} for binding to the biological receptors, thereby reducing the bioavailability of Pb^{2+} (Rogers et al., 2003). Similarly, the increase in alkalinity reduces the bioavailability and acute toxicity of lead in aquatic organisms. This occurs due to the fact that in alkaline water lead readily binds with inorganic ligands (e.g., HCO_3^- and CO_3^{2-}.) to form lead carbonate complexes, which are usually not available for uptake and thus not toxic to aquatic organisms (Davies et al., 1976; Holcombe et al., 1976). In contrast, the effect of low pH on lead toxicity in aquatic organisms is not well understood, although it is possible that acidic water can increase lead toxicity by increasing the solubility of lead complexes and thereby increasing the concentration of Pb^{2+} in water. In addition to inorganic complexation, organic complexation of lead by natural organic matter [NOM; also defined as dissolved organic matter (DOM)] can also reduce lead bioavailability and toxicity in aquatic organisms. NOMs have a large variety of weak metal-binding functional groups such as carboxyl and phenolic as well as strong metal-binding nitrogen and sulfur containing functional groups, which can bind with Pb^{2+} in the water (Buffle, 1988; Richards et al., 2001). It is believed that metal-NOM complexes are too large and too polar to cross the biological membrane, and thus are not available for uptake by the biota (Haitzer et al. 1999; Richards et al. 2001). The protective effects of NOMs against lead bioaccumulation and acute toxicity of lead have been demonstrated in freshwater fish (Richards et al., 2001; Macdonald et al., 2002)

1.3. Waterborne essential ion transport and regulation in freshwater animals

Many waterborne divalent metals such as copper, cadmium, zinc and lead have been reported to cause disruption of ionoregulatory (Na^+, Ca^{2+} and Cl^-) processes in freshwater animals, particularly during acute exposure (see Niyogi and Wood, 2004 for a comprehensive review). Therefore, it is important to review the mechanisms that regulate essential ion uptake from water and ionic homeostasis in freshwater animals.

1.3.1. Waterborne essential ion uptake in freshwater fish

The mechanisms of waterborne essential ion (Na^+, Ca^{2+} and Cl^-) uptake in freshwater fish have been well characterized in recent years (presented in Figure 1.1). The blood of freshwater fish is hypertonic to the ambient water which leads to the continuous loss of essential ions by passive diffusion primarily through the gill and the kidney. To compensate the loss and to maintain internal ionic homeostasis, they actively take up ions both from the water and the food. It is known that the waterborne essential ion uptake occurs *via* the mitochondria-rich chloride cells in the gills of freshwater fish (Galvez et al., 2002). The waterborne Ca^{2+} enters into the chloride cell *via* an apical voltage-insensitive Ca^{2+} channel (Flik and Verbost 1993). In the chloride cell, Ca^{2+} is transported *via* Ca^{2+}-binding proteins (e.g., calmodulin) to the basolateral membrane where it is actively extruded into the blood circulation by a high-affinity Ca^{2+}-ATPase (Flik et al., 1985; Verbost et al., 1994; Marshall, 2002) and/or a Na^+/Ca^{2+} exchange mechanism (Flik et al.,1994, 1997; Verbost et al., 1997). On the otherhand, the current understanding of branchial Na^+ uptake mechanism is controversial. It is generally believed that Na^+ enters into the chloride cell through a H^+ coupled Na^+ channel or in exchange of H^+ and/or NH_4^+ at the apical membrane. Subsequently, Na^+ is pumped into the blood circulation *via* a basolateral Na^+/K^+-ATPase (Wood, 2001). Cl^- uptake is believed to occur from water by a Cl^-/HCO_3^- exchange at the apical membrane of the chloride cell, and it enters into the blood circulation *via* an anion channel on the basolateral side. H^+ and HCO_3^- are produced in the chloride cell by the breakdown of carbonic acid (H_2CO_3), a reaction catalyzed by the cytosolic enzyme known as carbonic anhydrase (CA). Carbonic acid is formed due to the reaction of carbon dioxide, which diffuses into the chloride cell from the blood, and intracellular water.

1.3.2. Waterborne essential ion uptake in freshwater invertebrates

The mechanisms of waterborne essential ion (e.g., Na^+, Ca^{2+} and Cl^-) uptake in the freshwater invertebrates are not as well understood as in fish. Very recently, Grosell and Brix (2009) have proposed a conceptual model of waterborne essential ion uptake in freshwater pulmonate snail (gastropod), *Lymnea stagnalis*, based on the findings of a number of previous studies (presented in Figure 1.2). In this model, major cations (Na^+ and Ca^{2+}) and anion (Cl^-) are taken up at the integument via transport systems identical or similar to those in freshwater fish.

Namely, Ca^{2+} uptake occurs apically via a Ca^{2+} channel and pumped into the haemolymph by a basolateral Ca^{2+}-ATPase (Flik and Verbost, 1993).

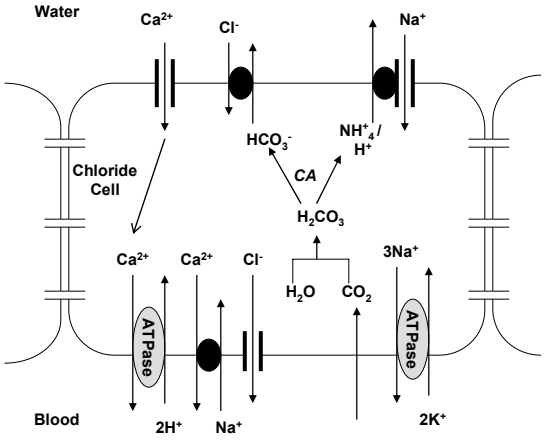

Figure 1.1: Mechanisms of branchial essential ion (Na^+, Ca^{2+} and Cl^-) uptake in freshwater fish (adopted from Niyogi and Wood, 2003).

Na^+ uptake is driven by an apical Na^+/H^+ exchanger, which is probably electrogenic in nature and relies in part on a V-type H^+-ATPase (Ebanks and Grosell, 2008). The model also proposed that Cl^- uptake in freshwater snail is also similar to fish, occurring apically via a Cl^-/HCO_3^- exchange and basolaterally via an anion channel (De With et al., 1980). Unlike fish, snails have an excreting epithelium, the mantle, which is responsible for shell formation. At the mantle, Ca^{2+} is excreted along with HCO_3^- resulting in the formation of $CaCO_3$ for the shell with a resulting H^+ as a byproduct. HCO_3^- needed for this process is probably provided, at least partially, by carbonic anhydrase(CA) facilitated hydration of metabolic CO_2. The H^+ produced in $CaCO_3$ formation is then transported back into the haemolymph to maintain an alkaline pH for shell formation. Subsequently, H^+ is likely exchanged for apical Na^+ uptake at the integument.

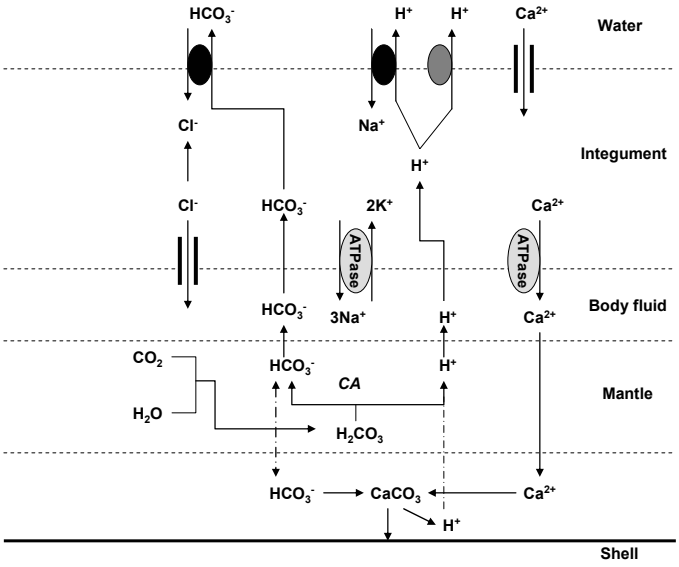

Figure 1.2: Mechanisms of waterborne essential ion (Na^+, Ca^{2+} and Cl^-) uptake in freshwater invertebrates (gastropods) (modified from Grosell and Brix, 2009).

The waterborne essential ion uptake processes have also been investigated to some extent in other groups of freshwater invertebrates such as crustaceans, which show considerable similarities to the snail model described above. Ahearn et al. (1999) suggested the existence of an apical Na^+/H^+ exchange process, which is electrogenic and amiloride (an epithelial Na^+ channel blocker) sensitive, in crustacean gill epithelial cells. Whereas the Na^+ transport mechanism at the basolateral membrane of gill epithelial cells of crustaceans is reported to involve Na^+/K^+-ATPase (Shetler and Towle, 1989). The existence of an apical Cl^-/HCO_3^- exchange process has also been reported in the crustacean gill epithelium (Towle, 1993). In addition to Na^+ and Cl^-, Ca^{2+} uptake is also very critical in freshwater crustaceans, not only for maintaining Ca^{2+} balance in the extracellular fluid but for generation/regeneration of calcareous exoskeleton as well. It is believed that waterborne Ca^{2+} transport in freshwater crustaceans occurs through the epithelial Ca^{2+} channel located in the integument (Ahearn et al., 1999), which is quite similar to the process reported in freshwater snail and fish. However, the importance of Ca^{2+} and its requirement are higher in crustaceans than other freshwater animals. The reason behind this is that the main content of their exoskeleton is $CaCO_3$. During their molting cycle, crustaceans shed their carapace and regenerate a new carapace which results into a huge demand

of calcium in their body. The Ca^{2+} taken up by the integument, antennal gland, gills and gut collectively contribute to regulate the Ca^{2+} requirement during the molting cycle (Ahearn et al. 1999). In relatively large crustaceans like lobsters, the calcium content of old exoskeleton is dissolved and transported to the haemolymph, and stored in the hepatopancreas and/or transferred to the stomach. In the stomach, Ca^{2+} is stored as granules in sacs called gastroliths. After the molt, soft new exoskeleton appears and becomes calcified very rapidly to protect the animal. At that time, stored Ca^{2+} is released from hepatopancreas and gastroliths into the haemolymph, which in combination with fresh Ca^{2+} entering into the haemolymph harden up the new carapace (Ahearn et al., 1999). However, small crustaceans such as *Daphnia* have very little capacity of storing Ca^{2+}, which can be reused during the molting process. Therefore, it is vital for them to maintain a high rate of Ca^{2+} uptake from the external environment.

1.4. Physiology of lead uptake and toxicity in freshwater animals

The physiology of lead uptake and toxicity in freshwater fish and invertebrates are described in the following section.

1.4.1. Lead uptake and toxicity in freshwater fish

The physiological effects of lead exposure in freshwater fish under both acute and chronic conditions have been studied extensively in recent years, which have provided considerable insights into the mechanisms of lead uptake and toxicity in fish. MacDonald et al. (2002) first speculated that lead disrupts Ca^{2+} uptake by competitive inhibition at apical Ca^{2+} channels in the fish gill. Circumstantial evidences from previous studies supported this notion of Pb^{2+}/Ca^{2+} antagonism in fish (Hodson et al., 1978; Varanasi and Gmur, 1978). Rogers et al. (2003) later demonstrated through direct experimental evidence that acute lead exposure causes significant inhibition of Ca^{2+} influx in rainbow trout (*Oncorhynchus mykiss*), which eventually leads to hypocalcaemia. In a related study, Rogers and Wood (2004) demonstrated by employing pharmacological techniques that Pb^{2+} shares the common uptake pathway with Ca^{2+} in fish gills. The uptake involves competitive inhibition of apical entry of Ca^{2+} *via* lanthanum-sensitive Ca^{2+} channels (voltage insensitive) and disruption of the function of the ATP-driven basolateral Ca^{2+} pump. Moreover, acute lead exposure has also been reported to enhance the efflux of Ca^{2+} in fish (Sayer et al., 1991). Recently, Patel et al. (2006) demonstrated that significant lead accumulation

occurs in the posterior kidney, containing the majority of functional renal tubules, of fish during waterborne acute lead exposure. They also observed that lead exposure disrupts the re-absorption of Ca^{2+} in the proximal renal tubule, possibly by inhibiting Ca^{2+}-ATPase as found in the gill. As a result, renal excretion of Ca^{2+} increases and becomes a significant contributor towards the development of hypocalcaemia.

Rogers et al. (2003) reported concurrent significant inhibition of Na^+ and Cl^-, in addition to Ca^{2+}, influx in the same study. In a follow up study, Rogers et al. (2005) reported that Pb^{2+}-induced disruption of Na^+ and Cl^- balance in fish results in part due to the rapid inhibition of CA activity and binding of Pb^{2+} with basolateral Na^+-K^+-ATPase in the gills, causing non-competitive inhibition of Na^+ and Cl^- uptake. The inhibition of Na^+/K^+-ATPase likely occurs due to the binding of lead with the carboxyl group at the active site of Na^+/K^+-ATPase as observed in human erythrocytes (Ong and Lee, 1980). Grosell et al. (2006a) reported that chronic waterborne lead exposure results in a general ionoregulatory disturbance affecting K^+, Na^+ and Ca^{2+} homeostasis in fathead minnow (*Pimephales promelas*). Overall, these studies suggest that waterborne lead exposure causes disruption of the normal physiological functions of both gill and kidney, which play a critical role in maintaining ionic homeostasis in freshwater fish. Interestingly, lead is a unique ionoregulatory toxicant in the sense that it shares the toxic mode of actions of two different types of ionoregulatory toxicants to freshwater fish. On one hand, it acts like copper and silver which affect Na^+ and Cl^- balance (Lauren and McDonald,1985; Wood et al., 1996; Morgan et al., 1997), and on the other hand it also behaves like other known Ca^{2+}-anatgonists (e.g., zinc and cadmium) which disrupt Ca^{2+} homeostasis (Verbost et al., 1987).

Waterborne lead can also act as a potential respiratory toxicant in freshwater fish, particularly during chronic exposure. It has been reported that sub-lethal lead exposure causes inhibition of δ-aminolevulinic acid dehydratase (ALAD) in the blood, spleen and renal tissue of fish, both in laboratory and field studies (Johansson-Sjobeck and Larsson, 1979; Rodrigues et al., 1989; Rabitto et al. 2005; Schmitt et al., 1993, 2002). ALAD is an enzyme that catalyzes the second step in heme biosynthesis (the synthesis of porphobilinogen from aminolevulinic acid), and its inhibition leads to lower hemoglobin level. ALAD is often used as a specific biomarker of lead exposure in higher vertebrates, particularly mammals (Hernberg et al., 1970). The mechanism of inhibition of ALAD activity by lead involves substitution of the co-factor Zn^{2+}

with Pb^{2+} and binding of Pb^{2+} to the functional –SH group of the enzyme (World Health Organization, 1995).

Chronic waterborne lead exposure has also been reported to cause oxidative stress in fish (Thomas and Juedes 1992; Chaurasia and Kar, 1999). Some of the documented effects included increased oxidized glutathione level and lipid peroxidation, and suppressed antioxidant enzyme activities in liver. However, the mechanism of lead induced oxidative damage is not quite clear since it is not a redox-active metal like iron or copper, and thus doesn't catalyze Fenton reaction to produce reactive oxygen species (ROS). Thomas and Juedes (1992) suggested that lead induced disruption of intracellular Ca^{2+} homeostasis is a potential cause for the induction of oxidative stress, however the actual mechanism remains unknown. In mammalian systems, it has been suggested that lead can increase auto-oxidation of haemoglobin which eventually generates ROS resulting in lipid peroxidation (Ribarov et al. 1981, 1982). Chaurasia and Kar (1999) also reported that chronic waterborne lead exposure leads to the reduced level of thyroid hormone (T_3) in fish. They suggested that it occurs as a result of membrane damage, induced by oxidative stress, which ultimately leads to the inactivity of iodothyronine 5'-monodeiodinase (a membrane bound seleno-enzyme that converts T_4 to T_3). Therefore, chronic waterborne lead exposure can also potentially affect the thyroid metabolism in fish.

