The Effect of Cadmium Chloride on the Biology of *Gammarus pulex*

(Crustacea: Amphipoda)

Submitted by

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature)...**……**

Abstract

Increased releases of cadmium to the aquatic environment have raised concern over the potential for adverse impacts on freshwater organisms in affected aquatic bodies. This thesis explored the responses of a common freshwater amphipod, *Gammarus pulex (G. pulex)* to sublethal concentrations of cadmium under different environmental conditions and at various stages of the lifestyle. Endpoints studied encompassed molecular, cellular, physiological and behavioural changes, to enable a comprehensive analysis of the effects of the organism.

Exposure to sublethal concentrations of cadmium (Cd) (0.001, 0.005 and 0.01 mg Cd L^{-1}) for 7 and 14 days, influenced the percent survival, induced lipid peroxidation and damaged DNA in haemolymph cells of *G. pulex*. These concentrations also reduced feeding and ventilation rates as well as the motility, in particular of the females, with increasing Cd concentration and time of exposure. Furthermore, Cd accumulation from water in the body tissues of the amphipods was lower than in their food, with increasing Cd concentrations. These results illustrate how concentrations of Cd below the Environmental Quality Standard for European waters can cause sublethal molecular and cellular damage after relatively short periods of exposure.

Histopathological effects of Cd on the gills and hepatopancreas (mid-gut gland) were examined using light, scanning and transmission electron microscopy. Multiple effects were observed on the tissues and cell organelles including external alternations in the muscular fibres of the hepatopancreas, lysis of microvilli and morphological changes in mitochondria. Exposure to 0.01 mg Cd L^{-1} altered the epithelial layer of the gill, causing vacuolation and lysis across the whole gill structure. Mitochondria showed damage to the inner membrane, shortened cristae and swelling, with an increase in apoptosis at concentrations of 0.005 and 0.01 mg Cd L^{-1}. Collectively, these results document the adverse effects of Cd on target organs at concentrations within the range found in freshwater bodies.

An investigation of the effects of water hardness on bioaccumulation and toxicity showed that hardness of water inhibited Cd toxicity and protected the juveniles during long term exposures, without affecting growth rate and food consumption. Soft water reduced the rate of survival, growth rate and food consumption during chronic exposure to low Cd concentrations, and facilitated Cd accumulation in the body parts compared with juveniles exposed in hard water. The results show that water quality plays a vital role in reducing or increasing detrimental effects of low Cd concentrations on the early life stage of amphipods which are a source of food for many species in aquatic environments.

Exposure to Cd led to an increase in metallothionein concentrations in the amphipods in both hard and soft water. It also caused disruption to ion/osmoregulation, which may represent one mechanism of compensation for the ions lost in the amphipods exposed to Cd in soft water.

In conclusion, these results add to the body of evidence describing the sublethal toxicity of Cd, a priority pollutant, to a common freshwater sentinel species. These results are of relevance for future environmental management and remediation approaches, because they provide scientific data to help in assessing, interpreting and understanding the effects of the heavy metal Cd in freshwater environments.

Dedication

To the spirit of my parents

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Table of contents

List of Tables

List of Figures

List of abbreviations

Å	Angstrom
AAS	Atomic absorption spectrometry
AIS	Apical Infolding System
Amu	Atomic mass unit
ANOVA	Analysis of Variance
BHT	Butylated hydroxytoluene
BIS	Basal Infolding System
BSA	Bovine Serum Albumin
^{o}C	Degree Celsius
Ca	Calcium
$CaCO_3$	Calcium Carbonate
$CaNa_2O_6.4H_2O$	calcium nitrate tetrahydrate
Cd	Cadmium
Cl	Chloride
cm	circular muscles
cm	centimeter
cm^3	cubic centimeter
DO	Dissolved Oxygen
DNA	Deoxyribonucleic Acid
DTNB	(5,5 dithiobis-2nitrobenzoic acid)
DTT	L-Dithiothreitol
d. wt	dry weight
E	Eosin
EDTA	Ethylenediaminetetraacetic Acid

$FeSO_4$	Ferrous Sulfate (iron (II) sulfate)
FR	Feeding rate
GSH	Glutathione
H	Hour
H	Hematoxylin
HL	Haemolymph
HC	Haemolymphatic canal
HL	Haemolymphatic lacuna
HO	Haemolymph osmolality
$HO^{\bullet}_2$	hydroperoxyl radicals
HNO_3	Nitric Acid
H_2SO_4	Sulfuric Acid
HP	Hepatopancreas
HW	Hard water
IARC	International Agency for Research on Cancer
IPCS	International Programme on Chemical Safety
IUPAC	International Union of Pure and Applied Chemistry
K	Potassium
K	Leaf change correction factor
KCl	Potassium Chloride
Kg	Kilogram
L	Litre
Lb	Initial dry weight of the leaf disc
LC_{50}	Median lethal concentration
LMA	low melting point agarose

lm	longitudinal muscles
M	Mitochondria
mA	Milliamp
mb	Millibar
MDA	Malondialdehyde
MFB	multispecies freshwater biomonitor
Mg	Magnesium
Mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
mm	Millimetre
mmol	Millimole
ml	milliliter
mOsm	Milliosmole
MT	Metallothionein
Mv	Microvilli
N	number of replicates
Na	Sodium
Na_2ATP	Adenosine 5`-triphosphate disodium salt hydrate
Na^+/K^+-ATPase	Sodium–potassium ATPase
Ng	Nanogram
nm	Nanometre
nmol	Nanomol
Nu	nucleus
$O^{\bullet -}_2$	superoxide radicals

OD	optical density
OECD	Organisation for Economic Co-operation and Development
$OH^{\bullet}$	hydroxyl radicals
Pbm	pleopod beat per minute
PBS	Phosphate Buffered Saline
PC	Pillar Cells
PCD	Programmed Cell Death
pH	Hydrogen ion concentration
Pi	Phosphate
ppm	Parts per million
PVC	Plastic polyvinyl chloride
PMSF	Phenylmethylsulphonyl Fluoride
RT	Remaining tissues
ROS	Reactive Oxygen Species
rpm	Rotation per minute
SCGE	Single cell gel electrophoresis
SEM	Scanning Electron Microscope
SEM	Standard Error
SH	Sulfhydryl group
SPSS	Statistical Package for the Social Sciences
SW	Soft water
TBA	Thiobarbituric acid
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic acid
TDS	Total Dissolved Solid

TEM	Transmission Electron Microscope
TH	Total hardness
TL	Thoracic leg
Tris	Trisaminomethane
UNEPA	United Nations Environment Programme Agency
µg	Microgram
µl	Microliter
µS/cm	Microsiemens per centimetre
WHO	World Health Organisation
w wt	wet weight
V	Volte

Chapter 1 General introduction

1.1 Heavy metals

Development in industrialisation and urbanisation has caused an increase in pollutants in the environment which have affected negatively on living organisms as well as human beings (Brzóska and Moniuszko-Jakoniuk, 1997; Vosyliene, 1999). Natural and industrial activities are also contributing factors to increasing heavy metals in atmospheric, terrestrial and aquatic systems (Babich and Stotzky, 1978; Landis and Yu, 2004; Tulonen, et al., 2006).

The term heavy metal has been used for more than two decades to describe a group of metals and semimetals having ecotoxic properties. International union of pure and applied chemistry (IUPAC) reported that the term is a "meaningless term". An alternative definition is based on a group of properties. Consequently, most heavy metals have a density (gravity) greater than 4 (e.g. cadmium, copper, iron, zinc), atomic number greater than 20, and atomic weight greater than sodium. Moreover, some prefer to classify them based on the chemical properties in the periodic table. These elements comprise more than 23 elements in periodic table such as aluminium (Al), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), and mercury (Hg). Some of them are essential for biological functions such as cobalt, copper, iron, and zinc (Duffus, 2002).

Heavy metals can accumulate in the tissues of organisms via food, water and suspended particulate materials. They may negatively influence biological processes and the stability and fate of flora and fauna in aquatic environments. Some of these elements such as Fe, Cu, and Cr have the ability to induce

reactive oxygen species (ROS). For instance, superoxide anions ($O^{\bullet -}_2$), and hydroxyl radicals ($OH^{\bullet}$), while, inactive redox metals such as Cd, Pb and Hg, indirectly induced ROS by depleting antioxidants such as glutathione, leading to disordered mechanical defence and an increase ROS in cells, which attack macromolecules of cellular components such as proteins, lipids, and nucleic acids, resulting in oxidative tissue damage (Halliwell and Gutteridge, 1989; Stohs and Bagchi, 1995; Ercal et al., 2001; Livingstone, 2001; 2003; Landis and Yu, 2004; Valavanidis, et al., 2006; Valko et al., 2006; Wang and Wang, 2009; Hirano and Tamae, 2010).

1.2 Cadmium

1.2.1 Properties and uses

Cadmium is a transition metal (group IIB) in the periodic table of elements with an atomic weight is 112.411 amu, and the density of Cd is 8.64 g/cm^3 at 20^{o}C. It is a rare element associated with zinc and lead ores, as complex oxide, sulphides and carbonates (Stohs and Bagchi, 1995; Martelli et al., 2006). Abundance in the earth's crust is estimated from 0.1 to 0.2 mg Cd/kg; non-polluted agricultural soil ranged from 0.01 to 0.7 µg/g, and increase than this when utilised phosphate fertilisers, and sewage sludge. In the non-polluted aquatic environment, it reached 0.05–0.2 µg Cd L^{-1} in freshwater, to more than 0.05 µg Cd L^{-1} in coastal seawater, in open seawater varied from 0.01 to 0.1 µg Cd L^{-1}. In atmosphere, the average of Cd in rural area is about 5 ng/m^3, in urban areas, from 2 to 15 ng/m^3, and 15 to 150 ng/m^3 in industrialised areas (Fleischer et al., 1974; Fassett, 1975; WHO, 1977; Babich and Stotzky, 1978; OECD, 1994; Hamada et al., 1997; Soegianto et al., 1999; Satarug et al., 2010).

The world production is estimated from 12,000 to 15,000 metric tons per year (Fleischer et al., 1974; Valko, et al., 2005).

It is utilised in several industrial products, due to the ability to resist the corrosion, it is utilised in coating, electroplating, galvanising. Furthermore, it is used as a coloured pigment in paints and a stabiliser in plastic polyvinyl chloride (PVC), the manufacture of nickel-cadmium batteries, fungicides and fertilisers, and utilised as a barrier to control nuclear fission in nuclear planting and cigarettes (IARC, 1993; Stohs and Bagchi, 1995; Newman, 2010; UNEPA, 2010).

1.3 Sources of cadmium in the environment

1.3.1 Natural sources

Cadmium is released to the atmosphere from volcanic activities, forest fires, weathering of rocks and erosion, sea pray, airborne soil particles and biogenic materials. The global Cd emission was estimated 15,000 and 88,000 tonnes per year, respectively (UNEPA, 2010).