Chronic waterborne lead exposure has been reported to affect the neurosecretory functions of the central nervous system in fish as well. Weber et al. (1991) observed increased level of dopamine and serotonin (neurotransmitters) in fathead minnow exposed to sub-lethal waterborne lead. Moreover, lead exposure has been reported to increase the expression of endocannabinoids (N-arachidonylethanolamine and 2-arachidonylglycerol) in fathead minnow, however this effect was found to be sex specific since it was recorded in males only (Rademacher et al., 2005). Endocannabinoids are a group of compounds known as 'retrograde' transmitters since it is released from the postsynaptic neuron and acts on presynaptic neuron. The increase of endocannabinoid expression can affect the transportation of neurotransmitter vesicles to the synaptic membrane or their fusion with the cell membrane. Therefore, the neurotoxic effect of lead can occur due to its ability to interfere with the release of neurotransmitter in the synapse and/or by changing endocannabinoid-mediated regulatory mechanisms.

1.4.2. Lead uptake and toxicity in freshwater invertebrates

Although the recent studies discussed above have provided considerable insights into the mechanisms of lead uptake and toxicity in freshwater fish, information about the physiological effects of lead exposure in invertebrates is quite limited. Ahearn and Morris (1998) examined the effects of chronic lead exposure on Na^+ uptake and homeostasis in freshwater crayfish (*Cherax destructor)*, and observed a disruption of sodium balance and a significant inhibition of Na^+-K^+-ATPase activity. Amado et al. (2006) reported osmoregulatory, but not ionoregulatory, disturbances in red crab (*Dilocarcinus pagei*) exposed to sub-lethal lead concentration in freshwater. Grosell et al. (2006b) reported the disruption of sodium homeostasis in the freshwater pulmonate snail (*Lymnaea stagnalis*) during chronic exposure to waterborne lead. Very recently, Grosell and Brix (2009) revealed in a more detailed mechanistic study that chronic waterborne lead exposure significantly inhibited Ca^{2+} influx rate and depleted the total calcium and sodium levels in the soft tissue of pulmonate snails. Interestingly, they also observed an increase in the pH of haemolymph, which indicated the occurrence of metabolic alkalosis. They suggested that chronic lead exposure causes toxicity in freshwater snails through a complex cascade of ionoregulatory and acid-base disturbance. Moreover, Berglind et al (1985) reported a concentration dependent inhibition of ALAD activity in water flea (*Daphnia magna*) exposed to waterborne lead. They also recorded a simultaneous decrease in wholebody haemoglobin concentration in the same study. Similar inhibition of ALAD activity was observed in both pigmented and non-pigmented freshwater gastropod (*Biomphalaria glabrata*) and oligochaete (*Lumbriculus variegates*) exposed to acute waterborne lead concentration, and the enzymatic activity was negatively correlated with the tissue lead accumulation in both animals (Aisemberg et al., 2005). Overall, these findings indicate that the waterborne lead exposure can produce ionoregulatory and/or respiratory disturbances in freshwater invertebrates, however further more in depth studies are required to enhance our knowledge regarding these aspects.

1.5. The experimental animal, *Daphnia magna*

Daphnia magna is classified under the phylum: Arthropoda, subphylum: Crustacea, class: Branchiopoda, and order: Cladocera. Daphnids are commonly called water fleas and represented by several species, among which *D. magna* is the largest in size. They are widely distributed in North America, Europe, Asia and Africa. They are important components of zooplankton diet of

fish, and thus represent an important link in food chains of virtually every inland water body. *Daphnia* are highly sensitive (10-100 fold more sensitive than fish) to a broad range of environmental contaminants including metals. They are universally accepted as a model species for various kinds of toxicity assessments in many environmental jurisdictions across the world. They are commonly used for monitoring the quality of industrial waste water so that it can be safely released into the environment by the industries. Because of the reasons described above, it is often regarded as the organism that drives the regulations for aquatic ecosystems (Meyer et al., 2007).

1.5.1. Morphology and physiology of *D. magna*

The adults of *D. magna* are usually 5-6 mm in length. The animal looks like a plump bird since the head projects posterio-ventrally from the trunk as a short beak. The noticeable part of the organism is its carapace which covers the trunk of the body. The carapace is made up of $CaCO_3$. The animal has 5-6 trunk appendages, and each appendage has flattened leaf like structure called endopodite which is divided into two parts, coxa and basis (like all other crustaceans). The coxa of the endopodite has a flattened epipodite that serves as the gill. This feature signifies the class Branchipoda, which means 'gill feet'. They are very sensitive to various water quality parameters such as pH, temperature and dissolved oxygen. Their abundance in natural waters varies with the season. Their population decreases in the winter, and increases in the spring (as much as 250-300 individuals/L at a temperature regime of 7-13^{0}C). The population declines again in the summer due to the rapid increase of temperature. Most of the species are highly ephemeral, i.e. short lived. *D. magna* has an average life span of 40 days at 25^{0}C and 55 days at 20^{0}C.It has four distinct stages in its life cycle: egg, neonate; juvenile and adult (gravid) (Pennak 1989). Under favorable environmental condition, *Daphnia* reproduce by parthenogenesis, and the population usually consists of females only. However, they switch to sexual mode of reproduction when the condition is unfavorable. The appearance of males in the population indicates deterioration of the habitat due to hostile temperature or oxygen regime, over crowding or lack of food availability. *D. magna* begins to molt every 2-3 days from the age of 9-10 days. The eggs are deposited in the brood chamber within minutes after molting (shedding off the carapace), the hatching of eggs occurs in the brood chamber. The neonates developed from the eggs are released into the water during the next molting. The maximum

growth of the animal occurs immediately after the molting because of the characteristic stretchiness of the new carapace.

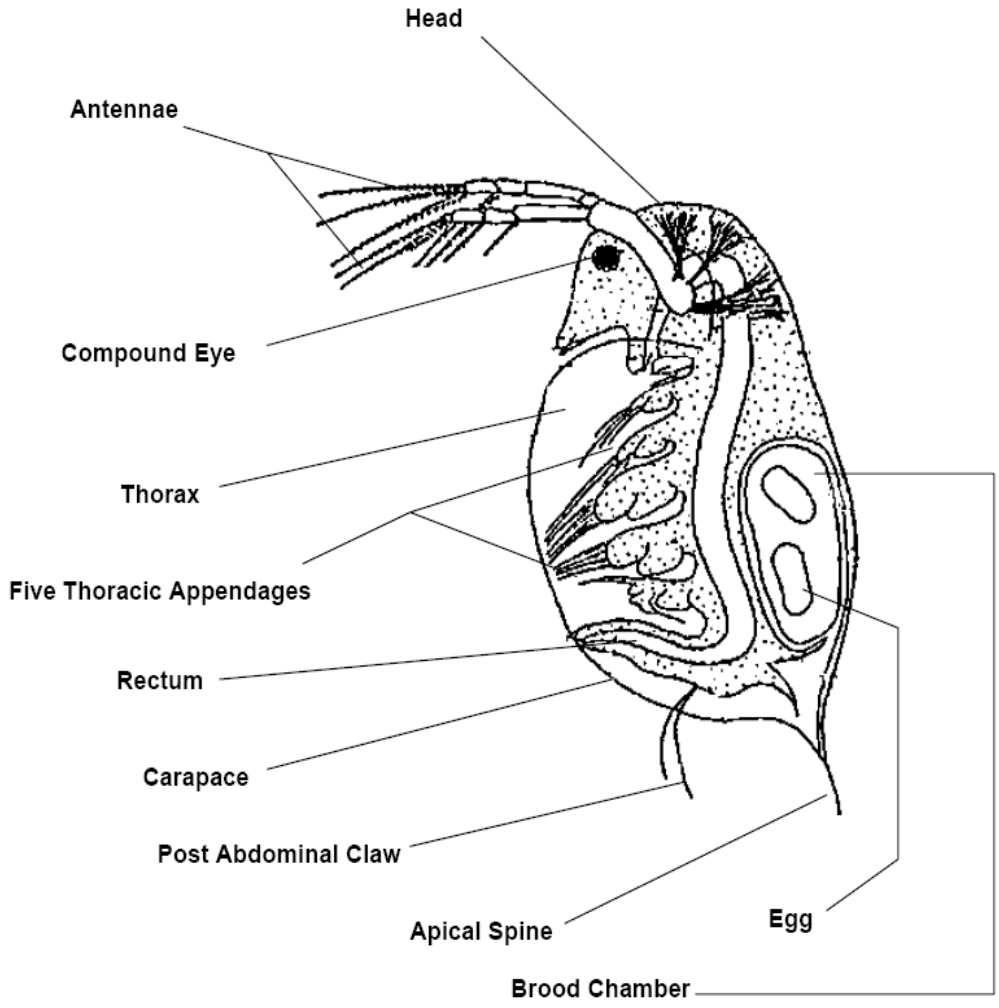

Fig. 1.3 The structure and morphology of *Daphnia* (Reproduced from FAO Fisheries Technical Paper, 1996)

Daphnia are genera of freshwater Cladocera which have haemoglobin as an oxygen carrying pigment (Berglind et al., 1985). During neonatal and juvenile stages, most of the gas exchange occurs through the body surface. The adults use hemoglobin as a supplementary mechanism to transport oxygen because the diffusive oxygen transport across the body becomes inadequate due to the reduced surface area-to-volume ratio (Wiggins and Frapppell, 1999).

Moreover; the pigmentation in *Daphnia* can differ depending on the ambient water quality condition. Their body is transparent in well aerated transparent water, but it becomes pink or red due to the presence of haemoglobin in stagnant hypoxic water. It is believed that the inner wall of carapace is their major site of gas and ion exchange, although their trunk appendages (gill feet) may also have similar functions. Haemolymph passes towards the carapace lacuna and becomes oxygenated during its flow through the ventral part of carapace.

1.6. Study objectives

Based on the information discussed above, it appears that the mechanisms of waterborne lead toxicity may be similar, at least partially, between freshwater fish and invertebrates. However, further investigations with a more comprehensive approach are required to elucidate the precise mechanisms of uptake and toxicity of waterborne lead in aquatic invertebrates. In this regard, the particular focus should be placed upon examining the ionoregulatory (especially the characterization of Pb^{2+}/Ca^{2+} interactions) and respiratory effects. It is also important to note here that species-specific differences in the toxic mechanisms of metals exist. For example, nickel at acute level is a respiratory toxicant (causes metabolic acidosis by disrupting CO_2 exchange) to freshwater fish (Pane et al., 2003b and 2004), whereas it is an ionoregulatory toxicant (causes disruption of Mg^{2+} balance) to the crustacean, *D. magna* (Pane et al. 2003a). Moreover, even in cases where the toxic mechanism is similar between freshwater fish and invertebrates (e.g., silver – disruption of sodium homeostasis), subtle differences exist. For example, the inhibition of Na^+ uptake by silver is competitive in nature in crustacean, *D. magna*, whereas in fish it is predominantly non-competitive (Morgan et al., 1997; Bianchini and Wood, 2003).

The main goal of my research was to improve our understanding of the mechanisms of lead uptake and toxicity in freshwater invertebrates using *D. magna* as a model organism. The two major aspects of my research project were:

(a) To elucidate the kinetic and pharmacological properties of lead uptake.

The two questions that I addressed here are:

(i) Is the interaction between waterborne Pb^{2+} and Ca^{2+} competitive or non-competitive in nature?

(ii) What type of Ca^{2+} channels are involved in waterborne Pb^{2+} uptake [lanthanum-sensitive (voltage-independent) and/or Verapamil-sensitive (voltage-dependent, L-type)]?

(b) To understand the physiological basis of acute lead toxicity.

The question that I addressed here is:

(i) Does waterborne lead exposure cause ionoregulatory and/or respiratory stress?

CHAPTER 2

MATERIALS AND METHODS

2.1. Culture and maintenance of *Daphnia magna*

The *Daphnia* colony was established in our laboratory using 1-day old neonates of *D. magna* obtained from the laboratory of Dr. Karsten Liber at the Toxicology Centre, University of Saskatchewan. They were cultured in 1L glass beakers (approximately 20 animals per L of water) using a static renewal system, with replacement of the culture water every 48h. The *Daphnia* colony was cultured in a synthetic water ($NaHCO_3$: 1.8 mM, $MgSO_4.7H_2O$: 0.7 mM, $CaSO_4.2H_2O$: 0.7 mM, KCl: 0.1 mM, Hardness: 120 mg/L as $CaCO_3$, pH: 7.8 – 8.0) [as recommended by the American Society for Testing and Materials (ASTM), 1993a]. The culture water was aerated continuously for 24h, and was supplemented with Vitamin B (2 μg/L) and selenium (2 μg/L) prior to its use. The stock cultures were maintained under a constant temperature (20 ± 2^0C) and photoperiod (16h light and 8h dark), and were fed with a mixture of 20 mL of algae (*Selenestrum capricornutum*, 3.2×10^6 cells/mL) and 1.5 mL of YCT (a slurry of yeast, cerophyll and trout chow) per L of culture water. The algae were cultured and the YCT was prepared in the laboratory following the methodology of ASTM (1993b). *Daphnia* stock cultures were maintained for only 4 weeks, and new cultures were set up every 4 weeks using neonates obtained from old stock cultures. For all experiments, 7-8 day old (~1 mg wet mass) Daphnids (prior to their first molting event) were used. Daphnids used in the experiments were collected only from the stock cultures producing a minimum of 15 neonates per adult (the indicator of a healthy colony). Moreover, Daphnids used in the experiments were isolated within a few hours of birth to ensure an exactly similar age at the time of experimentation.

2.2. Experimental exposure water

In general, Daphnids were not fed during the experimental exposures unless stated otherwise. All of the experimental assays were performed using synthetic water that was much softer (low salt content) and slightly acidic than the culture water. This was done primarily due to two reasons: (i) to ensure higher concentration of free Pb^{2+} (the most toxic and bioavailable form of lead) in the exposure water as a result of reduced inorganic complexation, and (ii) to increase the solubility of lead complexes in the exposure media since lead tends to form insoluble precipitates in alkaline pH range. The actual chemical composition of the experimental water was as follows: $Ca(NO_3)_2$: 0.25 mM, $MgSO_4.7H_2O$: 0.25 mM, KCl: 0.08 mM, NaCl: 0.08 mM, Hardness: 40 mg/L as $CaCO_3$, pH: 6.5 – 6.7). Similar to the culture water, the experimental water was also aerated for 24 h prior to its use in the experimental assays. At the beginning of the assays, the pH of the experimental water was adjusted to 7.0 by adding 2 mM $NaHCO_3$, thus all of the experiments were conducted at a circum-neutral pH. Daphnids from stock colonies were collected first in 1 L glass beakers containing the experimental water and subsequently transferred to the experimental beakers/chambers containing the same water. This two-step transfer process was performed to avoid contamination of experimental exposure water with the culture water. The animals were allowed to settle down in the experimental water for 30 minutes prior to the start of the experiments. All of the experiments described below were conducted at a temperature of 20 ± 2 ^{0}C and photoperiod of 16h light and 8h dark.

2.3. Experiment 1: Concentration and time dependent waterborne lead accumulation in *D. magna*

The basic objective of this experiment was to examine whether lead accumulation occurs in a concentration and time dependent manner in *Daphnia*. Daphnids (n = 10 per treatment, 2 replicates per treatment) were exposed to four different waterborne lead concentrations [0.02, 0.2, 0.3 and 0.6 μM; added as $Pb(NO_3)_2$] for 24 h. The lead exposure levels and exposure period were selected based on the result of previous experiments carried out in our laboratory, which indicated that the 48h LC_{50} (the concentration that is lethal to 50% of the test organisms at 48h) of lead for *D. magna* is ~1 μM [212 μg/L as $Pb(NO_3)_2$] under similar water chemistry condition (Hicks and Niyogi, unpublished data). Thus, the highest exposure concentration of lead used in the study represents about 60% of the measured LC_{50} value. For each experimental treatment, the

animals were exposed in 250 mL glass beakers containing 100 mL of experimental water. The desired lead concentrations in respective exposure waters were achieved by adding appropriate volumes of 1mM stock solution of $Pb(NO_3)_2$. At the end of the exposure, Daphnids were collected using disposable polyethylene pipettes, rinsed first in 1 mM EDTA solution for 30s to remove loosely bound metals, followed by two subsequent 30s wash in deionized Nanopure water, and blotted dry using Kimwipes. Each animal was transferred to a pre-weighed 1.5 mL microfuge tube, and the tube was weighed again to evaluate the weight of individual animal. An aliquot of 100 µL concentrated (15N) HNO_3 was added into each tube, and the animals were digested in a hot air oven at 60 ^{0}C for 24h. A 5 mL aliquot of water sample from each experimental treatment was also collected in 7 mL polyethylene vials both at the beginning and the end of the exposure, and acidified with 50 µL concentrated (15N) HNO_3. Both the water samples as well as the digested animal tissue samples were kept at 4 ^{0}C until their analysis for total lead concentration using a graphite furnace atomic absorption spectrometer (Analyst800, PerkinElmer, USA) (see section 2.10 for details).

For the examination of time-dependent lead accumulation, Daphnids (n = 10 per treatment, 2 replicates per treatment) were exposed similarly to 0.6 µM waterborne lead separately for 2h, 6h, 12h and 24h. After the exposure, the animals were sampled and processed as described above. A 5 mL aliquot of water samples were also collected from each experimental treatment and acidified with 50 µL of HNO_3. Both tissue and water samples were stored at 4 ^{0}C and later analyzed for total lead concentration using a graphite furnace atomic absorption spectrometer (Analyst800, PerkinElmer, USA) (see section 2.10 and 2.11for details).

2.4. Experiment 2: Effects of essential ions (Ca^{2+}, Mg^{2+} and Na^{+}) on waterborne lead accumulation in *D. magna*

The goal of this experiment was to investigate the effects of natural cations in water (Ca^{2+}, Mg^{2+} and Na^{+}) on wholebody lead accumulation in *Daphnia*. Daphnids (n = 10 per treatment, 2 replicates per treatment) were exposed to 0.6 µM of waterborne lead under an increasing range [0.25 (Control), 0.50, 1.0, 1.5 and 2 mM] of waterborne calcium [added as $Ca(NO_3)_2$], magnesium [added as $Mg(NO_3)_2$] and sodium [added as $NaNO_3$] concentration, separately, for 24h. For respective calcium, magnesium and sodium treatments, the animals were exposed in 250 mL glass beakers containing 100 mL of experimental water. Similar to

experiment 1, the desired calcium, magnesium, sodium and lead concentrations in the respective exposure mediums were achieved by adding the appropriate volume of 1mM stock solution of $Pb(NO_3)_2$, and 10 mM stock solutions of $Ca(NO_3)_2$, $Mg(NO_3)_2$ and $NaNO_3$, respectively. Following exposure, the animals were collected using disposable polyethylene pipettes, rinsed individually first in 1 mM EDTA solution for 30s followed by two subsequent 30s wash in deionized Nanopure water, and blotted dry using Kimwipes. As described previously, animals were weighed individually and acid digested at 60 ^{0}C for 24h. A 5 mL aliquot of water sample was collected at the beginning and the end of the exposure period, and acidified with 50 μL concentrated (15N) HNO_3. Both the water samples and tissue digests were stored at 4 ^{0}C until their analysis for total lead concentration by atomic absorption spectroscopy (see section 2.10 and 2.11 for details).

2.5. Experiment 3: Effect of waterborne lead exposure on waterborne Ca^{2+} influx in *D. magna*

The purpose of this experiment was to understand the effects of waterborne lead exposure on the kinetics of short-term (1h) waterborne calcium uptake in *Daphnia*. Daphnids (n = 10 per treatment, 2 replicates per treatment) were transferred to 250 mL polyethylene beakers containing 100 mL of experimental water representing an increasing range of calcium concentrations [0.25(control), 0.50, 1.0 and 1.5 mM, added as $Ca(NO_3)_2$ respectively]. The calcium influx at different calcium concentrations were examined both at the absence (0 μM) and presence (0.6 μM) of waterborne lead. The exposure waters were first spiked with appropriate volumes of 10 mM stock solutions of $Ca(NO_3)_2$ to achieve the desired calcium concentrations. Subsequently, the exposure waters were radio-labeled with ^{45}Ca (300 μCi/L as $CaCl_2$, specific activity = 12.2 mCi/mL, GE Healthcare, USA). For examining calcium influx at the presence of waterborne lead, the exposure waters were spiked additionally with appropriate volumes of 1mM stock solution of $Pb(NO_3)_2$. The calcium influx assay was conducted for 1h. At the end of the assay, Daphnids were collected using disposable polyethylene pipettes, first rinsed in 1 mM EDTA solution for 30s followed by two subsequent wash in deionized Nanopure water for 30s each, and blotted dry using Kimwipes. Subsequently, animals were placed individually in pre-weighed 1.5 mL microfuge tubes, weighed and digested in concentrated HNO_3 as described above. Each animal digest was then transferred into a 20mL glass scintillation vial and mixed

with 2 mL UltimaGold scintillation liquid (PerkinElmer, USA). Water samples (2mL, in duplicate) were collected before and after the exposure period 20 mL glass scintillation vials. One set of water samples was mixed with 10 mL of aqueous scintillation liquid (ACS, PerkinElmer, USA), and the other set was acidified with 20 μL of concentrated (15N) HNO_3 and stored at 4 ^{0}C for the analysis for total lead concentration by atomic absorption spectroscopy (see below for details). Both tissue digests and water samples, mixed with scintillation liquids, were counted for ^{45}Ca activity in a Packard Minaxi scintillation counter (LKB Wallac, USA). The digested tissue samples were analyzed for potential quenching by standard addition and recovery of known amounts of ^{45}Ca to tissue and water samples (see section 2.11.1 for influx rate calculation detail). No significant quenching was recorded, and therefore quench correction was not required for any samples.

2.6. Experiment 4: Effects of calcium channel blockers [Lanthanum chloride ($LaCl_3$) and verapamil] on waterborne calcium influx and lead accumulation in *D. magna*

The objective of this experiment was to understand the pharmacological properties of waterborne calcium and lead interaction in *Daphnia*. The effects of $LaCl_3$ (a voltage-insensitive calcium channel blocker) and Verapamil [a voltage-sensitive, L-type calcium channel blocker] on waterborne calcium influx and lead accumulation were examined separately. To study the effects on calcium influx, Daphnids (n = 8 per treatment, 2 replicates per treatment) were exposed for 1h in 100 mL of experimental water (0.25 mM of calcium) radio-labeled with ^{45}Ca (300 μCi/L as $CaCl_2$, specific activity = 12.2 mCi/mL, GE Healthcare, USA) at two different concentrations of $LaCl_3$ (5 and 10 μM) and Verapamil (100 and 200 μM) in addition to control (no channel blockers added). Following the exposure, the water and animal samples were collected, processed and analyzed for ^{45}Ca activity as described above in Experiment 3.

Similarly, to study the effects of calcium channel blockers on waterborne lead accumulation, Daphnids (n = 10 per treatment, 2 replicates per treatment) were exposed to 0.6 μM of waterborne lead for 24h at the same concentrations of $LaCl_3$ (5 and 10 μM) and Verapamil (100 and 200 μM) in addition to control (no channel blockers added). At the end of the exposure, the water and animal samples were collected and processed as described previously in Experiment 1, and stored at 4 ^{0}C until their analysis for total lead concentration by atomic absorption spectroscopy (see section 2.10 and 2.11for details).

2.7. Experiment 5: Time dependent effects of waterborne lead exposure on Ca^{2+} and Na^{+} influx in *D. magna*

The objective of this experiment was to understand how waterborne lead exposure influences waterborne calcium and sodium influx over time in *Daphnia*. For this experiment, the animals were fed with algae and YCT and maintained in experimental water supplemented with vitamin B and selenium (as mentioned previously in Section 2.1), during their exposure to 0.6 μM of waterborne lead over a period of 0 to 23h. Approximately 20 individuals (n= 10 per treatment, 2 replicates) were exposed to waterborne lead in 1 L glass beakers for 5h, 11h and 23h separately. The exposure started at 8o'clock in the morning. Subsequently, the animals were transferred to 250 ml polyethylene beakers containing the same experimental water (but without food) containing 0.6 μM of waterborne lead and labeled with either radioactive ^{45}Ca or ^{22}Na, and exposed for another 1h to evaluate the calcium or sodium influx rate. Another set of experimental animals were treated identically except waterborne lead exposure, which represented control. A subset of 20 animals (n= 10 per treatment, 2 replicates) collected randomly from a stock colony was also analyzed for 1h calcium and sodium influx rate, which represented time 0 (control). Animals were fed in this experiment because previous findings indicated that starvation can profoundly impact calcium handling in *Daphnia*. Porcella et al. (1969) reported 11-fold variation in calcium uptake depending on the molt stage of starved *Daphnia*. Therefore, I assumed that starvation could influence the waterborne calcium influx in such a way, which could make it difficult to interpret the effects of waterborne lead exposure.

For the evaluation of calcium influx, experimental water was radio-labeled with 300 μci/L of (as $CaCl_2$, specific activity = 12.2 mCi/mL, GE Healthcare, USA). At the end of the exposure period, the water samples and animals were collected, processed and analyzed for ^{45}Ca activity as described previously in Experiment 3. For the evaluation of sodium influx, experimental water was radio-labeled with 15 μci/L (as NaCl of specific activity = 19.2 mCi/mL, PerkinElmer, USA). At the end of the exposure, animals were transferred individually to 10 ml of 600mM NaCl solution with polyethylene pipettes and rinsed for 30s. The rinse in a cold NaCl solution was performed to remove the loosely bound ^{22}Na from the external surface of the animals. Subsequently, animals were rinsed twice individually in 10 mL of deionized Nanopure water for 30s each, and blotted dry in Kimwipes. Each daphnid was transferred to a pre-weighed 7 mL polyethylene vial, and the vial was weighed again to evaluate the weight of the animal. A 2 mL

aliquot of water samples were also collected in 7 mL polythene vials both at the beginning and the end of the exposure. The water and animal samples were counted for total ^{22}Na activity in a 1480 Wizard-3" gamma counter (PerkinElmer, USA). Using the same approach described in Experiment 3, the animal tissue samples were evaluated for quenching, again no quenching was recorded. Additionally, a 5 mL aliquot of water samples were also collected at the beginning and end of the exposure, acidified as described in Experiment 1, and kept at 4 ^0C until their analysis for total lead concentration by atomic absorption spectroscopy (see section 2.10 and 2.11for details).

2.8. Experiment 6: Effects of waterborne lead exposure on wholebody total calcium and sodium concentration in *D. magna*

The goal of this experiment was to evaluate the effects of waterborne lead exposure on wholebody total calcium and sodium concentration in *Daphnia*. Approximately 20 animals (n= 10 per treatment, 2 replicates) were exposed to 0 (control) and 0.6 μM lead in 1L experimental water for 6h, 12h and 24h, separately. The exposure started at 8 o'clock in the morning.They were fed during the exposure period and maintained in water supplemented with vitamin B and selenium as described in Experiment 5. At the end of the respective exposure periods, Daphnids were transferred individually to 10 mL of 1 mM EDTA solution. They were rinsed in the EDTA solution for 30s, which was followed by two subsequent rinses in deionized Nanopure water for 30s each. Animals were blotted dry in Kimwipes, and placed individually in a pre-weighed 1.5 mL microfuge tube. Each tube was weighed again to evaluate the weight of each animal. Each animal was then acid digested as described in Experiment 1, and stored at 4 ^0C. Similarly, a 5 mL aliquot of water sample was also collected from each treatment, acidified with 50 μL of 15N HNO_3, and kept at 4 ^0C. Later, the tissue digests were analyzed for total calcium and sodium, and the water samples were analyzed for total lead using atomic absorption spectroscopy (see section 2.10 and 2.11 for details). A subset of 20 animals (n= 10 per treatment, 2 replicates) collected randomly from a stock colony was also analyzed for total wholebody calcium and sodium concentration, which represented time 0 (control).

2.9. Experiment 7: Effects of waterborne lead exposure on O_2 consumption rate (respiration rate) in *D. magna*

The objective of this experiment was to evaluate whether waterborne lead exposure affects the respiratory capacity (rate of O_2 consumption) of *Daphnia*. Approximately 7-8 animals were exposed to 0 (control) and 0.6 μM of waterborne lead in 1 L of experimental water for 6h, 12h and 24h, separately. A subset of 7 animals collected randomly from a stock colony was also analyzed at the beginning of the exposure, which represented time 0 (control). At the end of the exposure periods, each animal was transferred to a pre-weighed 500 μL glass micro-respiration chamber (Unisense Ltd., Denmark) containing appropriate exposure water. The micro-respiration chamber was weighed again to evaluate the weight of the animal. The chamber was then fitted with a Clark-type O_2 micro-electrode (Unisense Ltd, Denmark), and the change in O_2 concentration within the respiration chamber was then recorded continuously (5 recordings/min) for 0.5h in a computer using the Micro-ox software (Unisense Ltd, Denmark). During the measurement, the entire respiratory set-up was kept immersed in a water bath to maintain a steady temperature (20±2 ^{0}C) and also to keep the micro-respiration chamber sealed from air. Prior to its use in the experiment, the O_2 micro-electrode was polarized overnight in an anoxic solution (0.1M sodium ascorbate and sodium hydroxide), then standardized with a 2-point calibration [0.1 M sodium ascorbate and sodium hydroxide solution (0% O_2) and 100% O_2 saturated solution]. The 100% saturated solution was prepared by aspirating deionized Nanopure water with 99.5% purified O_2 overnight. Micro-ox software recorded the gradual decline in aqueous O_2 concentration (as μmol/L) within the micro-respiration chamber over a period of 0.5h and presented the data in a linear regression. The most consistent 0.25h part of the entire recording from each measurement was used to calculate the rate of O_2 consumption for individual animals (see section 2.11.2 for details).

2.10. Analysis of total lead, calcium and sodium by Atomic Absorption Spectroscopy

Both the acid digested animal tissue and water samples were diluted appropriately with 1% HNO_3 solution (prepared in deionized Nanopure water). The samples were then analyzed for total lead concentration using the graphite furnace of an atomic absorption spectrometer (Analyst800, PerkinElmer, USA). A certified standard of lead (Fisher Scientific, Canada) was used to calibrate and standardize the employed analytical method. The detection limit of the

analytical method was 0.1 μg/L of lead. The quality control and assurance of the analytical method were checked by standard addition and recovery of known amounts of lead into the reference water and tissue samples. Approximately 98% recovery was recorded.

For the analysis of total wholebody calcium and sodium level in *Daphnia*, the tissue digests were diluted appropriately with 1% HNO_3 solution (prepared in deionized Nanopure water). The samples were then analyzed for total calcium and sodium using the flame burner of an atomic absorption spectrometer (Analyst800, PerkinElmer, USA). The certified standards of calcium and sodium (Fisher Scientific, Canada) were used to calibrate and standardize the employed analytical methods for calcium and sodium measurement, respectively. The detection limit of the analytical methods was 15 μg/L for calcium and 10 μg/L for sodium.