1.3.2 Anthropogenic sources

The majority of Cd is released to the environment as a result of human activities to air, water and soil (Fig 1.1). In 1983, the total anthropogenic Cd released to the aquatic environment was 1,200–13,400 tonnes coming from domestic waste water, non-ferrous smelting, manufacturing of metals and chemicals. Cadmium releases to the atmosphere from fossil fuel combustion, smelting, non-ferrous metals ores, and waste incineration. The global emission

to air in 1983 reached to 7,570 tonnes then declined to 2,983 tonnes in mid 1990 (UNEPA, 2010). It can be transported from source of emission long distances to deposit in soil and aquatic environments. Adding of phosphate fertilisers, domestic waste water and mining activities are contributing sources to increase Cd in soil and aquatic environments. Furthermore, storms and floods are also contributing factors to its spread to other places (WHO, 1977; Abel and Bärlocher, 1988; IPCS, 1992; IARC, 1993; Martelli et al., 2006, Newman, 2010; UNEPA, 2010; Valavanidis and Vlahogianni, 2010; Kang et al., 2012). Plants can take up an amount of Cd from the soil. It depends on soil chemistry (acidic or alkaline). For instance, acidity of soil increases Cd mobility, it was also found that calcareous soils are less toxic and bioavailability from non-calcareous soils is lower (IPCS, 1992; UNEPA, 2010). The toxicity of Cd in plants occurs at higher concentrations than those that cause toxicity to humans and animals. Using phosphate fertilisers in agriculture increase the rate of Cd in crops and vegetables (Nordic Council of Ministers, 2003; Satarug et al., 2003).

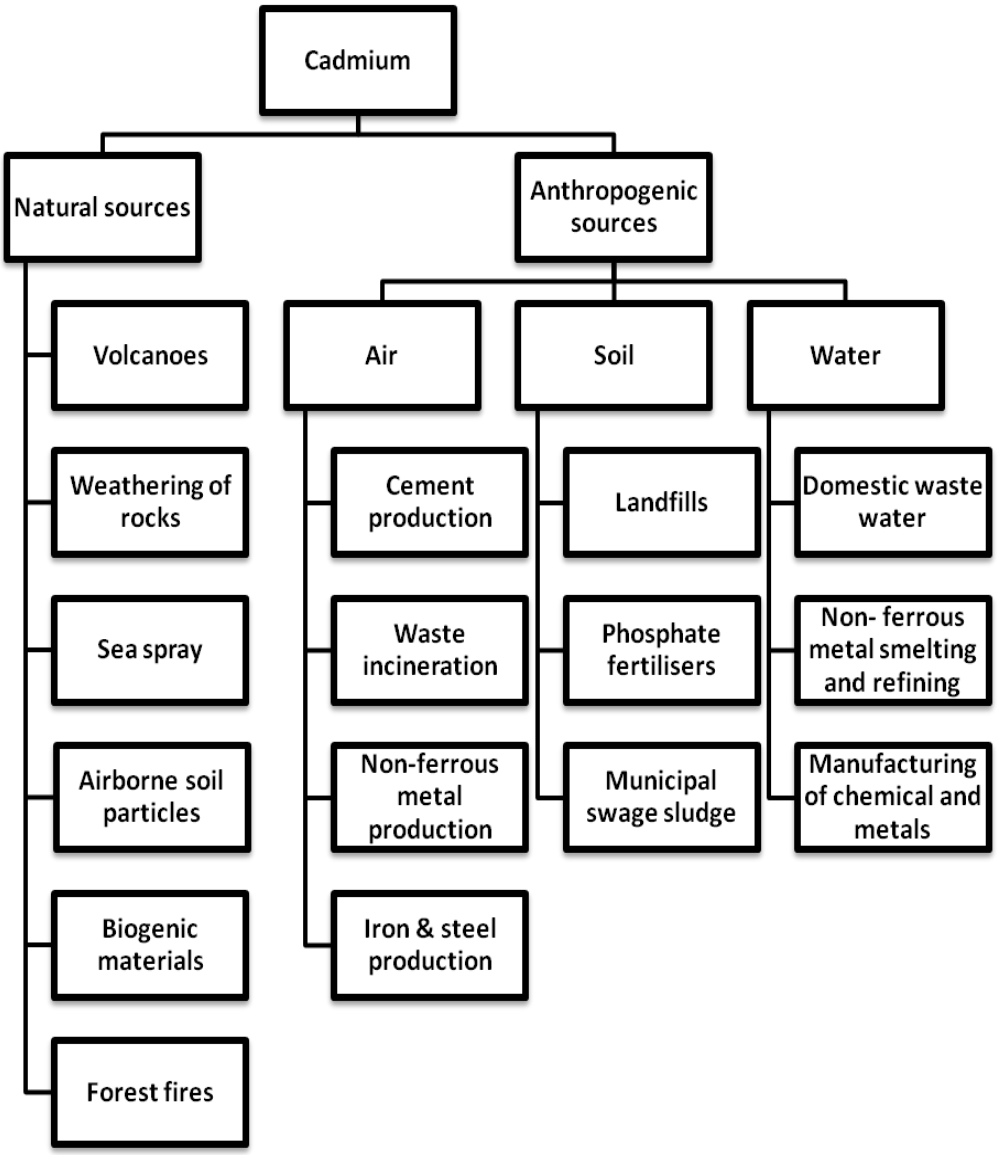

Figure 1.1 Schematic represents the sources of Cd in nature and from anthropogenic resources (according to UNEPA, 2010).

1.4 Effects of cadmium toxicity

1.4.1 Humans

Cadmium is a non-essential element, with no known biological function in the animal kingdom. In spite of that marine phytoplankton utilise it as a micronutrient by replacing Zn with Cd when Zn is limited, enhancing growth. It can also act as a catalytic metal in Cd carbonic anhydrase in the marine diatom

Thalassiosira weissflogii (Lee and Morel, 1995; Lane et al., 2000; Park et al., 2008; Xu et al., 2008).

Cadmium is highly toxic at low concentrations, classified in group 1 as one of six elements banned by the European Union. Furthermore, it is carcinogenic to humans in group B1, according to the International Agency for Research on Cancer (IARC) and National Toxicology Program (Waalkes, 2000; Liu et al., 2009; Strumylaite and Mechonosina, 2011). Biological half-life of this element is in excess of 25 years; it accumulates in liver and kidney of vertebrates, hepatopancreas of crustacea also a main organ to store toxic metals. It is incapable of being degraded to less toxic substances or being depurated rapidly from the body. Aquatic crustaceans, molluscs and algae accumulate a higher amount of Cd than other species (Fleisher et al., 1974; Hutton, 1983; IARC, 1993; Waalkes, 2003; UNEPA, 2010; Liu, et al., 2011).

It enters the human body via agriculture products such as crops, grains, cereals, herbal medicine and leafy vegetables (also tobacco leaves) which take it up from the soil, and seafood such as mussels and shellfish, or respiratory route such tobacco smoke and fine particles. Cadmium toxicity increased when the amount of Ca and vitamin D decreased, in addition to that deficiency of essential elements such as Zn and Fe contributes a factor (Freedman, 1989; Frust, et al., 1998; Stohs and Bagchi, 1995; Landis and Yu, 2004; Bertin and Averbeck, 2006). Safe intake of Cd was estimated to be 7 μg Cd/week/Kg body weight depending on the critical renal Cd concentration (UNEPA, 2010).

Acute exposure to Cd via inhalation caused irritation in the respiratory system and pulmonary edema, while chronic exposure caused osteoporosis, kidney dysfunction, anemia and bone fraction (Liu et al., 2009). Itai-itai disease in

Japan is one of the worst cases recorded since 1955 of environmental pollution. Discharged mining products contaminated with Cd from Toyama prefecture in the Jinzu River, were used by farmers in irrigation, fishing, washing, and drinking water (Inaba et al., 2005). It was found that due to the rate of consumption of rice by people in that area close to the mining area, the majority of them were suffering from symptoms of Itai-itai disease. The results showed that most of the victims were post-menopausal women suffering from osteoporosis, osteomalacia, associated with impairment of tubular glomerular function (Fleischer et al., 1974; Fassett, 1975; Landis and Yu, 2004; Duffus and Worth, 2006; Takashi and Wako, 2006). The average Cd in the body varies between countries and gender. It was observed to be higher in women than men in Australia (18.1 and 14.6 µg/g wet tissue weight, respectively). In contrast, in Japan Cd reached to 83.9 µg/g wet tissue weight in women and 72.1 µg/g wet tissue weight in men (Satarug, et al., 2002 and 2003; Uetani et al., 2006; Satarug, et al., 2010).

1.4.2 Aquatic crustaceans

Crustaceans are sources of food for many organisms in the food chain and can be highly sensitive to pollutants. Amphipods are used extensively in toxicological studies, because they are widely distributed in different habitats including freshwater, brackish and sea water, highly sensitive to pollutant as bioindicators and biomonitors to assess water pollutants and water quality (Dallinger, 1994; Rinderhagen et al., 2000).

Sea water organisms are more resistant to Cd toxicity than freshwater organisms, due to salinity and the abundance of inorganic anions such as

chloride. While, the rate of accumulation is higher in sea water species than in freshwater species. The mechanism of accumulation and elimination in the body of aquatic crustaceans is of high concern regarding the ability of metal to transport to the top of the food chain, in particular, early stages such as fish and crustacean are more sensitive (Engel, and Fowler., 1979; McCahon and Pascoe, 1988a; Meyer et al., 1991; Shuhaimi-Othman, 2012; and Rainbow and Dallinger, 1993).

Sensitivity of the amphipod *Gammarus pulex* to Cd has been studied at different concentrations from 1.9 µg Cd L^{-1} to 5 mg Cd L^{-1} by many researchers (Table 1.1). There are many factors contributing to enhance Cd accumulation in aquatic environments, such as low calcium in water, acidity, low salinity, and temperature (Chan, et al., 1992; Taylor, 1983). Direct exposure to Cd in water was more of a risk to the survival of *G. fossarum* than contaminated food, and was higher in soft than hard water (Abel and Bärlocher, 1988). Cadmium competes with Ca for the same sites within the cell so hardness of water plays an essential role to impair Cd toxicity and accumulation in many species such as alga, rooted plant, snails, catfish, and guppies (Kinkade and Erdman, 1975). Freshwater *G. pulex* (Wright, 1980b; Wright and Frain, 1981; Athar and Vohora, 2001) and also the freshwater fish *Puntius gonionotus* (Mungkung et al., 2001). Zinc as an essential element for crustacea interacts with Cd by reducing Cd accumulation, this was observed in the amphipod *G. pulex* exposed to 0.01 mg Zn L^{-1}, which was able to survive in containing 0.5 mg Cd L^{-1} (Howell, 1985).

Table 1.1 Representative cadmium concentrations used to expose *G. pulex* by several researchers.