2.11. Data analysis

2.11.1. Calculation of unidirectional Ca^{2+} and Na^{+} influx rate in *D. magna*

The unidirectional influx rate (J_{in}) of Ca^{2+} and Na^{+} (in μmol g^{-1} wet weight h^{-1}) was determined using the following equation (Pane et al., 2003a):

$$J_{in} = cpm / (SA.m.t)$$

Where cpm was either ^{45}Ca (β) or ^{22}Na (γ) counts per minute in the *Daphnia*, SA was the specific activity of the exposure water (cpm ^{45}Ca μM of total calcium or cpm ^{22}Na per μM of total sodium), m was the *Daphnia* wet mass (in g), and t was the time of exposure in hours (h).

2.11.2. Calculation of the rate of O2 consumption (respiration rate) in *D. magna*

The rate of O_2 consumption (MO_2, in μmol g^{-1} wet weight h^{-1}) was calculated using the following equation (Unisense Ltd., Denmark):

$$MO_2 = \Delta C.V/ W.t$$

Where ΔC was the difference between the initial and the final O_2 concentration (μmol) of exposure water in the micro-respiration chamber, V was the volume of total exposure water within the micro-respiration chamber, W was the wet mass of the *Daphnia* (in g), and t was the total elapsed time in hours (h). The final O_2 consumption rates were corrected for "blank" O_2 consumption rate by the experimental apparatus in the absence of *Daphnia*.

2.11.3. Calculation of wholebody lead, calcium and sodium concentration in *D. magna*

The wholebody lead, calcium and sodium concentrations (M) were calculated using the following equation:

$$M = A.V.\ df / W$$

Where A was the metal concentration in tissue digest (in μg L^{-1} for lead, and in μmol L^{-1} for calcium and sodium), V was the total volume of the tissue digest, df was the dilution factor, and W was the wet mass of the *Daphnia* (in g). The wholebody lead concentration was expressed as μg g^{-1} wet weight, and the wholebody calcium and sodium concentrations were expressed as μmol g^{-1} wet weight.

2.11.4. Statistical analyses and the determination of kinetics of waterborne calcium influx

Data were calculated and expressed as means ± SEM (standard error mean) (n = number of individuals). Statistical significance was determined by one-way or two way analysis of variance (ANOVA), followed by Tukey's multiple comparison test using SPSS for Windows (version 15; Chicago, IL, USA). Mean values were considered different at $p < 0.05$. The assumptions of ANOVA, i.e., normality of distribution and homogeneity of variances were examined by Shapiro-Wilk's test and Leven's test, respectively (both at $\alpha = 0.05$). All of the data met these assumptions.

Kinetic analysis of waterborne calcium influx in *Daphnia* was conducted using Michaelis-Menten model. Michaelis-Menten analyses of the relationship between waterborne calcium concentration and calcium influx rate at different waterborne lead exposure levels were performed using Lineweaver-Burke double reciprocal plots. Regressions were applied using SigmaPlot for Windows (version 9.0; Chicago, IL, USA). The parameter estimates of calcium influx kinetics (J_{max} = maximum rate of influx, and K_m = the substrate concentration at which the rate of influx is half of J_{max}) were determined using the equation of respective regression lines ($y = mx + c$). The inverse of the y intercept of the regression line provided the value of J_{max}, whereas inverse of the x intercept of the regression line provided the value of $-K_m$. Significance of regressions (control against individual treatments) were evaluated using a student's t test.

CHAPTER 3

RESULTS

3.1. Dose and time dependent lead accumulation in *D. magna*

The results indicated that the wholebody lead accumulation in *Daphnia* increased more or less in a concentration dependent manner over an exposure period of 24h and a concentration range of 0.02-0.6 μM waterborne lead (Figure 3.1). An increase in the waterborne lead concentration from 0.02 μM to 0.2-0.3 μM elevated the wholebody lead burden by approximately three-fold, followed by a five-fold increase of the same at 0.6 μM waterborne lead exposure.

Similar to the concentration dependent accumulation, wholebody lead burden in *Daphnia* increased significantly over time (2-24h) during an exposure to 0.6 μM waterborne lead (Figure 3.2). The wholebody lead concentration increased approximately five-fold and seven-fold at 6h and 12h, respectively, relative to that at 2h. However, lead accumulation seemed to reach a steady state at 12h since there was no further increase from 12h to 24h.

3.2. Effects of essential ions (Ca^{2+}, Mg^{2+} and Na^{+}) on waterborne lead accumulation in *D. magna*

Waterborne calcium had a significant effect on waterborne lead accumulation in *Daphnia* (Figure 3.3). The wholebody lead burden decreased steadily with increasing waterborne calcium level (0.25-2 mM) during an exposure to 0.6 μM lead for 24h. A two-fold and four-fold increase of calcium level in the exposure water, relative to the control (0.25 mM waterborne calcium), reduced wholebody lead burden by approximately 30% and 52%, respectively.

In contrast, waterborne magnesium (Figure 3.4) or waterborne sodium (Figure 3.5) did not influence the lead accumulation in *Daphnia*. Even an eight-fold increase (0.25 to 2.0 mM) in magnesium or sodium in water did not reduce wholebody lead burden in *Daphnia* exposed to 0.6 μM of waterborne lead for 24h.

3.3. Effect of waterborne lead exposure on waterborne Ca^{2+} influx in *D. magna*.

The results indicated that the rate of short-term (1h) waterborne calcium influx increased significantly with increasing range of waterborne calcium concentration (0.25-1.5 mM) both in the absence (control) and presence of waterborne lead (0.3 and 0.6 μM) (Figure 3.6). However, the rate of influx attained saturation at the higher range of calcium level (1.0-1.5 mM). Interestingly, waterborne lead decreased waterborne calcium uptake in a concentration dependent manner across the entire range of waterborne calcium concentration examined except at 1.0-1.5 mM of calcium and 0.3 μM of lead.

Since the waterborne calcium influx was saturable both in control and in the presence of waterborne lead, the Michaelis-Menten model of saturation kinetics was applied: (i) to determine the kinetic properties [apparent J_{max} (maximum rate of uptake) and K_m [substrate concentration at which uptake is half of J_{max} (an index of affinity)] of waterborne calcium uptake, and (ii) to evaluate how those kinetic properties were influenced by waterborne lead exposure. A double reciprocal plot was constructed (Figure 3.7) using the data presented in Figure 3.6, which allowed the estimation of J_{max} and K_m values of calcium influx at different waterborne lead concentrations (presented in Table 1). The J_{max} values at 0.3 μM and 0.6 μM of waterborne lead increased by 14% and 92%, respectively, relative to that in control (0.0 μM waterborne lead). In contrast, the K_m values at 0.3 μM and 0.6 μM of waterborne lead increased by 153% and 1136%.

Table 1. Apparent J_{max} (maximum rate of uptake) and K_m (substrate concentration at which uptake is half of J_{max}) for unidirectional waterborne calcium uptake at various waterborne lead concentrations (derived from Figure 10).

Waterborne lead (μM)	J_{max} (μmol Ca^{2+} g^{-1} wet wt. h^{-1})	K_m (mM Ca)
0.00 (control)	4.00	0.28
0.30	4.54	0.71
0.60	7.69	3.46

3.4. Effects of calcium channel blockers [Lanthanum chloride ($LaCl_3$) and Verapamil] on waterborne calcium influx and lead accumulation in *D. magna*.

Lanthanum chloride (a voltage-independent calcium channel blocker) in water significantly inhibited the rate of short-term (1h) waterborne calcium influx at 0.25 mM calcium concentration in water (experimental water) (Figure 3.8). However, the inhibitory effects were not dose dependent, since both 5 and 10 μM of lanthanum chloride in water produced about six-fold decrease of calcium influx rate in *Daphnia*. In contrast, verapamil (an L-type, voltage-dependent calcium channel blocker) also significantly inhibited the rate of waterborne calcium influx, and the effects were dose dependent (Figure 3.8). Approximately three-fold and five-fold decreases of waterborne calcium influx, in comparison to the control, were recorded at 100 μM and 200 μM of verapamil concentrations in the exposure water, respectively.

Similarly, both lanthanum chloride and verapamil decreased waterborne lead accumulation in *Daphnia* exposed to 0.6 μM of waterborne lead for 24h (Figure 3.9). 5 μM and 10 μM of lanthanum chloride reduced wholebody lead burden by approximately 23% and 33%, respectively. Likewise, 100 μM and 200 μM of verapamil decreased wholebody lead burden by approximately 27% and 40%, respectively.

3.5. Time dependent effects of waterborne lead exposure on Ca^{2+} and Na^{+} influx in *D. magna*

Waterborne lead exposure (0.6 μM) significantly decreased the rate of waterborne calcium influx in *Daphnia* in a time-dependent manner over 24h of exposure (Figure 3.10). The rate of calcium influx remained steady in the absence of waterborne lead (control) over 24h. In contrast, the rate of calcium influx decreased by 27-30% in *Daphnia* exposed to waterborne lead for 6-12h. At the end of the exposure period, the rate of calcium influx was about 45% lower in lead exposed *Daphnia* relative to that in the control population.

As observed in the case of calcium influx, waterborne lead exposure (0.6 μM) significantly decreased the rate of waterborne sodium influx as well in *Daphnia* (Figure 3.11), although the magnitude of decrease did not change after 6h until the end of the exposure (24h). The rate of sodium influx did not change in the control population over 0 to 24h of exposure period. However, the rate of sodium influx decreased by approximately 50% after 6h exposure to waterborne lead, and it remained at that level until the end of the exposure period.

3.6. Effects of waterborne lead exposure on wholebody total calcium and sodium concentration in *D. magna*

Waterborne lead exposure (0.6 μM) did not produce any effect on the wholebody calcium (Figure 3.12) and sodium (Figure 3.13) concentration in *Daphnia* over 24h of exposure. The wholebody calcium level in *Daphnia* exposed to waterborne lead decreased by approximately 30% after 24h of exposure, however the effect was not statistically significant. The wholebody sodium level remained unchanged in both control and lead exposed *Daphnia* populations over the entire range of exposure period.

3.7. Effects of waterborne lead exposure on O_2 consumption rate (respiration rate) in *D. magna*

The waterborne lead (0.6 μM) did not influence the rate of O_2 consumption in *Daphnia* over 24h of exposure (Figure 3.14). The rate of O_2 consumption remained unchanged in both control and lead exposed animals over the entire range of exposure period.

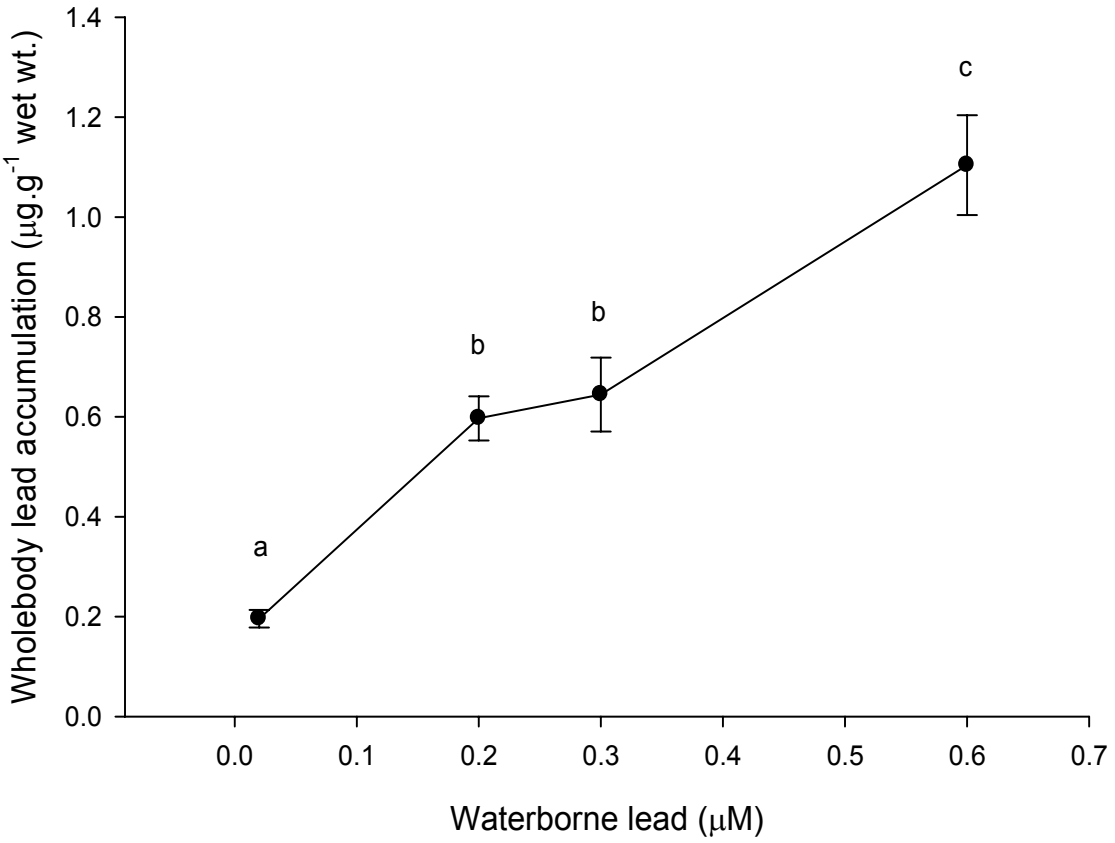

Figure 3.1 Lead accumulations in *Daphnia magna*, exposed to four different concentrations of waterborne lead [0.02, 0.2, 0.3 and 0.6 μM] for 24h. Data presented as mean ± SEM (n = 10). The data were analyzed for statistical significance by one way ANOVA followed by Tukey's multiple comparison tests. Significant differences in waterborne lead accumulation [$F_{(3, 36)}$ = 31.14, $p<0.001$] are indicated by different letters.

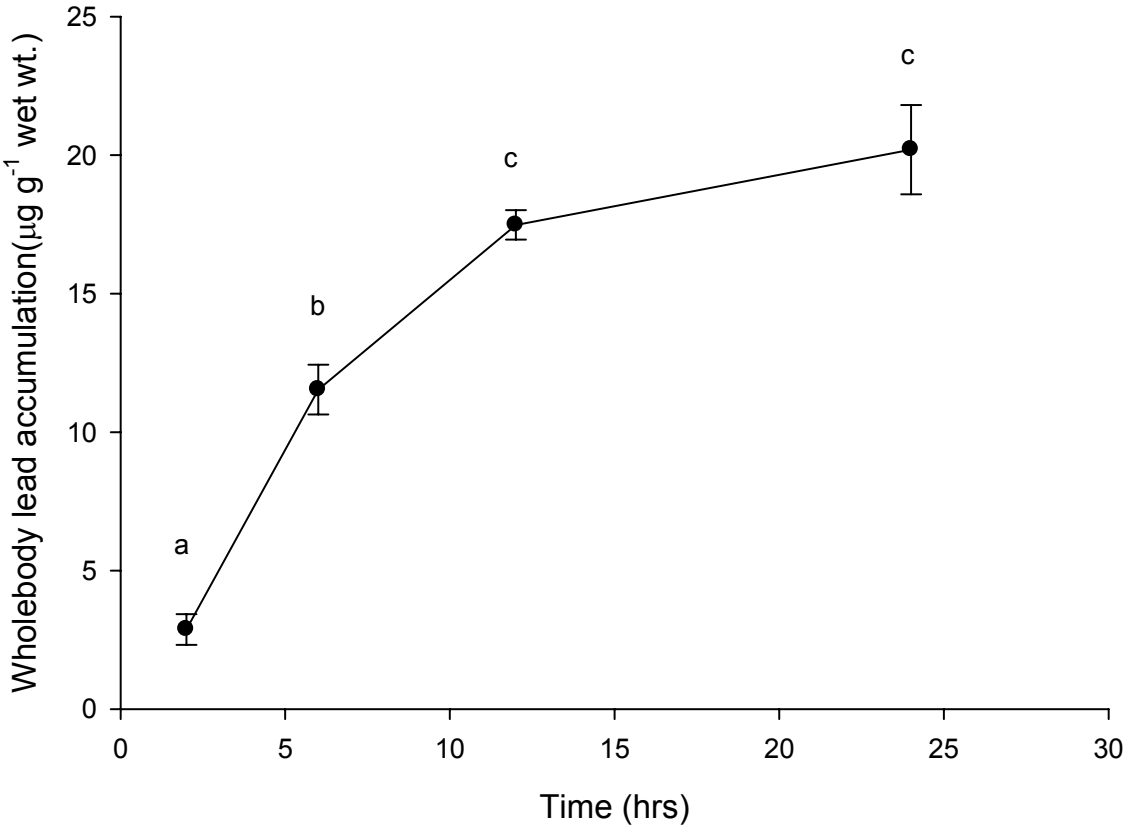

Figure 3.2 Lead accumulations in *Daphnia magna*, exposed to 0.6 μM waterborne lead for four different exposure periods (2h, 6h, 12h and 24h). Data presented as mean ± SEM (n = 10). The data were analyzed for statistical significance by one way ANOVA followed by Tukey's multiple comparison tests. Significant differences in wholebody lead accumulation with time [$F_{(3, 36)} = 58.91$, $p<0.001$] are indicated by different letters.

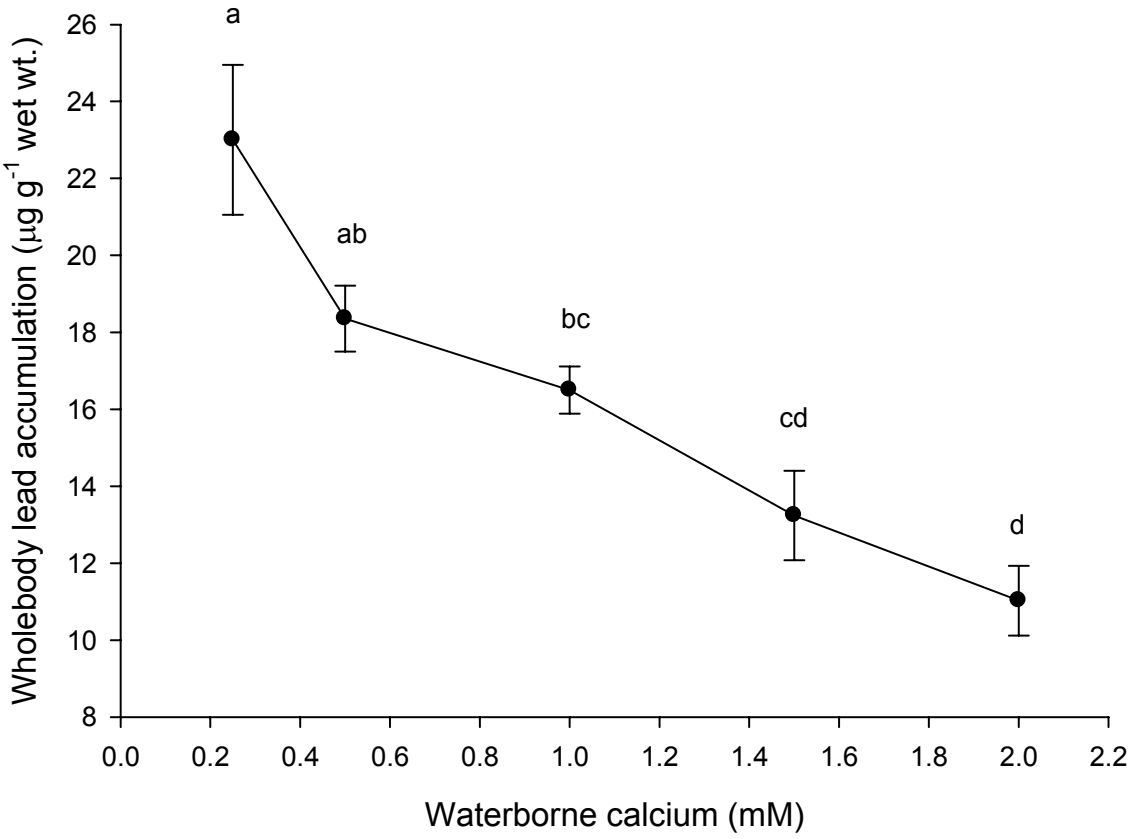

Figure 3.3 Lead accumulation in *Daphnia magna*, exposed to 0.6 μM waterborne lead for 24h at five different waterborne calcium concentrations [0.25 (control), 0.5, 1.0, 1.5 and 2.0 mM]. Data presented as mean ± SEM (n = 10). The data were analyzed for statistical significance by one way ANOVA followed by Tukey's multiple comparison tests. Significant differences in wholebody lead accumulation due to different waterborne calcium [$F_{(3, 36)} = 10.62$, $p<0.001$] are indicated by different letters.

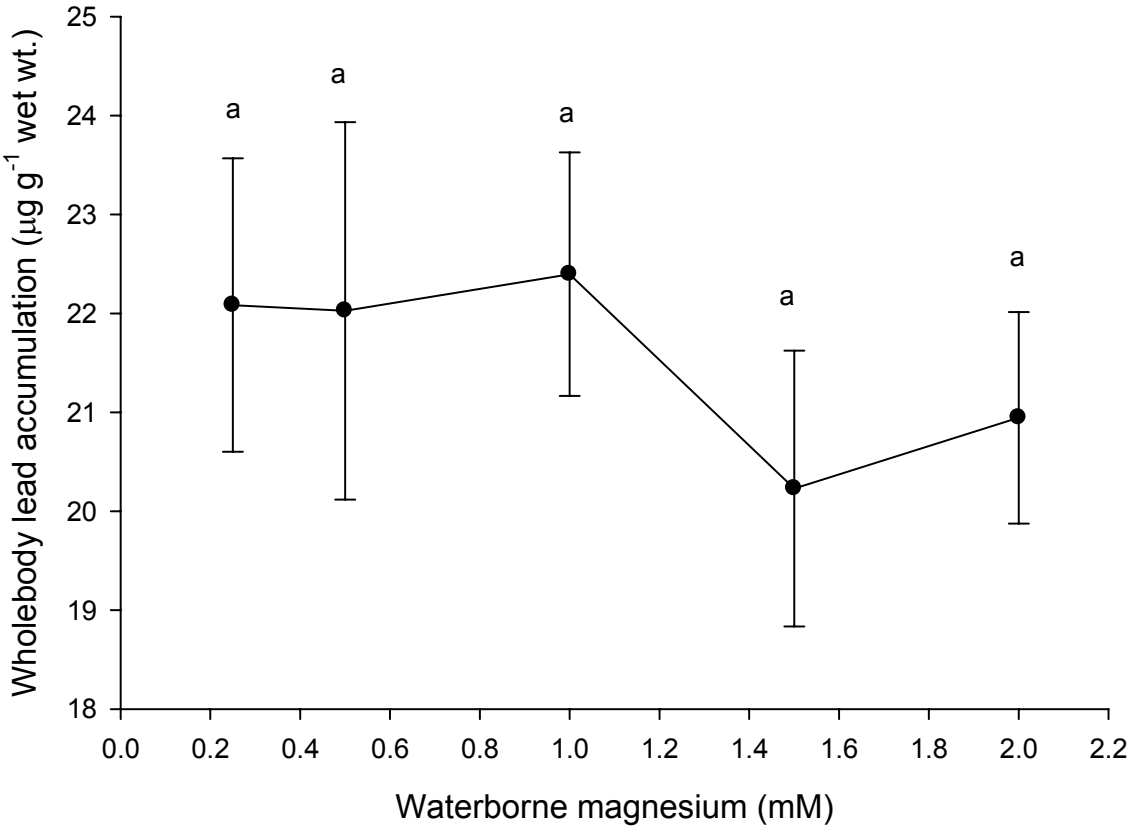

Figure 3.4 Lead accumulation in *Daphnia magna*, exposed to 0.6 μM waterborne lead for 24h at five different waterborne magnesium concentrations [0.25 (control), 0.5, 1.0, 1.5 and 2.0 mM]. Data presented as mean ± SEM (n = 10). The data were analyzed for statistical significance by one way ANOVA followed by Tukey's multiple comparison tests. No significant differences in wholebody lead accumulation due to different waterborne magnesium was recorded [$F_{(4, 45)}$ = 20.88, p=0.81].

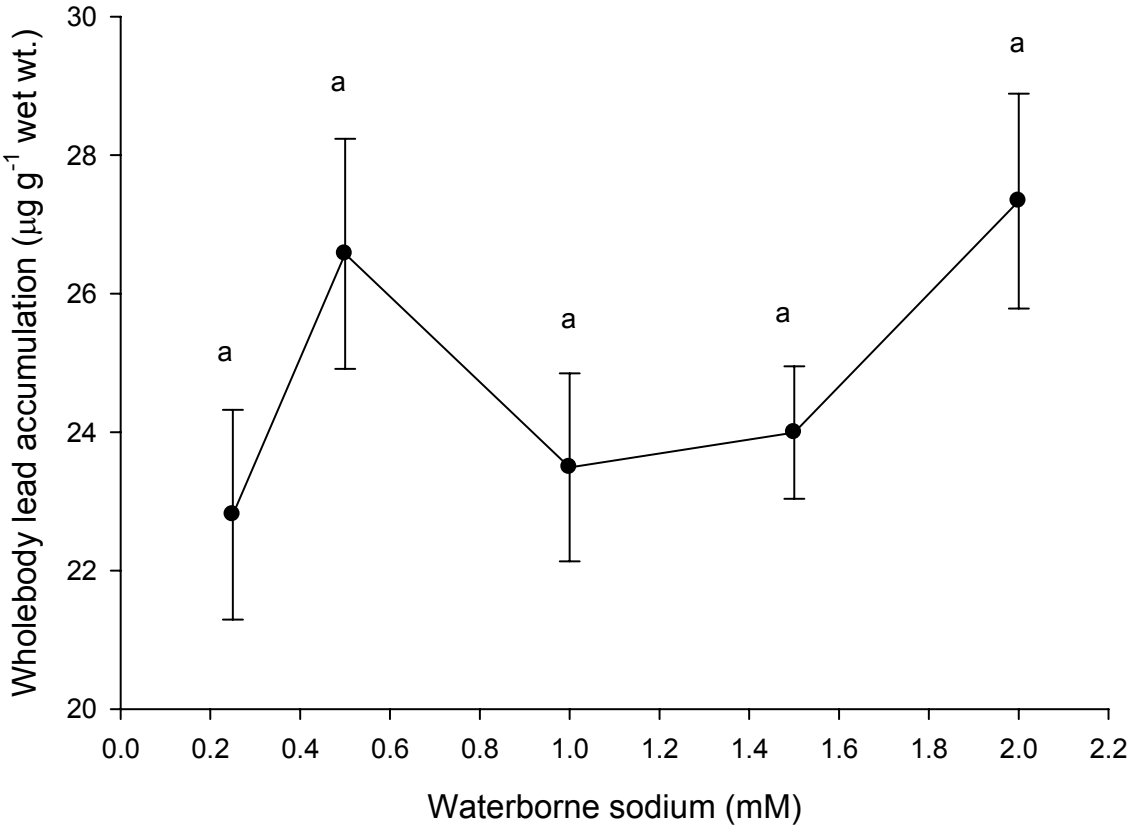

Figure 3.5 Lead accumulation in *Daphnia magna*, exposed to 0.6 μM waterborne lead for 24h at five different waterborne sodium concentrations [0.25 (control), 0.5, 1.0, 1.5 and 2.0 mM]. Data presented as mean ± SEM (n = 10). The data were analyzed for statistical significance by one way ANOVA followed by Tukey's multiple comparison tests. No significant differences in wholebody lead accumulation due to different waterborne sodium was recorded [$F_{(4, 45)} = 20.43$, p=0.12].

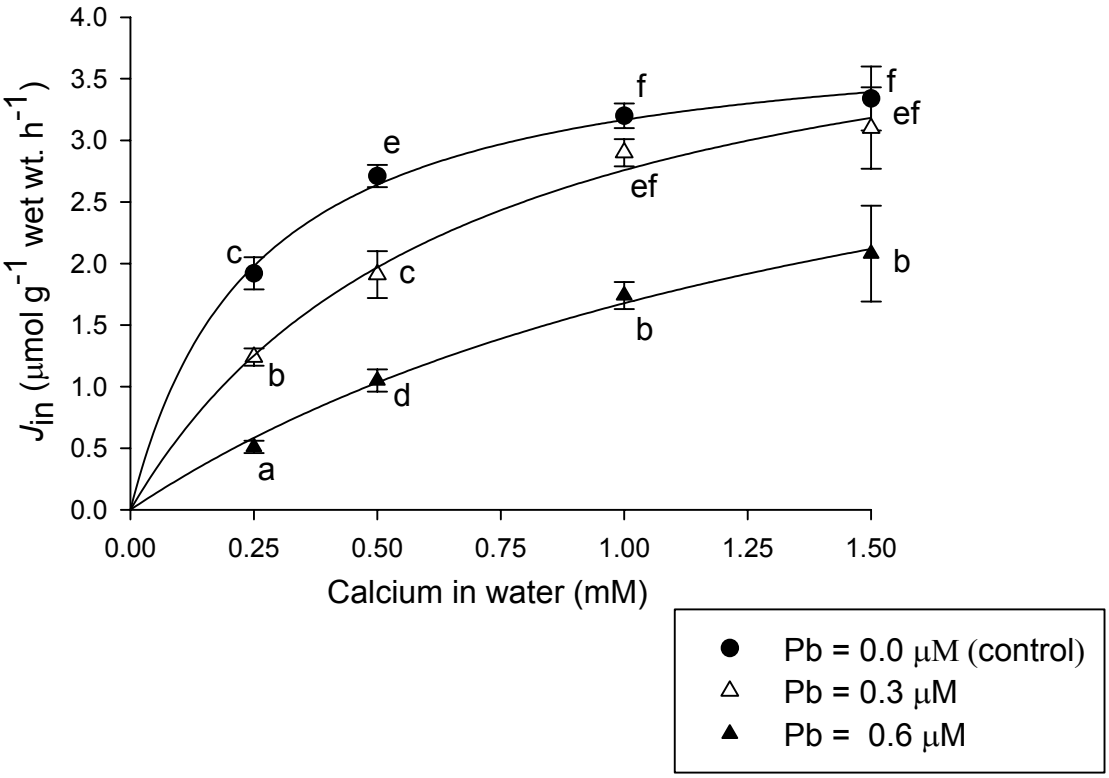

Figure 3.6 Short-term (1h) unidirectional Ca^{2+} influx rate in *Daphnia magna* at various waterborne calcium concentrations [0.25, 0.50, 1.0 and 1.5 mM], and the effect of three different waterborne lead concentrations [0.0 (control), 0.3 and 0.6 µM] on concentration dependent Ca^{2+} influx. The data were analyzed for statistical significance by two way ANOVA followed by Tukey's multiple comparison tests. Significant differences in Calcium influx due to different waterborne calcium [$F_{(3, 72)} = 34.95$, $p<0.001$] and due to different waterborne lead [$F_{(2, 72)} = 24.25$, $p<0.001$] are indicated by different letters. Significant interaction was observed between waterborne lead and waterborne calcium [$F_{(6, 72)} = 2.21$, $p\leq0.05$]