Concentrations	Endpoint	Water quality	Citation
0.01; 0.1; 0.5 mg Cd L^{-1}	LC_{50} (0.12 mg Cd L^{-1}) adult intermoult, after 96 h	Artificial stream water	Wright and Frain, 1981
0.5; 1; 2; 5 mg Cd L^{-1}	LC_{50} (0.1 mg Cd L^{-1}) after 14 days	Conditioned tap water (mean total hardness was 216 ppm as $CaCO_3$)	Abel and Garner, 1986
0.01; 0.03; 0.05; 0.1; 0.3; 1 mg Cd L^{-1}	LC_{50} (0.221 mg Cd L^{-1}) after 96 h (220 days old).	Dechlorinated tap water (hardness 115 ppm as $CaCO_3$).	McCahon and Pascoe, 1988a
0.01; 0.03; 0.1; 0.3; 1 mg Cd L^{-1}	LC_{50} (0.014 and 0.021 mg Cd L^{-1}) after 48 h.	Dechlorinated tap water (hardness 94.6 ppm as $CaCO_3$).	McCahon and Pascoe, 1988b
0.01; 0.03; 0.1; 0.3; 1 mg Cd L^{-1}	LC_{50} (0.018 and 0.021 mg Cd L^{-1} immediate post-moult and intermoult respectively) after 96 h	Dechlorinated tap water (hardness 115 ppm as $CaCO_3$).	McCahon and Pascoe, 1988c
10; 20; 30; 40 µg Cd L^{-1}	EC_{50} (2.315 mg Cd L^{-1}) after 48 h	Artificial pond water (92 mg Ca L^{-1})	Stuhlbacher and Maltby, 1992
7.5; 15 µg Cd L^{-1}	LC_{50} (10.5 µg Cd L^{-1}) after 264 h	Well-water (mean values of Ca^{2+} and Mg^{2+} were 99.3 and 5.17 ppm respectively).	Felten et al., 2008a
1; 3; 9 µg Cd L^{-1}	7 days	Mineral water and three Ca concentrations (3.5; 8.8; 174 ppm	Pellet et al., 2009

0.01; 0.03; 0.05 mg Cd L^{-1}	NOEC was 0.03 mg Cd L^{-1} and LOEC was 0.05 mg Cd L^{-1} after 48 h	DSW (Dutch Standard water)	Alonso et al., 2009
0.10; 0.20; 0.35 mg Cd L^{-1}	LC_{50} range from 0.02 to 0.06 mg Cd L^{-1}) after 96 h	DSW (Dutch Standard water)	Alonso et al., 2010a
0.3; 0.5; 0.7; 1; 2 mg Cd L^{-1} *	LC_{50} was 8 mg Cd L^{-1}	DSW (Dutch Standard water)	Alonso et al., 2010b
0.1; 0.3; 0.5; 0.7; 1 mg Cd L^{-1} **	LC_{50} was 2 mg Cd L^{-1}	DSW (Dutch Standard water)	Alonso et al., 2010b
300 µg Cd L^{-1}	13 days	Moderately hard synthetic water (80-100 ppm as $CaCO_3$)	Khan et al., 2010
1.9; 3.7; 7.5; 28.5 µg Cd L^{-1}	LC_{50} (10.5 µg Cd L^{-1}) after 240 h	Mineral water (Volvic, France).	Vellinger et al., 2012b
2; 3.8; 8; 14.9; 27; 56.7 µg Cd L^{-1}	LC_{50} (10.5 µg Cd L^{-1}) after 240 h	Mineral water (Volvic, France).	Vellinger et al., 2012a
27; 52 µg Cd L^{-1}	LC_{25} (54.1 µg Cd L^{-1}) after 240 h	Mineral water (Volvic, France).	Vellinger et al., 2012c
3.4; 6 µg Cd L^{-1}	LC_{25} (3.7 µg Cd L^{-1}) after 240 h.	Mineral water (Volvic, France).	Vellinger et al., 2013

Adult*, juveniles **

1.4.3 Oxidative stress

Oxidative stress represents an imbalance between pro-oxidants and antioxidant defence systems (Gutteridge, 1995). Cadmium is an inactive element in redox reactions, unlike Fe and Cu. However, it induced free radical for example, ROS comprising of hydroperoxyl radicals ($HO^{\bullet}_2$), hydroxyl radicals ($OH^{\bullet}$), and superoxide radicals ($O^{\bullet -}_2$) (equation 1-4) indirectly by depletion of glutathione (GSH) in the cell (Hartwig, 1994; Hamada et al., 1997; Stohs and Bagchi, 1995; Stohs et al., 2000; Błasiak, 2001; Wang et al., 2004; Bertin and Averbeck, 2006; Halliwell and Gutteridge, 1989; Liu, et al., 2011; Wang et al., 2011). Failure of antioxidant defences to reduce ROS concentrations can lead to protein degradation, DNA damage and lipid peroxidation (Ercal et al., 2001; Halliwell and Gutteridge, 1989; Kang, et al., 2012) (Fig 1.2).

Oxidative stress associated with apoptosis was observed after exposure to different Cd concentrations in diverse cells and tissues including human fetal lung fibroblasts (Yang et al., 1977), gills and hepatopancreas of freshwater crab *Barytelphusa guerini* (Venugopal et al., 1997), the cortical neurons of fetal rat (Lòpez, et al., 2006), human fetal germ cells (Angenard, et al., 2010), rat testes (Ognjanović et al., 2010), heart of freshwater crab *Sinopotamon yangtsekiense* (Lei et al., 2011), hemocytes, gills and testis of freshwater crab *Sinopotamon henanense* (Qin et al., 2012; Wang et al., 2011; 2012).

$$O_2 + e + H^+ \longrightarrow HO_2^{\bullet} \text{ (hydroperoxyl radical)} \quad (1)$$

$$HO_2^{\bullet} \longrightarrow H^+ + O^{\bullet -}_2 \text{ (superoxide radical)} \quad (2)$$

$$O^{\bullet -}_2 + H^+ + e \longrightarrow H_2O_2 \text{ (hydrogen peroxide)} \quad (3)$$

$$H_2O_2 + e \longrightarrow OH^- + OH^{\bullet} \text{ (hydroxyl radical)} \quad (4)$$

Equation 1–4 represents formation reactive oxygen species (ROS), (Gutteridge, 1995).

1.4.4 Genotoxicity of cadmium

Cadmium is not directly genotoxic, because it is unable to bind DNA, and it is an inactive redox metal, and does not participate in Fenton reactions. As noted above, Cd causes an imbalance between antioxidants and free radical generation via depletion of antioxidant enzymes such as glutathione and an increase of free radicals such as ROS in the cell which leads to lipid peroxidation, alterations in cell division, gene expression, DNA strand breaks, chromosome aberrations and DNA repair (Waalkes, 2000, 2003; Bertin and Averbeck, 2006).

Genotoxicity studies were applied as a sensitive tool to detect the effects of toxicants such as Cd on haemocytes, oocytes and spermatozoa of the freshwater crustacean *G. fossarum*, the spermatozoa were the most sensitive to Cd and pollutants (Lacaze et al., 2010, 2011), human lymphocytes exposed to 3; 30; 150 μM of Cd (Błasiak, 2001), and leukocytes of Swiss Albino mice exposed to range of concentrations from 0.5 to 128 mg/kg. b. wt at 24 h (Devi,

et al., 2001). It induced DNA and lysosomal damage in a rat hepatoma cell line exposed to range of concentration from 0 to 100 μM for 5 and 8 h (Fotakis et al., 2005). Exposure to 4.25 and 8.50 μ mol L^{-1} caused DNA damage and oxidative stress in the haemocytes and hepatopancreas cells of the white shrimp *Litopenaeus vannamei* after 6 h (Chang et al., 2009). Increase Cd accumulations led to induce DNA degradation in gill cells of the clam *Corbicula japonica* after 4 days exposure to 300 μg Cd L^{-1} (Slobodskova, et al., 2010).

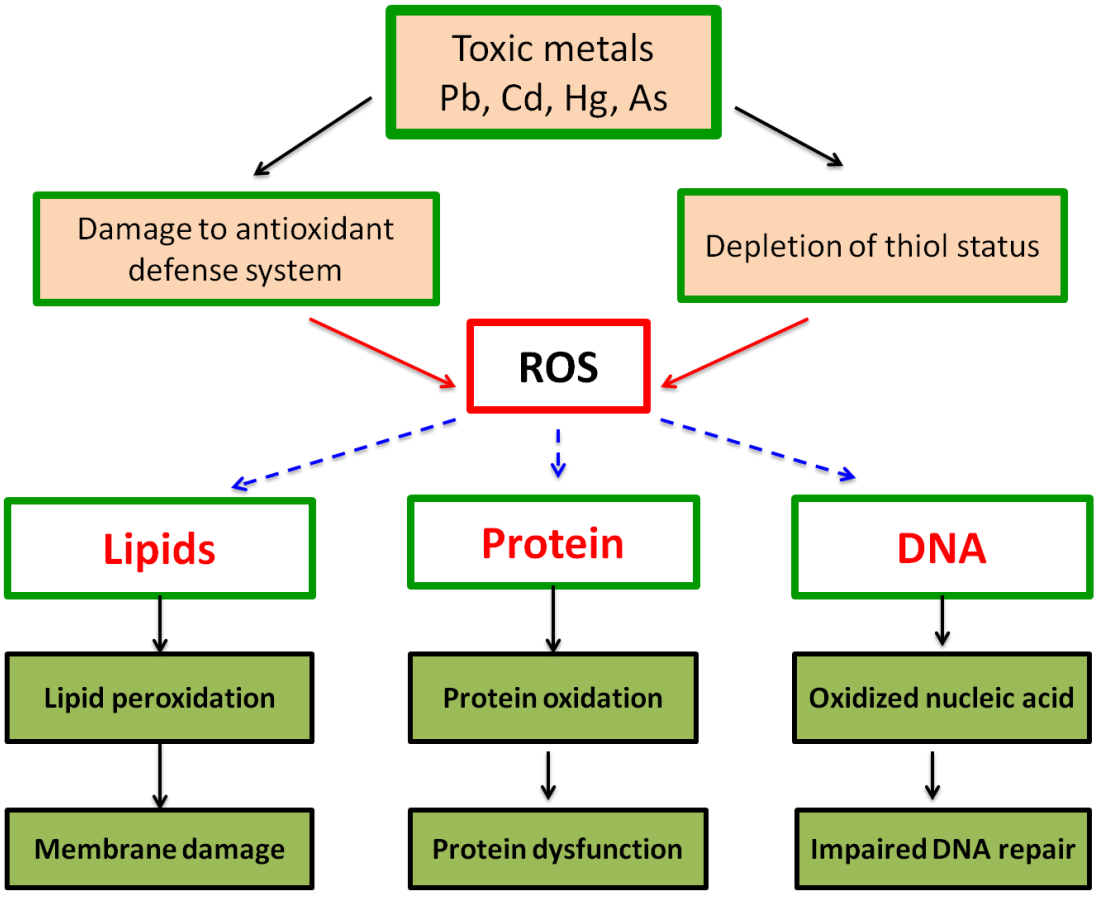

Figure 1.2 Schematic represents metals – induced oxidative stress (Ercal et al., 2001).

1.4.5 Behaviour

The impact of toxicants on behaviour has been studied as a tool and indicator in ecotoxicity testing to assess the effect of pollutants on aquatic organisms, and water quality. Behavioural changes have a negative impact on the survival and adaptation of organisms to pollutant environments (Blockwell, et al., 1998; Maltby et al., 2002; Felten, et al., 2008a). Measurement of locomotor activity and ventilation during exposure to pollutants was applied on the amphipod *G. pulex* (Maltby, 1995; Felten, et al., 2008a; Alonso et al., 2009), estuarine amphipod *G. lawrencianus* (Wallace and Estephan, 2004) and locomotor activity rhythms in *G. aequicauda* (Morillo-Velarde et al., 2011).

Feeding bioassays are also used as a biomonitor to assess water quality and the health conditions of animals during exposure to pollutants. Several laboratory and field studies have shown that the feeding rate, growth and reproduction in freshwater *G. pulex* were impaired as a result of exposure to pollutants (Maltby, 1999; Maltby et al., 2002). Increase the level of metalliferous effluents decreased digestive enzyme activities (esterase, β-glucosidase, β-galactosidase, amylase and endoglucanase) and feeding rate of the amphipod *G. fossarum* in Amous River (France) (Dedourge-Geffard et al., 2009). The metalliferous effluents which contained high concentrations of iron and manganese in the West Okement River (North Devon) reduced feeding rate on caged *G. pulex* for 6 days (Maltby and Crane, 1994).

1.4.6 Tissues and cell structure

In crustaceans, the mid-gut gland (hepatopancreas) and the gills are highly sensitive to toxicants in comparison to other organs. The gills of aquatic

crustacea are responsible for ion regulation, acid-base balance, gas exchanges, and direct contact with toxicant in the media (Felten et al., 2008a; Issartel et al., 2010). Cadmium damages the fine gill cell structure, disrupting several enzyme activities related with respiration activity and osmoregulation such as the Na^+/K^+-ATPase and carbonic anhydrase in numerous species of crustaceans. Histological studies have shown effects of Cd on cellular structure and function of gills in pink shrimp *Penaeus duorarum* (Couch, 1977), the brown shrimp *Crangon crangon* (Papathanssiou, 1985), *Palamon serratus* (Papathanassiou and King, 1983), late juvenile *Penaeus japonicas* (Soegianto et al., 1999), and white shrimp, *Litopenaeus vannamei* (Wu, et al., 2009).

The mid-gut gland of crustacea is also used as a bioindicator in toxicological studies, because it is a site for the synthesis and secretion of enzymes, and is responsible for detoxifying the toxicants (Schultz, 1976; Icely and Nott, 1980; Muskò, 1988; Correia, et al., 2002b). Alteration in function and structure of hepatopancreas cells was observed in several studies as a result of the pollutants (Papathanassiou and King, 1986; Blockwell, et al., 1996; Kutlu, et al., 2002; Schuwerack and Lewis., 2003; Liu, et al., 2011; Ozalp, et al., 2011). Damage to hepatopancreas cells is related to reduction of feeding and growth rate (Blockwell, et al., 1998; Maltby, et al., 2002).

Cell organelles are more sensitive to Cd toxicity; it was clearly observed in different tissues of crustaceans. Mitochondria are the first organelles targeted by Cd toxicity, associated with alterations in structure and disrupting a vital role to produce energy. Injury to the cell induced mitochondria to generate ROS which can induce programmed cell death (Hamada, et al., 1997; Cannino, et al., 2009; Lei, et al, 2011; Liu, et al., 2011).

1.4.7 Life stages

Early life stages of aquatic freshwater invertebrates are more sensitive to Cd than adults (Mebane, 2006). There is many factors that affected the life cycle of *G. pulex* during the exposure to toxicant such as temperature and water quality (Stephenson, 1983), season (Duran, 2007), life stage (Hobrough, 1973; McCahon and Pascoe, 1988a), moult cycle and the reproduction stage (McCahon and Pascoe, 1988b & c).

Post-moult stage in most crustaceans is at more risk during exposure to Cd in contrast with other stages (Wright, 1980a; Wright and Frain, 1981). Juveniles *G. pulex* were more sensitive to cadmium than adults (McCahon and Pascoe, 1988a; Alonso et al., 2010b).

1.4.8 Ion regulation

There are different organs responsible for regulating the movement of ions between the fluids inside the body and external media in marine, brackish, and freshwater crustaceans. Gills are responsible for osmoregulation between external and internal fluids in the majority of aquatic crustacea, whether in seawater, brackish water and freshwater. There are many enzymes that have been used as a tool, to assess the physiological conditions during exposure to toxicants such as carbonic anhydrase and Na^+/K^+-ATPase, which play a substantial role in acid-base balance and osmoregulation during the life of organisms (Brook and Mills, 2003; 2006).

Previous studies have shown that Cd causes disturbance of enzyme activities and ion regulation in the gills of the freshwater crustacean *G. pulex* (Felten, et al., 2008a), gill bluegill sunfish, fathead minnow and golden shiner

(Watson and Benson, 1987), the gills and hepatopancreas of the crab *Scylla serrata* (Dhavale et al., 1988), dogfish rectal gland and rabbit kidney outer medulla (Kinne-Saffran, et al., 1993), gills and intestine of European eel *Anguilla anguilla* (Lionetto et al., 1998, 2000) and gills of the sand fiddler crab *Uca pugilator* (D'orazio and Holliday, 1985). It also affected haemolymph ion composition (Bjerregaard and Vislie, 1985).

1.4.9 Metallothionein

There are several mechanisms of defence to protect living organisms against Cd toxicity such as metallothionein (MT), glutathione, and vitamins (E & C). Metallothionein works on both sides as antioxidant and metal–detoxification. It is a sulphur-rich low molecular weight, small cysteine-rich protein and is heat stable. It is found in most invertebrates and vertebrates. In crustacean, MT sequence includes 58- 60 amino acids (Lerch et al., 1982; Pedersen et al., 1996 &1998). It has high affinity to bind with many metals such as cadmium (Cd), zinc (Zn), copper (Cu), and silver (Ag) from low concentrations via cysteine –SH. Not only in the detoxification of non-essential metals, but also in the storage of heavy metals in a non toxic form, as a scavenger of free radicals such as $OH^{\bullet}$ and $O^{\bullet -}_2$ by cysteine residues from MT, it plays an important role in the metabolism of essential elements such as Zn and Cu (Cherian and Goyer, 1978; Singhal, et al., 1987; Halliwell and Gutteridge, 1989; Roesijadi, 1992; Klaassen et al, 1999; Ahearn, et al., 2004, Nordberg and Nordberg, 2009). Zn^{2+} release from zinc-metallothionein acts to reduce lipid peroxidation and Cd is accumulated by binding with MT (Howell, 1985; Halliwell and Gutteridge, 1989; Brzóska, and Moniuszko-Jakoniuk, 2001).

Furthermore, MT is used as a biomarker in toxicological studies to assess metal toxicity in living organisms such as crustaceans and molluscs. Geffard, et al (2007) reported that abiotic and biotic factors might affect MT in the field studies. For example, weight, sex, reproduction, season and age of animals.

Glutathione is an important scavenger for free radical and primary defence against Cd toxicity (Singhal et al., 1987). It contains cysteine sulfhydryl group which combined with Cd to form (GS-Cd) as steady complex. However, increase Cd concentrations led to deplete glutathione synthesis in hepatopancreas of freshwater crab *Sinopotamon yangtsekiense* (Wang et al., 2008).

1.4.10 Metal speciation and complexation

There are many metals in different chemical forms and oxidation state in water bodies can combine directly with endogenous compound to form metal speciation and complexes. In aquatic systems, very small percent of heavy metals dissolved as free metal ion. Most of them combined in complexes or adsorbed to colloidal particles (Florence et al., 1992). These complexes may be transport metal ions in environmental systems non toxic form (Carter, 1995). Bioavailability and toxicity of metal ions in aquatic organisms depend on the presence of organic ligands (amino acid, carboxylic acid, fulvic acid, humic acid) and an inorganic ligands (HO, Cl) and the concentrations of competitive cations like H^{+}, Ca^{2+} and Mg^{2+} (Allen and Hansen, 1996., Pellet et al., 2009., Newman, 2010). The interaction between toxic metal ions and carbonate minerals in aquatic environments can provide the important pathway to reduce toxicity in water column and sediments (Korfali and Davies, 2004). The speciation of element in water utilised to understand the biological cycling (bioaccumulation,

Bioconcentration, bioavailability and toxicity) and the transport, precipitation and adsorption of metal in aquatic system. The form of chemical species for toxic metal could be used to determine the fraction of total dissolved metal and predict the bioavailability to aquatic organisms. In recent years, many models has been developed to predict the metals speciation and chemical equilibrium in water bodies such as Biotic ligand model (BLM), Windermere Humic-Aqueous Model (WHAM) for metal speciation, free ion activity model (FIAM) and MINTEQ were designed to calculate metal speciation in surface, ground waters, sediments and soils (Florence et al., 1992; Newman, 2010).

1.5 The freshwater amphipod *Gammarus pulex* (L.)

The freshwater *G. pulex* (Linnaeus, 1758) belongs to Amphipod crustaceans; it is widespread in Europe, British Isles, North Africa and Northern Asia in many rivers, streams and stagnant ponds under leaves, stones, and wood. It usually lives in flowing water. The average length of males is about 21 mm, while females are approximately 14 mm (Pinkster, 1970; Sutcliffe, 1992; Maltby and Crane, 1994).

The body is curved, laterally compressed and divided into 4 main parts: head, peron, pleon and urosome. The head has two pairs of antenna and a pair of compound-eyes; the peron has 7 pairs of jointed legs which are used for swimming, crawling and grasping; the gills attaches the thoracic legs in the peron; the pleon consists of three pairs of pleopods, used for circulating water and swimming; the urosome has three pairs of uropods for swimming as well (Fig 1.3). The male guards the female during reproduction for several weeks (Fig 1.4). Female *G. pulex* produce two generation each year, which can become fully developed (Willoughby, 1976; Crane, 1994).

The gut of *G. pulex* includes the main gut; mid-gut and hindgut, on both sides of the gut are two pairs of caeca. This is the so-called mid-gut gland or hepatopancreas, each caecum is end-shaped (Chapter 3). The caecum plays an essential role in the synthesis and secretion of enzymes and detoxification of toxicants (Monk, 1977; Icely and Nott 1980).

Figure 1.3 Male freshwater *G. pulex* carrying a female.