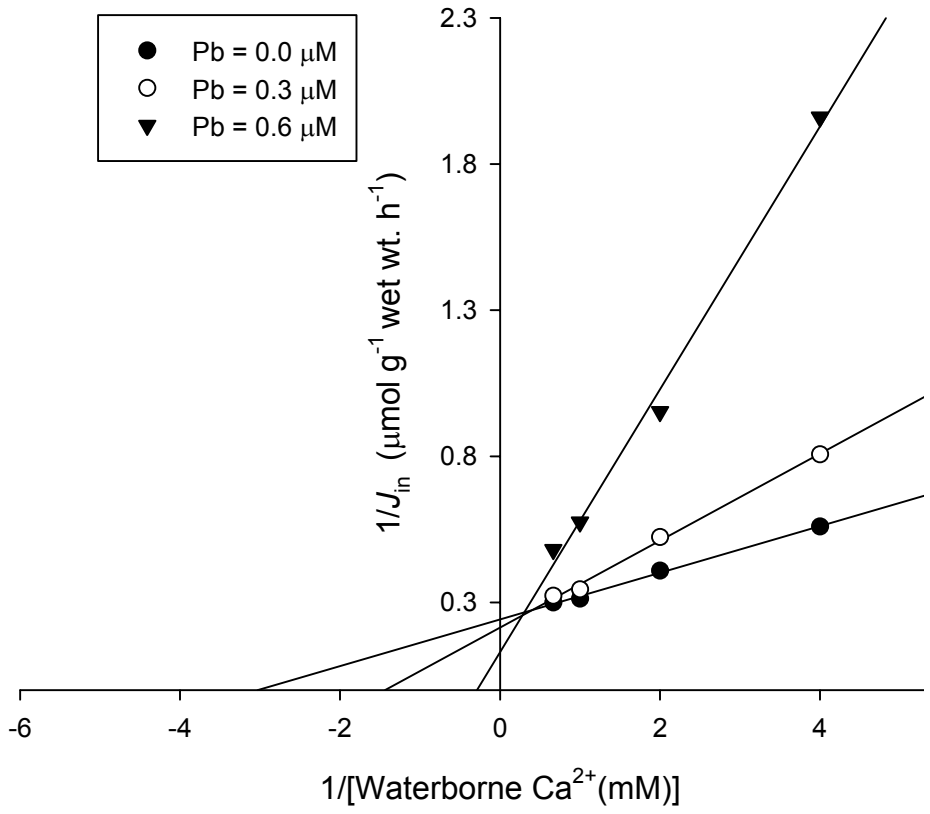

Figure 3.7 Michaelis-Menten analyses by Lineweaver-Burke (double reciprocal) regression plot of data presented in Figure 6. The equation ($y = mx + c$) of the three regression lines were: y = 0.07x + 0.25 ($R^2 = 0.99$) for 0.0 μM waterborne lead (control), y = 0.15x + 0.22 ($R^2 = 0.99$) for 0.3 μM waterborne lead, and y = 0.45x + 0.13 ($R^2 = 0.99$) for 0.6 μM waterborne lead. Significant difference ($p \leq 0.05$) was found at 0.6μM waterborne lead compared to the control (0.0μM) lead.

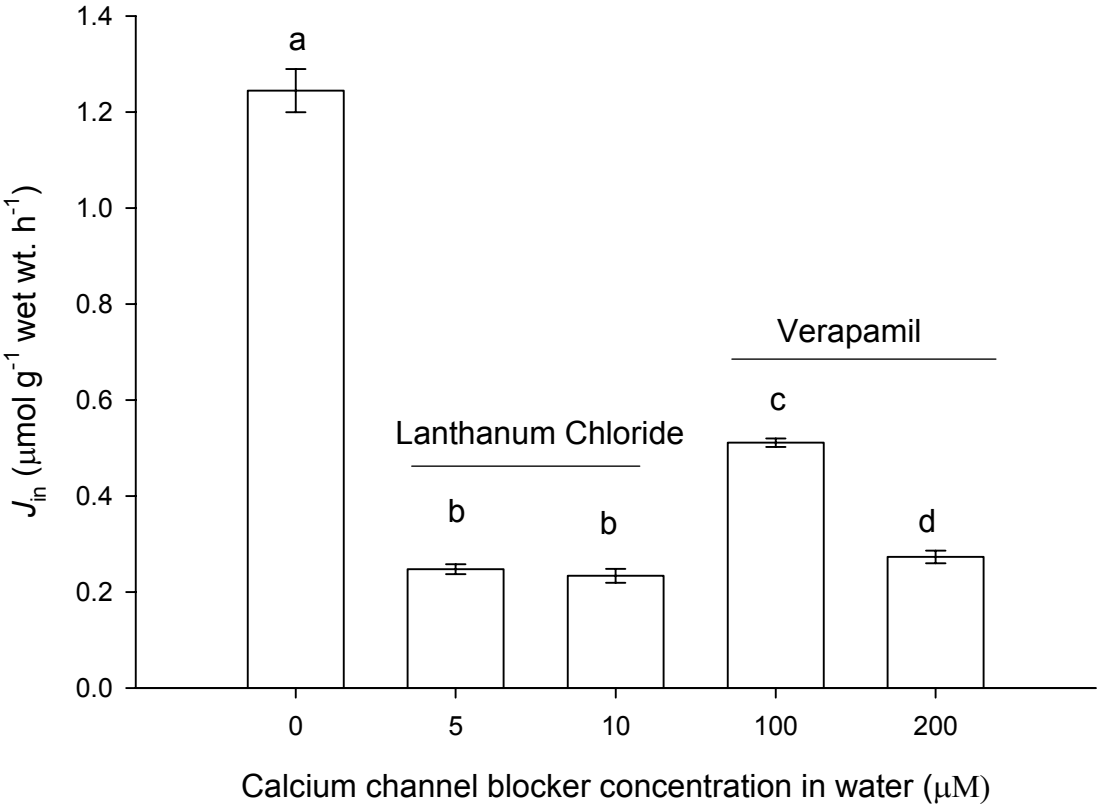

Figure 3.8 Short-term (1h) effects of calcium channel blockers (lanthanum chloride and verapamil) on Ca^{2+} influx rate in *Daphnia magna*, exposed to 0.25 mM of waterborne calcium. Data presented as mean ± SEM (n = 8). The data were analyzed for statistical significance by one way ANOVA followed by Tukey's multiple comparison tests. Significant differences in wholebody lead accumulation in different waterborne calcium channel blockers [$F_{(4, 35)} = 44.60$, $p<0.001$] are indicated by different letters.

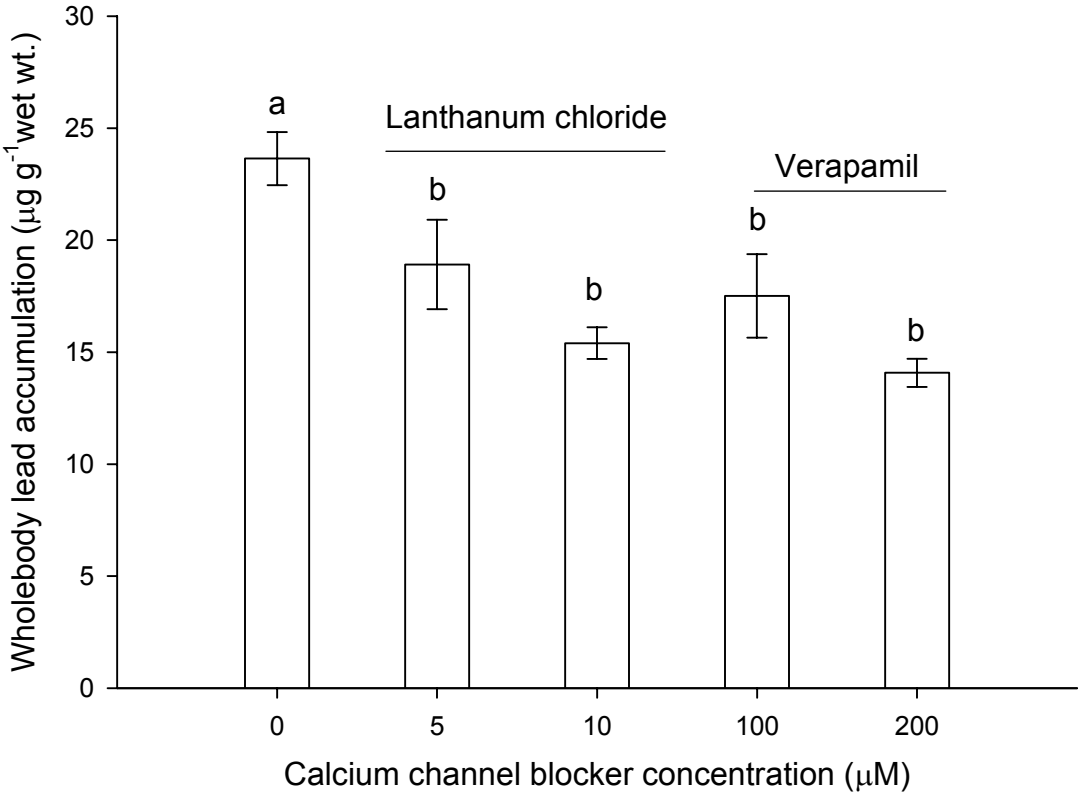

Figure 3.9 Effect of calcium channel blockers (lanthanum chloride and verapamil) on lead accumulation in *Daphnia magna*, exposed to 0.6 μM of waterborne lead for 24h. Data presented as mean ± SEM (n = 10). The data were analyzed for statistical significance by one way ANOVA followed by Tukey's multiple comparison tests. Significant differences in wholebody lead accumulation in different waterborne calcium channel blockers [$F_{(4, 45)} = 7.04$, $p<0.001$] are indicated by different letters.

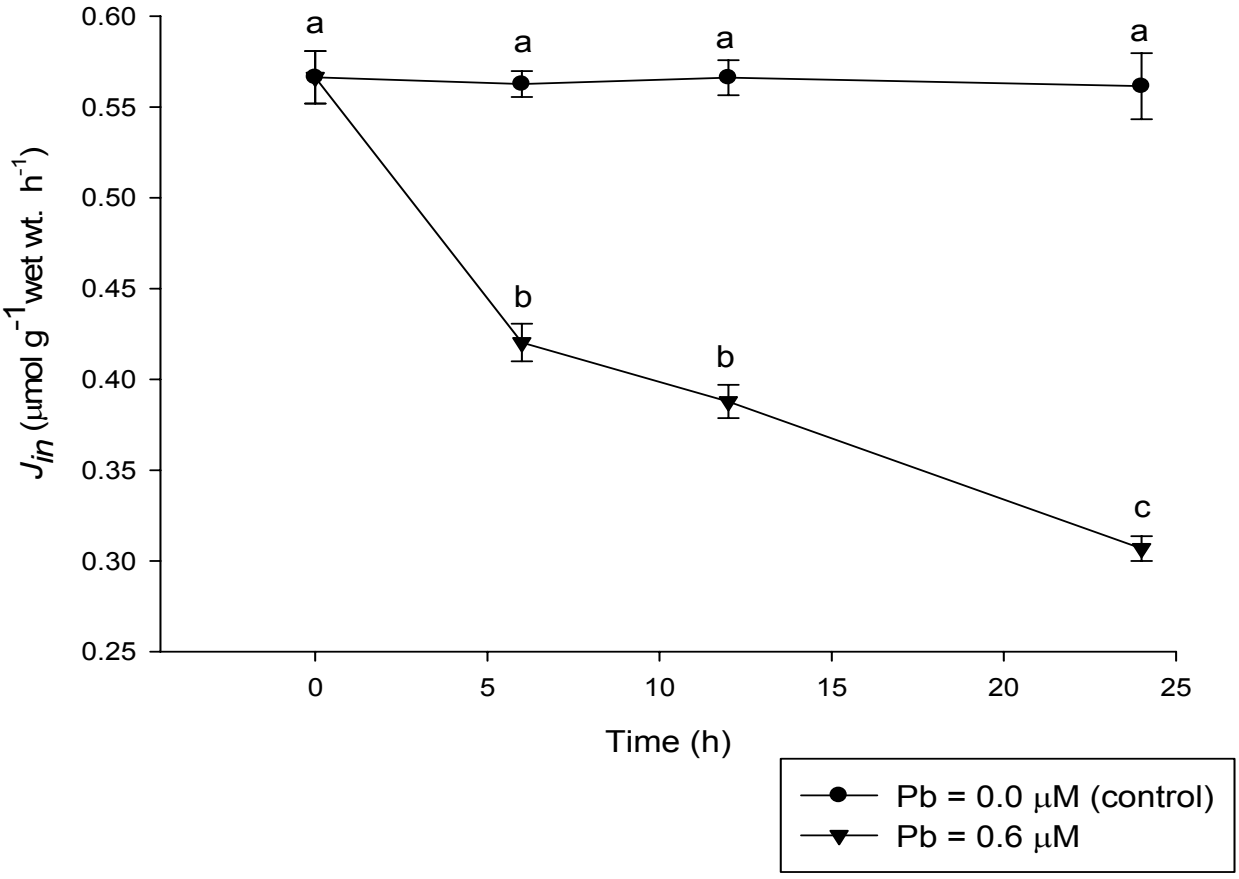

Figure 3.10 Unidirectional Ca^{2+} influx rate in *Daphnia magna*, exposed to two different waterborne lead concentrations [0.0 (control) and 0.6 µM] for various exposure periods (0h, 6h, 12h and 24h). Data presented as mean ± SEM (n = 10). The data were analyzed for statistical significance by two way ANOVA followed by Tukey's multiple comparison tests. Significant differences in wholebody lead accumulation with reference to their respective controls [$F_{(1, 63)}$ = 75.28, $p<0.001$] are indicated by different letters. There was significant differences within the treatment group after 24 hrs [$F_{(2, 65)}$ = 4.61, $p=0.014$]