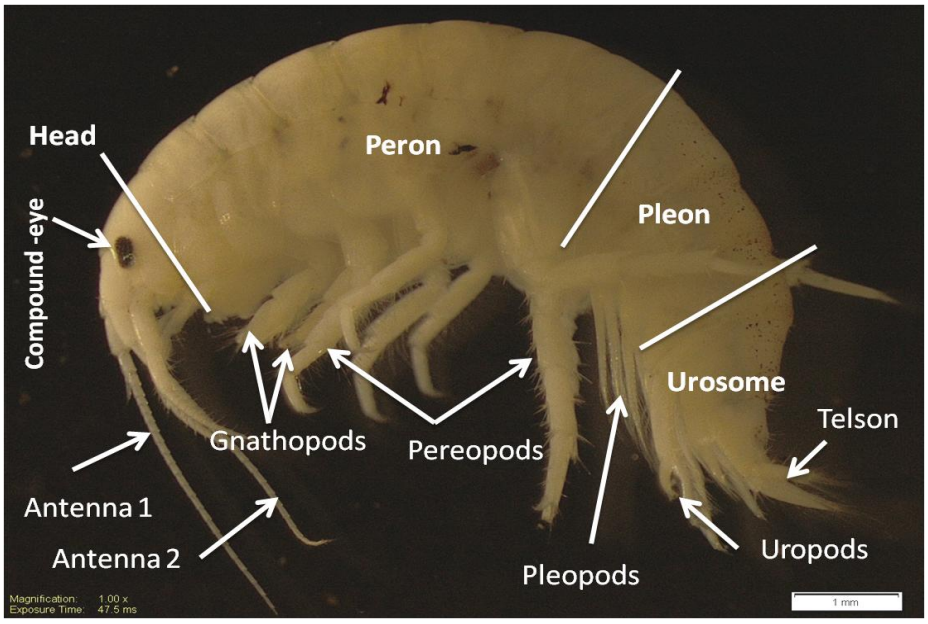

Figure 1.4 External structures the body of female *G. pulex.*

The previous studies has reported that the freshwater *Gammarus pulex* is easy to collect in spring and autumn, and can culture and survive under lab conditions at temperature 5-20^{o}C, hardness (> 60 ppm as $CaCO_3$) (Nilsson, 1977; Welton and Clarke, 1980; McCahon and Pascoe, 1988a & d; García et al., 2010), and it is sensitive to a range of stresses. Furthermore, *G. pulex* is ecologically relevant to study as it is a major food item for many types of fish, amphibian, birds and macroinvertebrates. As a detritivore, *G. pulex* plays a vital role to decompose litter leaves in rivers and streams as a source of energy (Graça et al., 1993a; b; Maltby and Crane, 1994; Forrow and Maltby, 2000; Felten et al., 2008a).

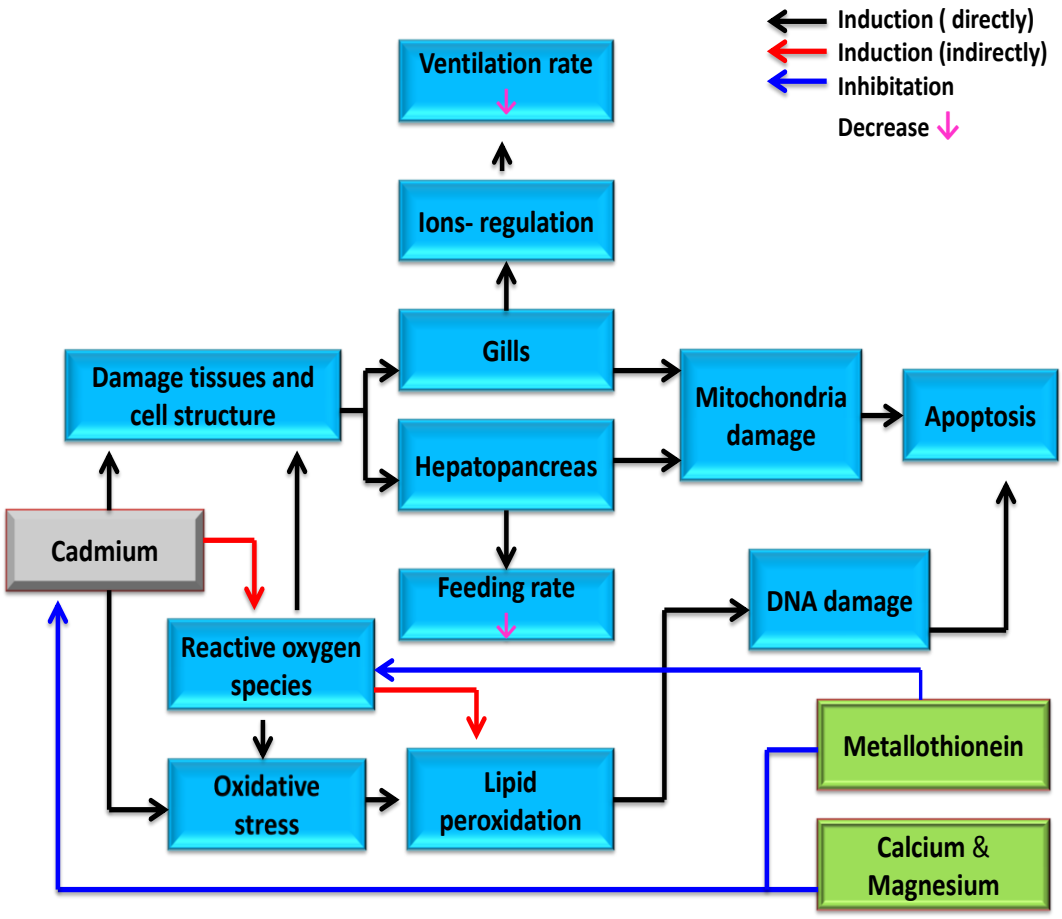

Figure 1.5 Schematic represents of hypothetical interactions between Cd toxicity, antioxidant and cations (Ca & Mg) on physiological, histopathological and behaviour endpoints in organisms.

1.6 Aims of thesis

The study tries to focus on new aspects to understand the effects of Cd toxicity on the freshwater amphipod *G. pulex.* Several factors that are likely to affect the toxicity of this metal, such as gender, life stage and the hardness of the water were studied. Endpoints related to oxidative stress were measured in combination. The overall aim was to enhance understanding of the toxicity of this pollutant to a key ecological species and to provide information that may enhance environmental protection and the setting of appropriate limits for water quality.

This was achieved through the following specific objectives

- To investigate the effects of Cd on biological endpoints, including survival, lipid peroxidation, DNA damage and behaviour at lethal and sublethal concentration (Chapter 2 and 3).
- To investigate the effects of Cd on tissue and cell structures (chapter 4).
- To investigate the effects of long-term exposure to Cd on the survival and physiological function of juvenile *G. pulex* in hard and soft water (Chapter 5).
- To investigate the effects of exposure of adult *G. pulex* to Cd on haemolymph ion concentrations, osmolality, Na^+/K^+-ATPase and metallothionein synthesis in hard and soft water (Chapter 6).

1.7 Hypothesis

1. Exposure to Cd at sublethal doses causes genotoxicity, and inducing lipid peroxidation (Chapter 2). The sublethal doses have inhibitory effects on feeding rate, ventilation rate and locomotor activity on males and females (Chapter 3).

2. Exposure to Cd at sublethal doses causes damage to cell organelles in the gills and hepatopancreas (Chapter 4).

3. The toxicity of Cd to freshwater organisms is dependent on the concentrations of other essentials elements such as calcium and magnesium (Chapter 5).

4. Exposure to Cd at sublethal doses in hard and soft water disrupt haemolymph ions regulation and Na^+/K^+-ATPase in the gills and hepatopancreas (Chapter 6).

Chapter 2 Cadmium uptake by the freshwater amphipod *Gammarus pulex* and its sublethal effects on lipid peroxidation, and genotoxicity.

2.1 Introduction

The effect of Cd toxicity varies from species to species in aquatic environments and depends upon many factors such as water quality, gender, age, moulting cycle and seasonal variation (temperature and salinity) (Wright and Frain, 1981; Ravera, 1984; Alonso et al., 2010a). The features of freshwater which is characterised by containing low concentrations of dissolved such as Ca, Cl and Mg in some sites make living organisms in those habitats less tolerant and resistance to Cd toxicity than those living in marine environments (Wright and Frain, 1981; Marsden and Rainbow, 2004). Cadmium concentrations in unpolluted freshwater is less than 0.1 µg Cd L^{-1} (USEPA, 2001). According to the Canadian Environmental quality guideline (Marine, 1999) the levels of Cd should not exceed 0.017 µg Cd L^{-1} in freshwater and 0.12 µg Cd L^{-1} in marine and estuarine water, above this would cause detrimental effect to natural biota. In aquatic environments, transportation and accumulation of Cd could occur through the food chain, especially primary producers (phytoplankton) and primary consumers as a source of food for many species (Marsden and Rainbow, 2004; Ruangsomboon and Wongrat, 2006).

The main pathway of Cd uptake in aquatic organisms is via food and/or water, or through the biological membranes such as calcium (Ca) channels (Giesy et al., 1980; Hinkle et al., 1987; Abel and Bärlocher. 1988; Wang, 2002; Marsden and Rainbow, 2004). Uptake of Cd through contaminated food has been shown to be less detrimental than contaminated water (Abel and

Bärlocher, 1988). There are many factors which act on reducing or raising Cd toxicity, for example, an abundance of Ca, organic ligands and humic acid in hardwater inhibit Cd toxicity on the freshwater organisms (Pellet et al., 2009). Furthermore, low Ca concentrations, acidity and temperature have a more drastic effect on survival of the freshwater organisms when exposed to Cd (Abel and Bärlocher, 1988; Felten et al., 2008b; Vellinger et al., 2012c), it was found that increase temperature is related to rise Cd toxicity (McLusky et al., 1986), in the freshwater crayfish *Procambarus clarkia* (Del Ramo et al., 1987), and increase mortality rate in *G. pulex* (Vellinger et al., 2012c).

Amphipods have been used as a bioindicator for estimating metal concentrations and water quality in toxicological studies due to their sensitivity to pollutants (Marsden and Rainbow, 2004). The majority of crustaceans can regulate essential elements such as Cu and Zn, although there is no evidence to suggest regulation of non-essential elements such as Cd and Hg (Rainbow, 2007).

Cadmium belongs to redox-inactive metals, such lead and mercury, it indirectly induces the generation of reactive oxygen species (ROS) by inhibition of antioxidant defence systems or by displacement of redox active metals (zinc and copper) in the Fenton reaction, induced ROS lead to oxidative stress (Livingstone, 2003; Waisberg et al., 2003; Bertin and Averbeck, 2006), which causes damage to macromolecules such as lipid, proteins and DNA (Stohs and Bagchi, 1995; Ercal et al., 2001; Wang et al., 2011). Furthermore, induction of ROS such as hydroxyl radical and superoxide radical cause excess permeability in biological membrane to H^+ and other ions; which decreases membrane potential and loss of fluidity (Gutteridge, 1995). The effect of lipid peroxidation also relies on the level of antioxidant enzymes and fatty acids in

the organisms (Correia et al., 2003). In previous studies it has been reported that exposure to Cd elevated lipid peroxidation. This has been shown in the hepatopancreas and gill cells of the freshwater crab *Barytelphusa guerini* exposed to 0.62 mg Cd L^{-1} (Venugopal et al., 1997), human fetal lung fibroblasts after 16 h to 35 mM of Cd (Yang et al., 1997), the testis of freshwater crab *Sinopotamon henanense* at 116 mg Cd L^{-1} (Wang et al., 2011), alimentary system of the freshwater crab *S. henanense* exposure to 14.5 and 29 mg Cd L^{-1} (Wu et al., 2012) and testes of cultured frogs *Rana limnocharis* after 14 days to 10 mg Cd L^{-1} (Zhang et al., 2012).