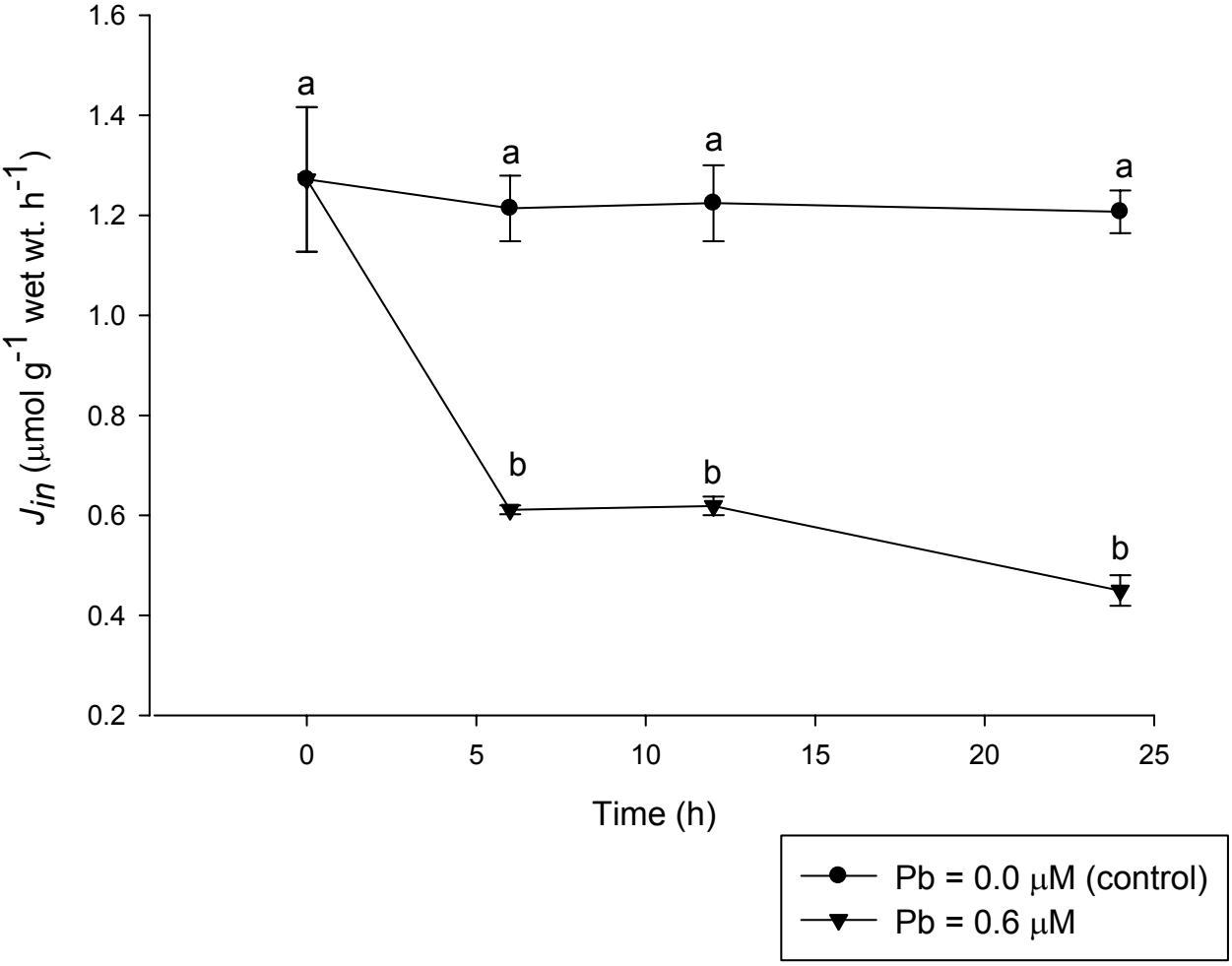

Figure 3.11 Unidirectional Na^+ influx rate in *Daphnia magna*, exposed to two different waterborne lead concentrations [0.0 (control) and 0.6 µM] for various exposure periods (0h, 6h, 12h and 24h). Data presented as mean ± SEM (n = 10). The data were analyzed for statistical significance by two way ANOVA followed by Tukey's multiple comparison tests. Significant differences in wholebody lead accumulation with reference to their respective controls [$F_{(1, 63)}$ = 38.24, $p<0.001$] are indicated by different letters. There was significant differences within the treatment group after 24 hrs [$F_{(2, 63)}$ = 1.53, $p=0.23$]

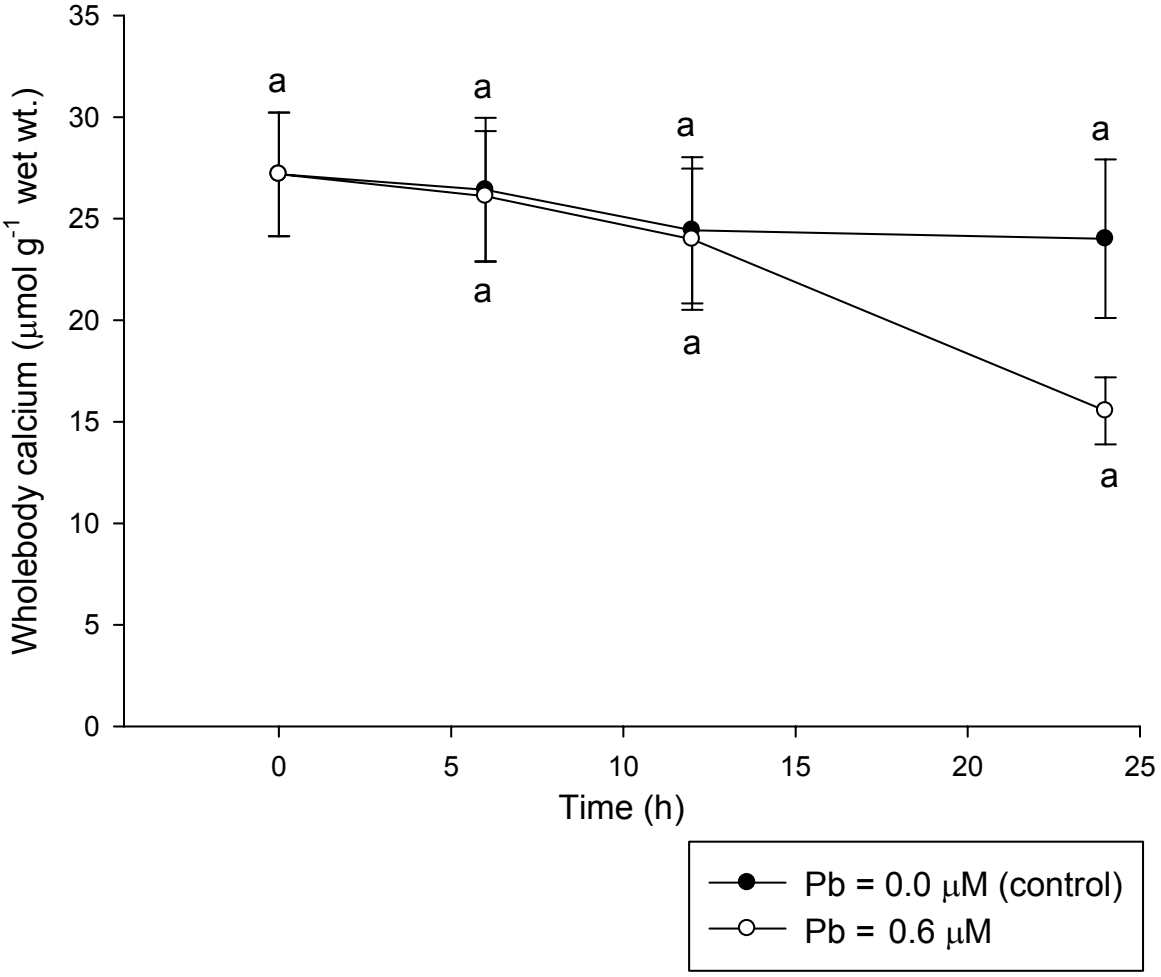

Figure 3.12 Wholebody calcium concentration in *Daphnia magna*, exposed to two different waterborne lead concentrations [0.0 (control) and 0.6 μM] for various exposure periods (0h, 6h, 12h and 24h). Data presented as mean ± SEM (n = 10). The data were analyzed for statistical significance by two way ANOVA followed by Tukey's multiple comparison tests. There was no significant differences in wholebody calcium accumulation with time [$F_{(1, 63)} = 1.005$, p=0.32]. There was no significant interaction between time and treatment [$F_{(1, 63)} = 1.180$, p=0.31].

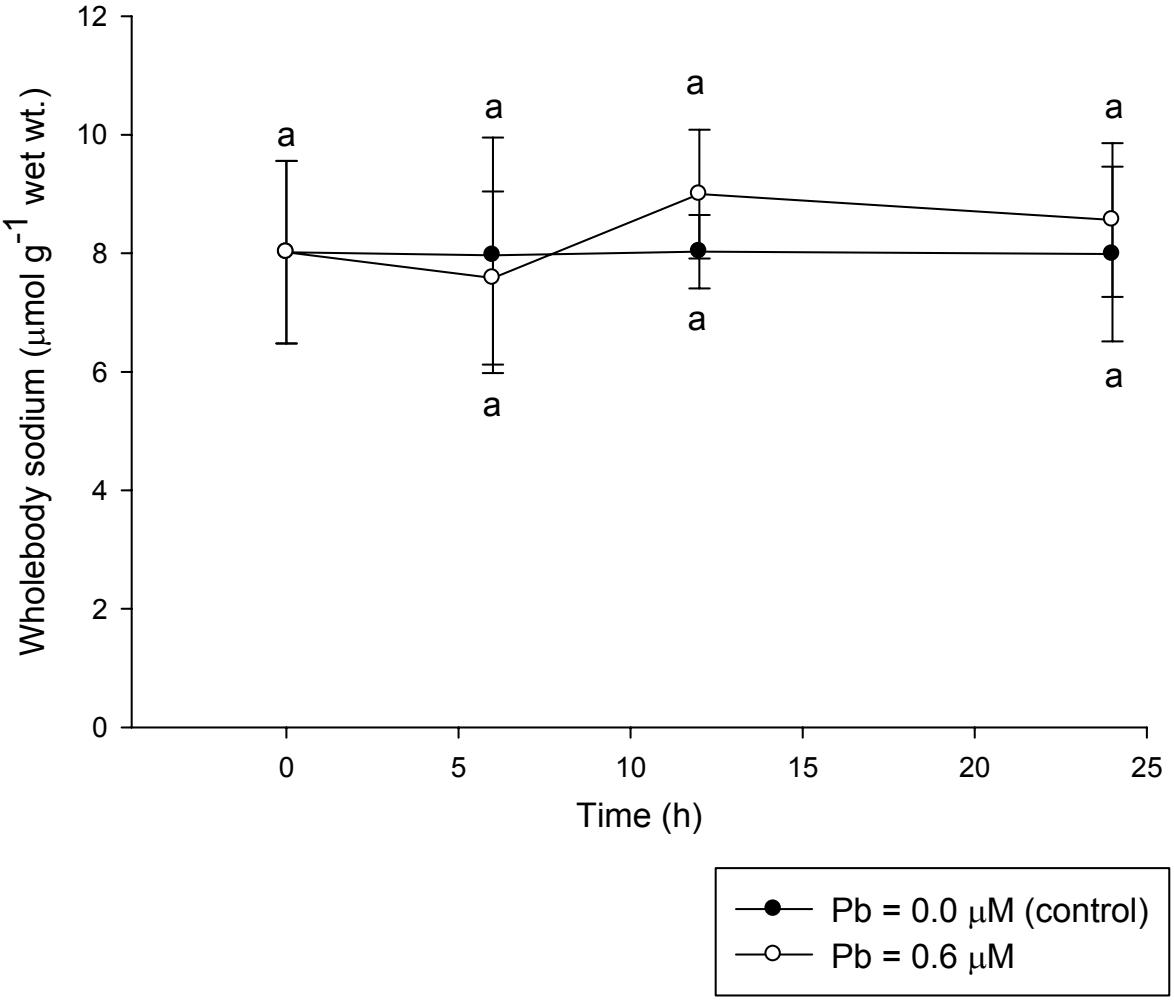

Figure 3.13 Wholebody sodium concentration in *Daphnia magna*, exposed to two different waterborne lead concentrations [0.0 (control) and 0.6 μM] for various exposure periods (0h, 6h, 12h and 24h). Data presented as mean ± SEM (n = 7). The data were analyzed for statistical significance by two way ANOVA followed by Tukey's multiple comparison tests. There was no significant differences in wholebody sodium accumulation with time [$F_{(1, 42)} = 1.58$, p=0.73]. There was no significant interaction between time and treatment [$F_{(2, 42)} = 1.69$, p=0.88].

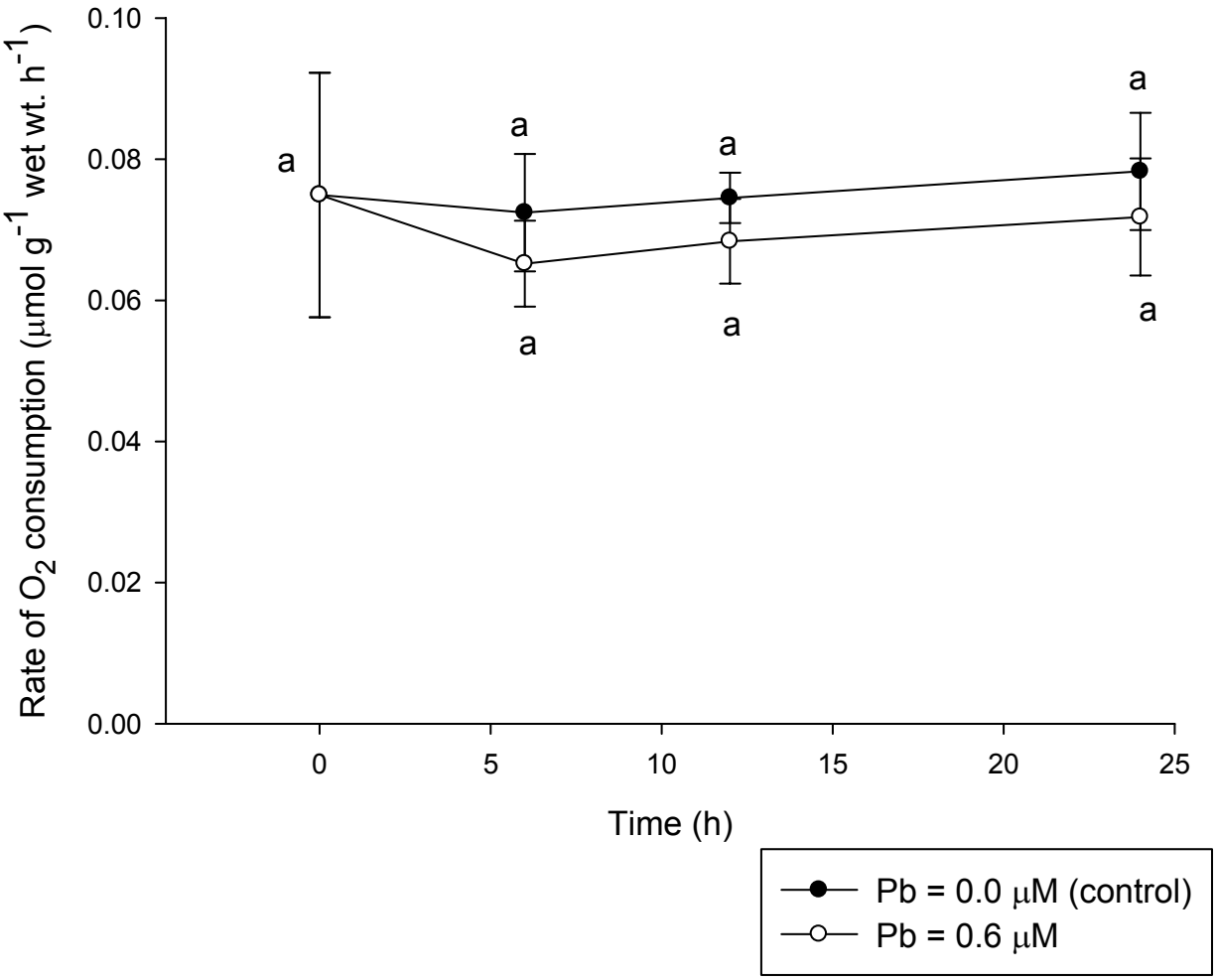

Figure 3.14 The O_2 consumption rate (respiration rate) in *Daphnia magna*, exposed to two different waterborne lead concentrations [0.0 (control) and 0.6 μM] for various exposure periods (0h, 6h, 12h and 24h). Data presented as mean ± SEM (n = 7). The data were analyzed for statistical significance by two way ANOVA followed by Tukey's multiple comparison tests. There was no significant differences in respiration (rate of oxygen consumption) with time [$F_{(1, 42)} = 0.511$, p=0.48]. There was no significant interaction between time and treatment [$F_{2, 42)} = 0.078$, p=0.93].

CHAPTER 4
DISCUSSION

4.1. Characterization of waterborne lead uptake and accumulation in *Daphnia magna*

The results of my experiments indicated that waterborne lead accumulation in *Daphnia* occurs in a concentration and time dependent manner. The lead accumulation seems to reach a steady-state after 12h of exposure to 0.6 μM of waterborne lead, which suggests that the lead uptake pathway in *Daphnia* may be biologically mediated. Grosell et al. (2006b) reported a concentration dependent increase in waterborne lead accumulation in freshwater snail, *L. stagnalis*, exposed to 0-1.2 μM waterborne lead for 30 days. A time dependent increase of lead accumulation in the gill and the wholebody has also been observed in freshwater fish (*P. promelas*) exposed 0.12 μM waterborne lead for 30 days (Grosell et al., 2006a). Thus, the results of my study are in good agreement with the findings of previous studies.