The single cell gel electrophoresis (SCGE)/Comet assay has been used successively in genetic toxicology as a tool to detect DNA damage at level single cell as it is a sensitive, inexpensive, and rapid technique (Frenzilli et al., 2009; Tice et al., 2000; Lacaze et al., 2011). Indirect induction of ROS by Cd leads to DNA damage, especially mitochondrial DNA, it occurs before any damage in the nucleus (Chang et al., 2009). In aquatic invertebrates, Cd in the mg/L range caused DNA damage to hepatopancreas cells of the white shrimp *Litopenaeus vannamei* (Chang et al., 2009), spermatozoa the freshwater amphipods *G. fossarum* (Lacaze et al., 2010 and 2011), and the gills of marine mussel *Mytilus edulis* and the clam *Corbicula japonica* (Emmanouil et al., 2007; Slobodskova et al., 2010). In vertebrates, Cd caused DNA damage to leukocytes of mice (Davi et al., 2001), human lymphocytes (Błasiak, 2001), and the testes of frog *Rana limnocharis* (Zhang et al., 2012). However, most of these studies have been performed at concentration of Cd in excess of environmental limits and there is less information on the effects of Cd at lower concentrations.

This research aimed to estimate the sublethal effects of Cd at low environmentally realistic concentrations on the amphipod *G. pulex* after 24, 48, 96, and 114 h. Effects on survival, lipid peroxidation, and genotoxicity of Cd on haemolymph cells were investigated. Bioaccumulation of Cd was also assessed in the bodies of gammaridean and leaves (food source).

2.2 Materials and methods

2.2.1 Amphipods collection and acclimatisation

G. pulex were collected in October 2010 from the lower Hoopern valley stream (50^{o} 43`N; $30^{o}$ 31`W), using a hand net and sieves (Fig 2.1). The organisms were transported in plastic containers to the laboratory, placed in trays containing dechlorinated tap water, with constant aeration by using plastic pipette. Decaying leaves (*Quercus cerris*) were used as a food source, and were collected from the same site.

Organisms were kept at constant temperature (15 ± 1^{o}C) and under a light-dark regime (photoperiod of 16 h light and 8 h of darkness) under laboratory conditions for 2 days before exposure to Cd to acclimatize.

2.2.2 Experimental design - Acute toxicity

Prior to the experiment all glassware was acid washed (10% HCl). An initial exposure was performed in order to investigate the dose-response curve for $CdCl_2$. Juveniles and gravid females were excluded, twenty adult organisms (length: 13.76 ± 0.39 mm), were placed in each crystallising dishes (10 cm diameter) containing 150 ml of constantly aerated dechlorinated freshwater of

Hatherly laboratories (hardness: 73 ppm as $CaCO_3$; DO, 7.31 ± 0.2; pH 8.4 ± 0.19).

Four replicates were done per treatment. The stock $CdCl_2$ (Acrōs-organics) solution was added to each dish to give a range of nominal Cd concentrations of 0, 0.01, 0.03, 0.1, and 1 mg Cd L^{-1} and were covered. Observations for mortality (i.e. failure to respond to mechanical stimulation) were made after 24, 48, 96 and 144 h. The organisms were not fed during the course of exposure and the test solution was not changed. Dead animals were removed immediately.

Figure 2.1 Location of Exeter in south west (UK), the low Hoopern valley in Exeter (↘), and Exeter University (✵). Images from Google Earth (2013).

2.2.3 Experimental design - Chronic toxicity

From data obtained from the acute 4 days exposure, groups of 15 *G. pulex* were placed in each dish (4 replicates per treatment) and exposed to sublethal

concentrations of Cd to 0.001; 0.005; 0.01 mg Cd L^{-1} for 7 and 14 days. During the exposure, one pre-weighed leaf was placed as a source of food in each experimental dish. Water change and re-dosing were done every five days. Each dish was covered to reduce water evaporation. Live animals were counted daily, and dead animals were removed immediately.

2.2.4 Single-cell gel electrophoresis (Comet Assay)

DNA damage was measured in individual haemolymph cells from non-exposed and exposed organisms to lethal and sublethal concentrations, according to the method described by Singh et al. (1988). Three replicates were done per treatment. Haemolymph was sampled by inserting a hypodermic needle attached to a 1 ml microsyringe in the telson of the organisms. Due to the low volume of haemolymph and thus low number of cells, the haemolymph of four test individuals was pooled from each replicate. Haemolymph was transferred into microcentrifuge tubes containing 20 µl of phosphate buffer saline (PBS) pH 7.4 on ice. Each sample (200 µl) was centrifuged (3 min, 1000 rpm) and the supernatant removed. The pellet (cell concentrate) was gently mixed with 1% low melting point agarose (LMA), heated at 37^{o}C and placed onto 1% agarose-coated slides. The Comet assay which measures both double and single stranded DNA breaks was performed using alkaline conditions. Briefly: The slides were placed in lysis solution (0.1 mM EDTA, 2.5 M NaCl and 10 µM Tris) for 1 h at 4^{o}C and immersed for 45 mins in electrophoresis buffer (0.3 M NaOH and 1mM). They were placed in the electrophoresis tank, an electric field was applied (25 V and 300 mA) for 30 mins. Thereafter, the slides were neutralised with 0.4 M Tris, pH 7.5 for 5 mins each time, stained with 20

mg/l ethidium bromide, and analysed with a fluorescent microscope (420–490 nm excitation filter and 520 nm emission filter). The percentage of DNA in the comet tail (quantity of DNA with strand breaks) for each cell was quantified (n = 100) using Kinetic V COMET Software.

2.2.5 Lipid peroxidation

To measure the degree of lipid peroxidation caused by Cd, the thiobarbituric acid reactive substances (TBARS) assay was used in exposed and unexposed organisms. This assay measures lipid peroxidation in the form of malondialdehyde (MDA) and was modified from Camejo et al (1998). Samples of whole animal were directly snap frozen in liquid nitrogen to avoid any oxidation, and stored at –80^{o}C. The level of lipid peroxidation due to oxidative stress was assessed in tissue. In brief, tissue was homogenized with a hand-homogenizer with 400 µl of PBS. To 80 µl of tissue homogenates, volumes of 300 µl a mixture of (5 tables of PBS + 372.2 mg of EDTA), 20 µl BHT (butylated hydroxytoluene), 150 µl TBA (thiobarbituric acid dissolved in 0.3% w/v of NaOH) and 100 µl (TCA) trichloroacetic acid were added to each sample. All samples were then vortexed and incubated at 60^{o}C for an hour. Thereafter, samples were centrifuged for 5 mins at 10000 rpm, the supernatant was removed carefully, and 200 µl from each sample was added to a 96-well plate in duplicate. The absorbance was measured at 530 and 630 nm (spectrophotometer). Standard curve were prepared from 1,1,3,3 tetraethoxypropane (0.01 mol/l) to estimate TBARS concentrations.

2.2.6 Cadmium uptake

Cadmium accumulation in the whole body of exposed animals as well as the amount of Cd accumulated in leaves which were used as a source of food was also undertaken. The animals were exposed to sublethal concentrations (0, 0.001, 0.005, and 0.01 mg Cd L^{-1}) of $CdCl_2$ in triplicate for 120 h. Ten animals were placed in each crystal dish (10 cm diameter), with 150 ml dechlorinated tap water, and fed on oak leaves (*Q. cerris*).

The initial dry weight of leaves was taken. During the exposure water was not changed, and the dishes were covered to prevent water evaporation. The remaining leaves were dried in an oven at 60^{o}C for 48 h, weighed, and digested in 3 ml ultrapure nitric acid (65%) (Fluka) and heated until dry. After this 3 ml ultrapure nitric acid with 0.5 ml of hydrochloric acid (v/v) was then added and heated until the brown fume of nitrogen oxide started to appear. After this the digest was diluted in 25 ml Milli Q water and measured by atomic absorption spectrometry (AAS).

After 120 h of exposure, the amphipods were rinsed in deionised water, dried between two sheets of tissue paper, weighed, snap-frozen in liquid nitrogen, and then stored at –80^{o}C until chemical analysis. Approximately, ten pooled animals or more were weighted to get 0.5 g. A mixture of 3 ml nitric acid and 0.5 ml of hydrochloric acid was added, digested at 60^{o}C until dried, transferred to a centrifuge tube after mixing with ultrapure water, and centrifuged at 2500 rpm for 20 min at room temperature. The first supernatant was transferred to the volumetric flask. The remaining digest in the centrifuge tube was then centrifuged at 2500 rpm for 20 minutes, and the second supernatant was transferred to the volumetric flask with the first supernatant and made up to 25 ml with Milli Q water (Amiard et al, 1987; Felten et al, 2008a). Cadmium

levels were determined by AAS at 228.8 nm. The Cd accumulation in the gammaridean and leaves was expressed in mg/g dry weight.

2.2.7 Statistical analysis

Statistical analysis was performed by One-way ANOVA and Tukey for multiple comparisons by using SPSS, 19. The results are represented as mean ± standard error, to compare statistical difference between controls. Significance was set at $p < 0.05$. Regression and probit was used for estimate Median lethal concentration (LC_{50}) by using SPSS.

2.3 Results

2.3.1 Water samples

Parameters of water collected from the field showed (Table 2.1) that the Hoopern stream was more alkaline and Cd during sampling was less than 0.001 mg Cd L^{-1} and pH was 9.11 ± 0.20.

Table 2.1 Physio-chemical parameter of water collected from the lower Hoopern valley stream in 2010.

Water parameters	Mean ± SEM
Water temperature (^{o}C)	12.8 ± 0.0
pH	9.11 ± 0.20
Conductivity (µS/cm)	415 ± 0.10
Dissolved oxygen (ppm)	6 ± 0.10
Calcium (ppm)	37.62 ± 1.31
Sodium (ppm)	25.25 ± 0.25
Potassium (ppm)	4.60 ± 0.12
Cadmium (ppm)	< 0.001

2.3.2 Acute exposure

The percent of survival of *G. pulex* decreased significantly at 0.03 mg Cd L^{-1} ($p < 0.001$) after 24 and 48 h exposure (Fig 2.3A). At concentration 1 mg Cd L^{-1} only a 10% of the population survived after 24 h, after 48 h there was 100% mortality. After 48 h exposure the median lethal concentration (LC_{50}) was 0.073 mg Cd L^{-1} according to probit analysis (Fig 2.2). In the lowest concentrations of Cd tested (Fig 2.3B), the percent of mortality was not significantly different from nonexposed animals, the median lethal concentration (LC_{50}) was estimated after 24, 48, 72, 96 and 114 h during exposure to different Cd concentrations (Table 2.1).

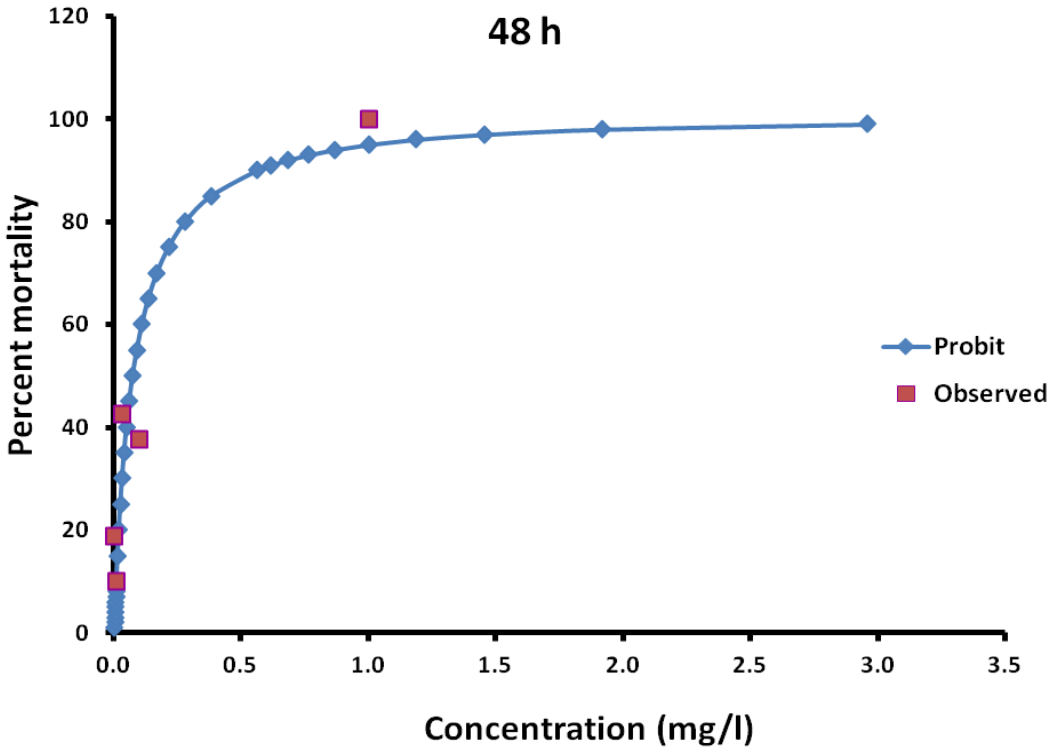

Figure 2.2 Toxicity of Cd to *G. pulex.* Median lethal concentration (LC_{50}) after 48 h exposure.

Table 2.2 Lethal concentrations (LC_{50}) based on time exposure during acute exposure to cadmium.

Period of exposure (h)	(% survival) LC_{50} (mg Cd L^{-1})	Probit analysis (SPSS) LC_{50} (mg Cd L^{-1})
24	0.9	0.175
48	0.4	0.073
72	0.018	0.015
96	0.014	0.010
114	0.012	0.021

2.3.3 Chronic exposure

The lowest concentrations tested did not show a significant mortality in comparison to control population. Whereas the highest concentration tested of 0.01 mg Cd L^{-1} did show significant difference in survival after 7 and 14 days where 79 ± 0.27% ($p < 0.01$) and 59 ± 0.9% ($p < 0.05$) respectively survived after this time (Fig 2.4).

2.3.4 Genotoxicity

Initial results of acute toxicity show that a 48 h exposure to 0.01 mg Cd L^{-1} increased DNA damage (as shown by tail intensity) to 16.56 ± 6.11% ($p > 0.05$) compared to the control. Exposure to 0.03 and 0.1 mg Cd L^{-1} decreased the damage slightly to 14.56 ± 6.11% and 10.23 ± 7.67% (ANOVA; $p > 0.05$) respectively (Fig 2.5A).

Cadmium had a significant ($p < 0.001$) effect on DNA damage of haemolymph cells at the highest concentration tested of 0.01 mg Cd L^{-1} after 14

days (Fig 2.5B), whereas the DNA damage in the lowest concentrations tested were not significantly different from control organisms.

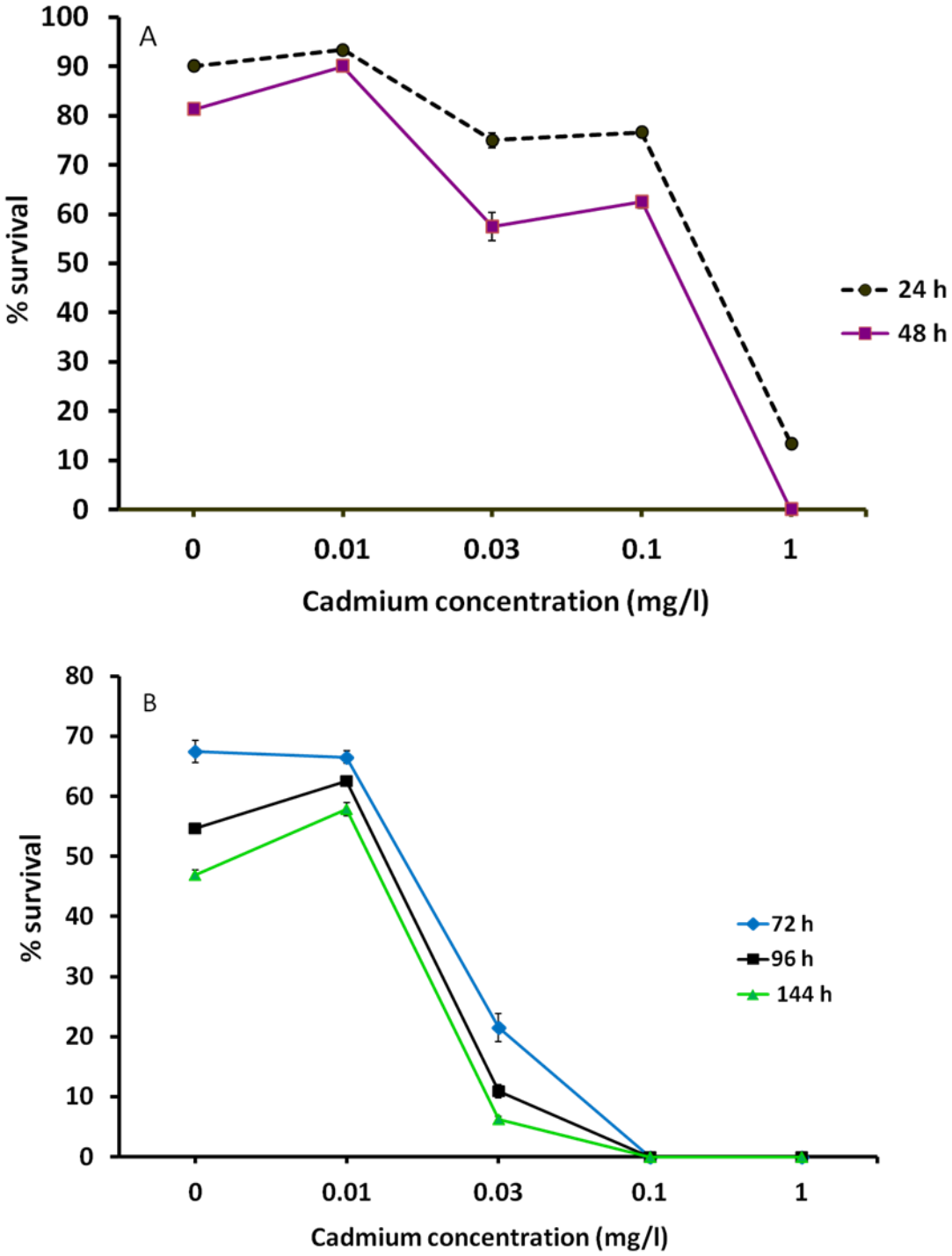

Figure 2.3 Percentage survival of *G. pulex* exposed to Cd for 24 and 48 h (A) and 96 and 144 h (B). Mean ± SEM, n = 20 each treatment).

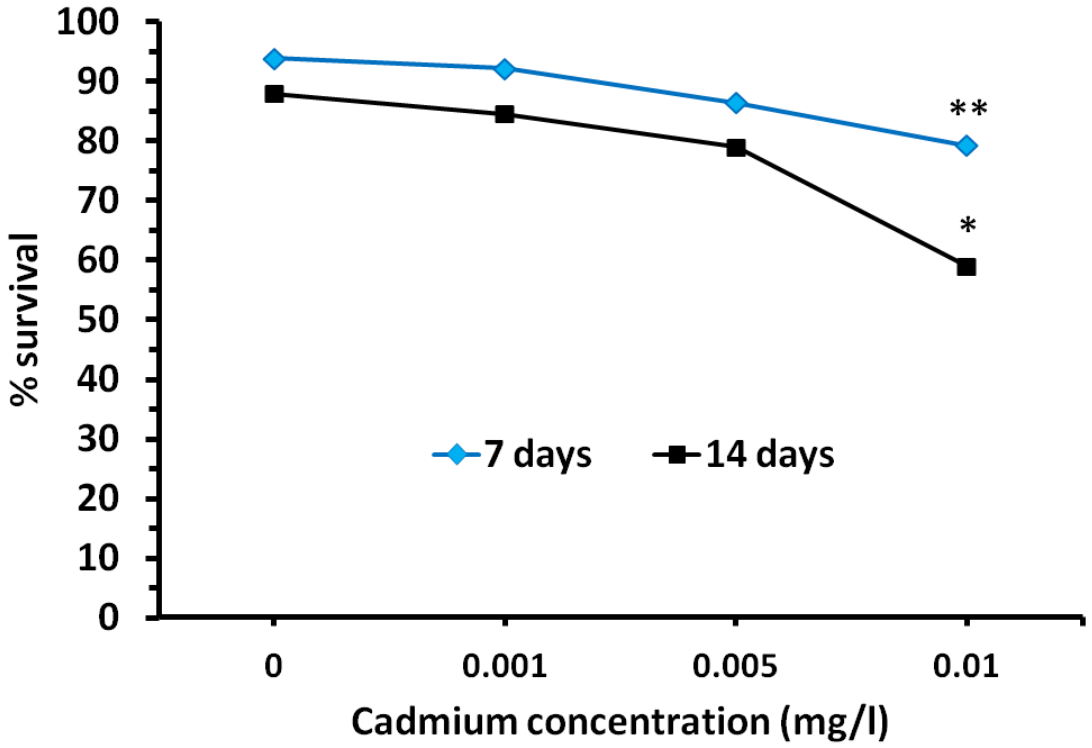

Figure 2.4 Percentage survival of *G. pulex* exposed to Cd for 7 and 14 days. Asterisks show significance difference between each treatment and control, *p < 0.05, **p < 0.01; (mean ± SEM, n = 15 each treatment, ANOVA and Tukey post hoc, p < 0.05).