Interestingly, I observed that among the three natural cations (Ca^{2+}, Mg^{2+} and Na^{+}) examined, only Ca^{2+} significantly inhibited waterborne lead accumulation during an exposure to 0.6 μM of lead for 24h. At present, there is no other information available describing the effects of natural cations on the waterborne lead accumulation in any aquatic invertebrates. Macdonald et al. (2002) examined the effects of waterborne Ca^{2+}, Mg^{2+} and Na^{+} on short-term (3h) waterborne lead accumulation in the gills of freshwater fish (*O. mykiss*). They also reported a strong concentration dependent inhibitory effect of waterborne Ca^{2+} (concentration range: 0.018 – 6.0 mM) on lead accumulation in the gills. Similar protective effect of waterborne Ca^{2+} on branchial lead accumulation in freshwater fish has been reported by Rogers and Wood (2004) as well. In addition, MacDonald et al. (2002) reported no notable effect of Mg^{2+} on branchial lead accumulation which is similar to the findings of my experiment. However, they observed inhibitory effects of waterborne Na^{+} on branchial lead accumulation, which is in contradiction with my findings. It is to be noted though that they found inhibitory effects only at a waterborne sodium level of >6.0 mM, whereas the sodium concentration range in my study was 0.2-2.0 mM. The effect of Na^{+} on waterborne lead accumulation reported by Macdonald et al. (2002) has limited physiological significance since the sodium level rarely exceeds 2-2.5 mM in natural freshwater environments (Meyer et al., 2007). Overall, the findings of my study suggest that

waterborne Ca^{2+}, and not Mg^{2+} or Na^{+}, compete with Pb^{2+} at the epithelial uptake sites in *D. magna*.

I recorded a saturable profile of unidirectional waterborne Ca^{2+} influx in *Daphnia* exposed to waterborne calcium levels of 0.25-1.50 mM, both in control (0 μM lead) as well as 0.3 and 0.6 μM of lead in the exposure water. However, the rate of Ca^{2+} influx decreased with increasing lead concentration in the water at almost all waterborne calcium levels examined, indicating a strong interaction between waterborne Ca^{2+} and Pb^{2+}. The kinetic analysis of waterborne Ca^{2+} influx rate using Michaelis-Menten model revealed that the J_{max} values (the maximum rate of Ca^{2+} transport) increased moderately (14-92%) with increasing lead concentration in the water, whereas a large increase (153-1136%) of the K_m values (affinity of Ca^{2+} transport) were observed under the same conditions. In Michaelis-Menten model, a competitive interaction between the two substrates is characterized by an increase of K_m and no change in J_{max}. Alternatively, an altration in J_{max} and no change in K_m signify a non-competitive interaction. Therefore, it appears that the interaction between waterborne Ca^{2+} and Pb^{2+} in *D. magna* is predominantly competitive in nature , although the possibility of a small but notable non-competitive component cannot be ruled out particularly at the highest waterborne lead level tested (0.6 μM). Rogers and Wood (2004) reported a K_m value of 0.16 mM waterborne calcium for branchial Ca^{2+} uptake in control (0.0 μM lead) in rainbow trout exposed to a similar range of waterborne calcium concentration (0.2–1.4 mM). In contrast, I found an almost two-fold higher K_m value (0.28 mM waterborne calcium) of waterborne Ca^{2+} uptake in *Daphnia* under control condition (0.0 μM lead). The difference in the K_m values between the two species suggests that the affinity of the waterborne Ca^{2+} uptake pathway in *Daphnia* is considerably higher relative to freshwater fish. Similarly, the J_{max} value in Daphnia was found to be about thirty-fold higher than that in fish (Rogers and Wood, 2004) under the same experimental conditions. These observations are not unreasonable since the requirement of Ca^{2+} in the crustaceans are much higher relative to the fish because of their calcareous carapace and molting activity (Ahearn et al., 1999). Moreover, Rogers and Wood (2004) also reported a significantly large increase (186-1051%) of K_m and a relatively small increase (7-15%) of J_{max} in fish exposed to 0-4.8 μM of waterborne lead. Thus, the kinetic properties of waterborne Ca^{2+} and Pb^{2+} interaction recorded in my study are in good agreement with their findings, thereby suggesting that waterborne Ca^{2+} and Pb^{2+} interact in a similar way in both *Daphnia* and fish.

In my study, the examination of the effects of calcium channel blockers (lanthanum and verapamil) on waterborne Ca^{2+} uptake in *D. magna* revealed that it was inhibited by both of the calcium channel blockers tested, although the effects of lanthanum was somewhat stronger than verapamil. Similarly, the waterborne lead accumulation was also inhibited significantly by both lanthanum and verapamil, and the effects of both calcium channel blockers were similar in magnitude. These findings suggest that waterborne Ca^{2+} and Pb^{2+} enter into the body of *Daphnia* through a common pathway (i.e., epithelial calcium channels). It is believed that the mechanism of action of lanthanum is quite different from that of verapamil. Lanthanum is known to inactivate the voltage-independent calcium channels by binding to the Ca^{2+}-binding sites of the ion channels and thereby blocking the passage of Ca^{2+} through the channel (Weiss, 1974). In contrast, verapamil is known to block L-type, voltage-dependent calcium channels by binding to a specific receptor domain of the ion channels (Hosey and Lazdunski, 1988). My results are in contradiction, in part, with similar studies conducted in freshwater fish. Rogers and Wood (2004) observed that waterborne lead accumulation in rainbow trout was significantly inhibited by lanthanum (0.001 to 1 μM), however they did not find any reduction in branchial lead accumulation by either verapamil or nifedipine (another L-type, voltage-dependent calcium channel blocker) (both at 100 μM). The relevance of waterborne lead uptake apparently *via* an epithelial L-type, voltage-dependent calcium channels is not clear, however it is to be noted that verapamil has been reported to block voltage-independent calcium channels as well in mammalian systems (Hughes et al., 1986). Therefore, it is possible that the inhibitory effects of both lanthanum and verapamil on waterborne Ca^{2+} and Pb^{2+} uptake observed in *Daphnia* involved voltage-independent calcium channels only. Craig et al. (1999) examined the effects lanthanum and verapamil on cadmium uptake in freshwater insect, *Chironomus staegeri*, exposed to 50 nM waterborne cadmium for 36h. Cd^{2+}, like Pb^{2+}, is a known Ca^{2+} antagonist and shares a common uptake pathway with Ca^{2+} in fish gill (Verbost et al., 1987; Niyogi and Wood, 2004). Interestingly, Craig et al. (1999) observed about 73-92 % inhibition of waterborne cadmium accumulation by lanthanum (10 and 100 μM) and about 59-85% inhibition of the same by verapamil (100 and 300 μM). Thus, it appears that the pharmacological properties of waterborne Cd^{2+} and Pb^{2+} uptake are similar in freshwater invertebrates. Overall, the findings of my study suggest that waterborne Pb^{2+} uptake in *D. magna* occurs *via* both lanthanum-sensitive and verapamil-sensitive epithelial calcium channels.

4.2. Physiological basis of waterborne lead toxicity in *Daphnia magna*

The rate of waterborne Ca^{2+} and Na^{+} influx remained steady in the control (0 μM lead) over a period of 24h. These results are consistent with the previous observations in *D. magna* reported by Pane et al. (2003a). They found that waterborne Ca^{2+} and Na^{+} influx remained unchanged under control condition for up to 48h in Daphnia. Interestingly, they observed that unlike in *Daphnia* supplied with food as in my study, waterborne Ca^{2+} influx rate decreases significantly over time in starved *Daphnia*. However, Pane et al. (2003a) recorded about 2-4 fold higher rate of both waterborne Ca^{2+} and Na^{+} uptake in *D. magna* compared to the rates observed in my study. There may be two reasons for that: (i) the animals used in my study were older in age (7-8 day) relative to theirs (4 day), and (ii) the animals used in my experiment were cultured in a water containing much higher calcium and sodium level (Ca: 0.7 mM; Na: 1.8 mM) than theirs (Ca: 0.25 mM; Na: 0.4 mM). Both of these factors may have contributed towards lower Ca^{2+} and Na^{+} requirement in Daphnia used in my experiments.

The results of my study indicated that waterborne lead inhibited both waterborne Ca^{2+} and Na^{+} influx rates in *D. magna* during an acute exposure for 24h. However, the magnitude of the inhibition appeared to increase with time for Ca^{2+} uptake only. In contrast, the magnitude of decrease in the rate of Na^{+} uptake remained at a steady level (approximately 50%) from 6h until the end of the exposure. The gradual time-dependent decrease in the rate of waterborne Ca^{2+} influx occurred with a simultaneous time-dependent increase in the wholebody lead accumulation, which further supported the observation that waterborne Ca^{2+} and Pb^{2+} directly compete with each other for uptake in *Daphnia*. The comparison of the effects of waterborne lead exposure on time-dependent Na^{+} uptake rate and wholebody lead accumulation suggests that Pb^{2+} probably interacts with Na^{+} in an indirect manner. No statistically significant change was recorded in either wholebody calcium or sodium level in *D. magna* during the acute exposure to waterborne lead for 24h, although the reduction of wholebody calcium concentration recoded at the end of the exposure was notable. These findings suggest that while waterborne Ca^{2+} and Na^{+} influx were disrupted in *Daphnia*, Ca^{2+} and Na^{+} homeostasis were maintained during the acute lead exposure.

The effects of chronic waterborne lead exposure on essential ion uptake and homeostasis in freshwater animals (both invertebrates and fish) were investigated by other workers. Recently, Grosell and Brix (2009) demonstrated that Ca^{2+} influx in freshwater snails (*L. stagnalis*) was

inhibited by about 39% after 21 day chronic exposure to waterborne lead (0.1 μM), although they did not record any significant change in waterborne Na^+ influx rate. They also reported a significant decrease of total calcium and sodium levels in the soft tissue of snails exposed to very low levels (0.007 and 0.04 μM) of waterborne lead, which suggested the disruption of Ca^{2+} and Na^+ homeostasis in their body. Similar observations were also reported in freshwater crayfish (*C. destructor*) by Ahearn and Morris (1998). They recorded a 50% decrease of waterborne Na^+ influx and a concomitant 40% inhibition of gill Na^+-K^+-ATPase in crayfish exposed to 2.4 μM of waterborne lead for 21days, which indicated that lead disrupts Na^+ uptake from the water by inactivating the Na^+-K^+-ATPase enzyme. Ahearn and Morris (1998) did not examine the effects of waterborne lead exposure on waterborne Ca^{2+} influx, nonetheless they observed a 37% decrease in haemolymph calcium concentration in lead-exposed crayfish. However, they did not find any significant change in any other haemolymph ion concentrations (e.g., sodium, potassium, magnesium and chloride). The effects of waterborne lead exposure on waterborne Cl^- uptake was not evaluated in my study, but waterborne lead has been reported to stimulate Cl^- influx in freshwater snails (*L. stagnalis*) after exposure to 0.1 μM of waterborne lead for 21 days (Grosell and Brix 2009). Interestingly, a significant increase in haemolymph pH (metabolic alkalosis) was also recorded in the snails following exposure to lead. Grosell and Brix (2009) argued that the Cl^- uptake was stimulated to remove the excess HCO_3^- (resulted from higher CO_2 production) from the body by epithelial HCO_3^-/Cl^- exchange. It is unlikely that lead exposure might have produced similar induction on Cl^- influx in *D. magna*, since lead exposure did not influence the metabolic rate (rate of O_2 consumption) of *Daphnia* in my study.

In freshwater fish (*O. mykiss*), a 65% inhibition of waterborne Ca^{2+} influx along with a simultaneous 40-50% inhibition of Na^+ and Cl^- influx were recorded during 0-48h exposure to acute waterborne lead concentration (5.3 μM) (Rogers et al., 2003). Rogers and Wood (2004) later demonstrated that waterborne lead disrupts waterborne Ca^{2+} uptake and homeostasis in fish by a competitive inhibition of apical Ca^{2+} entry in fish gills through lanthanum-sensitive Ca^{2+} channels and inactivation of basolateral ATP-driven Ca^{2+}-pump. Similarly, Rogers et al. (2005) demonstrated that lead-induced disruption of Na^+ and Cl^- homeostasis in fish occurs in part due to rapid binding of lead to Na^+-K^+-ATPase and carbonic anhydrase enzymes, causing a non-competitive inhibition of Na^+ and Cl^- uptake. Overall, the lead-induced disruption of Ca^{2+} and Na^+ influx observed in my study are quite consistent with similar observations in other

freshwater animals, both invertebrates and fish. The lack of effect on wholebody calcium and sodium level in Daphnia exposed to acute lead concentration may be due to the relatively shorter exposure period used in my study. It is also plausible that lead exposure induced the mobilization of calcium and sodium from internal storage pool in *Daphnia*.

The rate of oxygen consumption in *D. magna* (both control and lead-exposed) recorded in my experiment was comparable to the rate previously reported by Pane et. al., (2003a). The rate did not change significantly in lead-exposed *D. magna* relative to control (0 μM lead) population during the entire exposure period (24h). This observation indicates that no acute respiratory toxicity occurs in *Daphnia* following acute waterborne lead exposure. Berglind et al. (1985) observed an inhibition of ALAD activity as well as low haemoglobin content in *D. magna* exposed to 1.2 μM of waterborne lead after 48 hrs, however they did not examine whether the low hemoglobin level had any effect on their respiratory capacity. *Daphnia* have no specialized respiratory epithelium; instead they exchange gases across the entire integument with O_2 uptake facilitated by hemoglobin in the open haemocoele (Wiggins and Frapppell, 1999). The lack of specialized respiratory epithelium and juvenile stage (high surface area-to-volume ratio) of the individuals used in my experiments may have rendered *Daphnia* less vulnerable to respiratory damage by lead, a potential respiratory toxicant.

4.3. Conclusion and environmental perspectives

To the best of knowledge, this is the first study to characterize the pathway of waterborne lead uptake and to elucidate the mechanism of acute toxicity of lead in a freshwater invertebrate. Overall, my study has revealed that *Daphnia* have the ability to rapidly accumulate lead from water during acute exposure. Waterborne Pb^{2+} inhibits waterborne Ca^{2+} uptake in *Daphnia* in a concentration dependent manner, and this inhibition occurs at least partially through a direct competitive interaction between Pb^{2+} and Ca^{2+} at the epithelial surface. The entry of waterborne Pb^{2+} in *Daphnia* occurs *via* both lanthanum-sensitive and verapamil-sensitive epithelial calcium channels. Acute waterborne lead exposure severely disrupts both Ca^{2+} and Na^{+} uptake from water. Such effects can ultimately lead to the disruption of Ca^{2+} and Na^{+} homeostasis in *Daphnia*, despite the present study didn't reflect that notion. Acute exposure to lead however

does not cause acute respiratory toxicity in *Daphnia*. Thus, it can be concluded that waterborne lead causes toxicity to *Daphnia* during acute exposure by acting as an ionoregulatory toxicant.

Daphnia is regarded as one of the most preferred species for the risk assessment of metals in natural waters. Presently, *Daphnia* based predictive models such as the biotic ligand models (BLM) are currently used for deriving site-specific AWQC for metals like copper and zinc in many environmental jurisdictions. The BLM is a physiologically based mechanistic model which relates metal bioavailability in the ambient water to the toxicity in resident animals. Unlike the conventional approaches which only accounts for the water hardness, the BLM provides a framework which allows incorporation of all aspects of water chemistry to the water quality regulation for metals, and not just the water hardness alone. The development of BLM for any specific metal requires knowledge of the mechanisms of its uptake and toxicity. At present, there is no BLM available for performing water quality regulations for lead. Therefore, I believe that the findings of my study will help towards developing a *Daphnia* BLM for lead.

CHAPTER 5
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