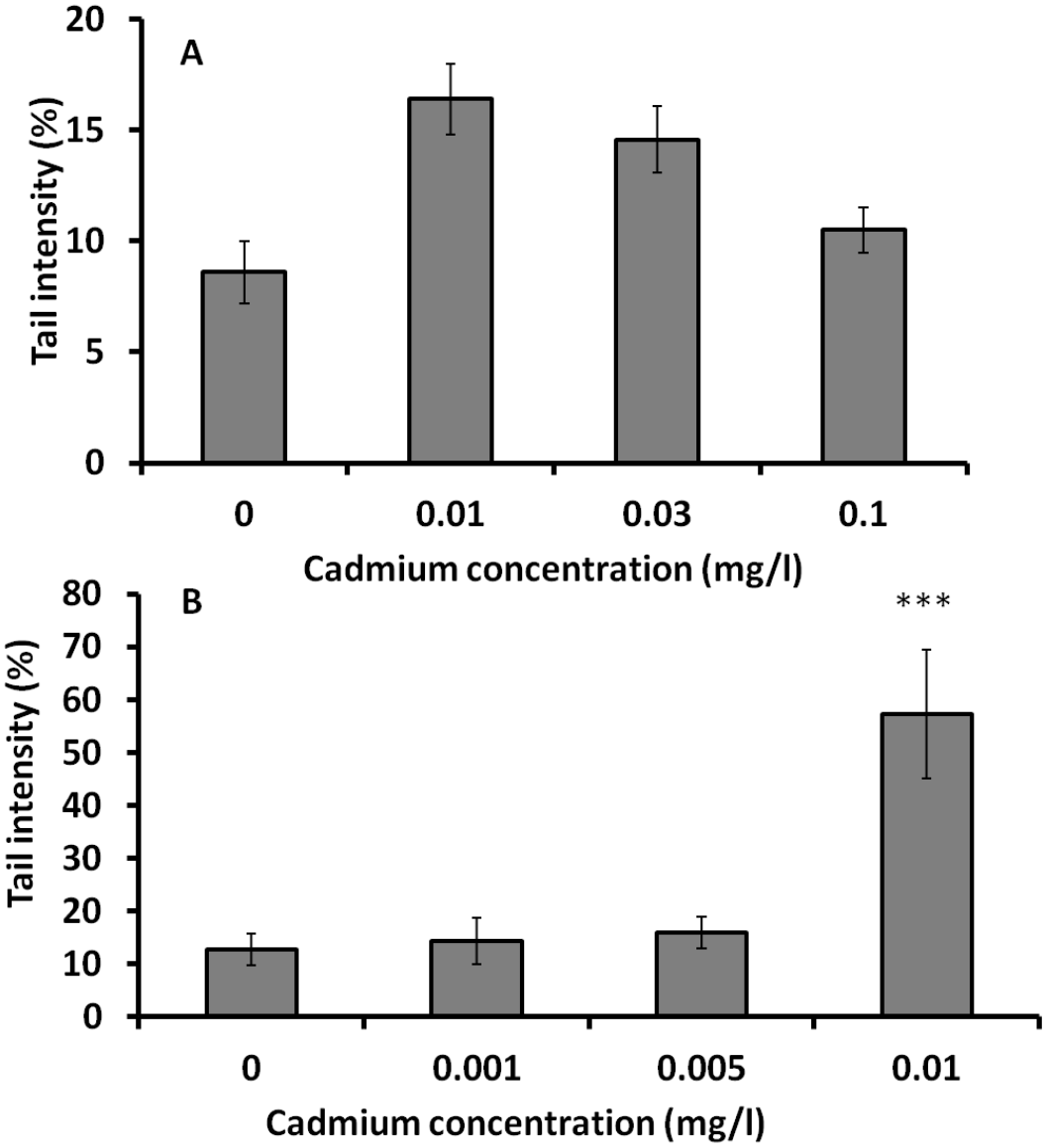

Figure 2.5 DNA damage quantified as the percentage (%) of DNA in the tail of haemolymph cells of *G. pulex* after 24 h (A) and 14 days (B) exposure to waterborne cadmium. Asterisks show significance difference between each treatment and control (mean ± SEM of 4 replicates, ANOVA and Tukey post hoc, ***$p < 0.001$).

2.3.5 Lipid peroxidation

The results were estimated from the standard curve (Fig 2.6A) and showed that Cd induces lipid peroxidation with increased Cd concentrations in the whole body of test animals (Fig 2.6B). It increased gradually from 0.69 ± 0.039 nmol

MDA/g wet weight tissue in control to 0.86 ± 0.004 nmol MDA/g wet weight tissue at the of concentration 0.001 mg Cd L^{-1}. It increased significantly in exposed population to 0.005 mg Cd L^{-1} from 1.22 ± 0.052 nmol MDA/g wet weight tissue ($p < 0.001$) to 2.61 ± 0.078 nmol MDA/g wet weight tissue at 0.01mg Cd L^{-1} ($p < 0.001$).

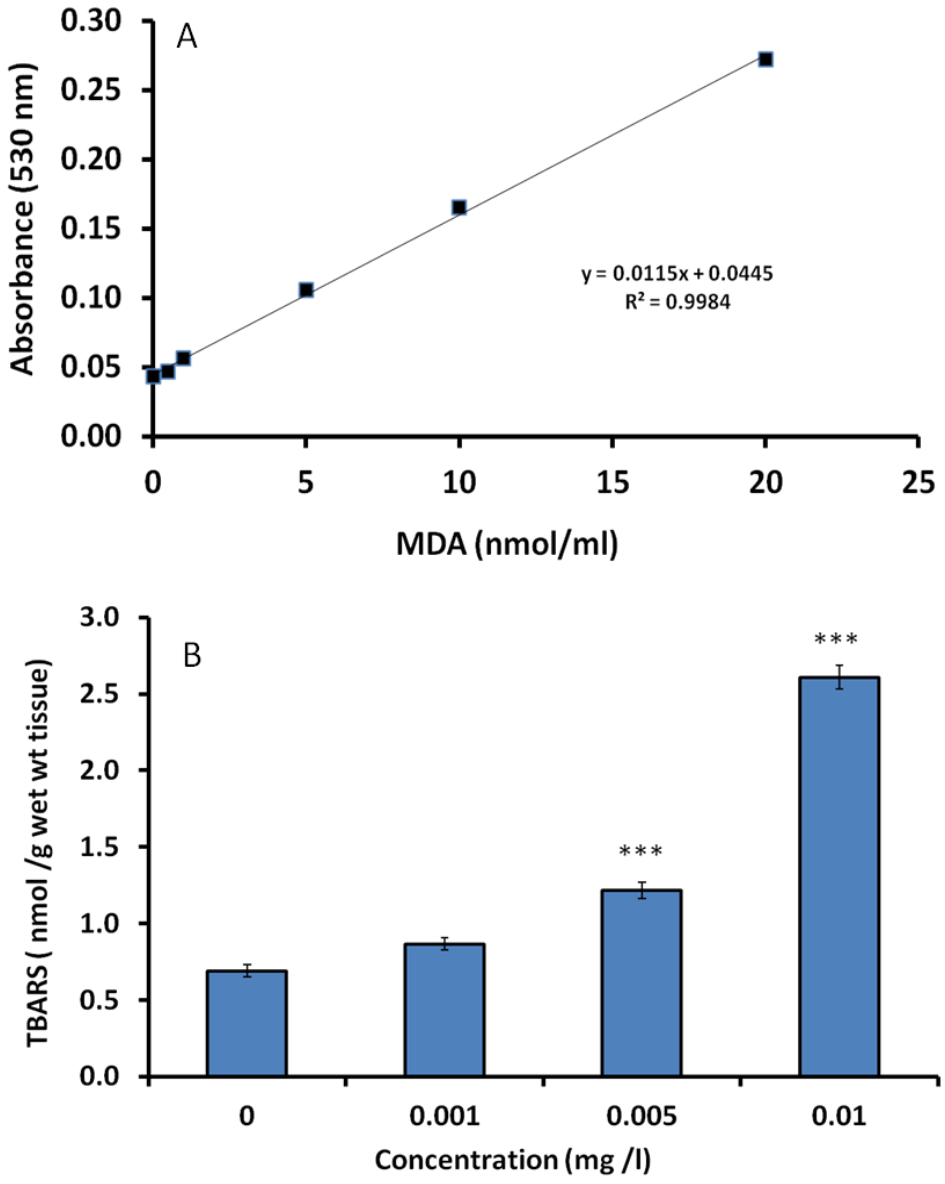

Figure 2.6 Standard curve of malondialdehyde (A), lipid peroxidation (B) in 4 replicates. Asterisks show significance difference between each treatment and control, ANOVA and Tukey post hoc, *$p < 0.001$. The results represent as mean ± SEM.

2.3.6 Cadmium uptake

The mean temperature of water during the experiment was 12^{o}C, pH 9.45 ± 0.20, and dissolved oxygen was 6.56 ± 0.56 ppm. The data showed that the leaves (0.037 ± 0.007 mg/g dry weight, p = 0.029) accumulated significantly more Cd than *G. pulex* (0.030 ± 0.002 mg/g dry weight, p = 0.003) at the same concentration 0.01 mg Cd L^{-1} (Fig 2.7). The present result showed that the leaves exposed to 0.005 and 0.01 mg Cd L^{-1} accumulated proportionately 27.67% and 46.54% respectively. It was 0.007 ± 0.000 mg/g dry weight in control, and then increased to double in leaves exposed to 0.001 mg Cd L^{-1} to 0.014 ± 0.004 mg/g dry weight. While, the exposure concentration 0.005 mg Cd L^{-1} led to 0.022 ± 0.002 mg/g dry weight. Cadmium accumulated in the whole body of *G. pulex* and the leaves in a dose-dependent manner; from the results the leaves accumulated higher amounts of Cd than the animals. It was 0.006 ± 0.002 mg/g dry weight in the whole body of control and reached to 0.011 ± 0.003 mg/g dry weight at concentration 0.001 mg Cd L^{-1}, in exposed amphipods to 0.005 and 0.01 mg Cd L^{-1} were more significant (0.021 ± 0.002 mg/g dry weight, p = 0.019, 0.030 ± 0.002 mg/g dry weight, p = 0.003, respectively). The percentage of Cd in the whole body of exposed to 0.005 and 0.01 mg Cd L^{-1} ranged from 31.06 to 44.77% respectively (Table 2.3).

Table 2.3 Percent of cadmium accumulation in exposed animals and leaves as food (mg/g dry weight).

Nominal concentrations (mg Cd L^{-1})	Measured water concentration %	Gammarus %	Leaves %
0	16.26	8.33	8.81
0.001	20.93	15.91	16.98
0.005	29.17	31.06	27.67
0.01	33.63	44.77	46.54

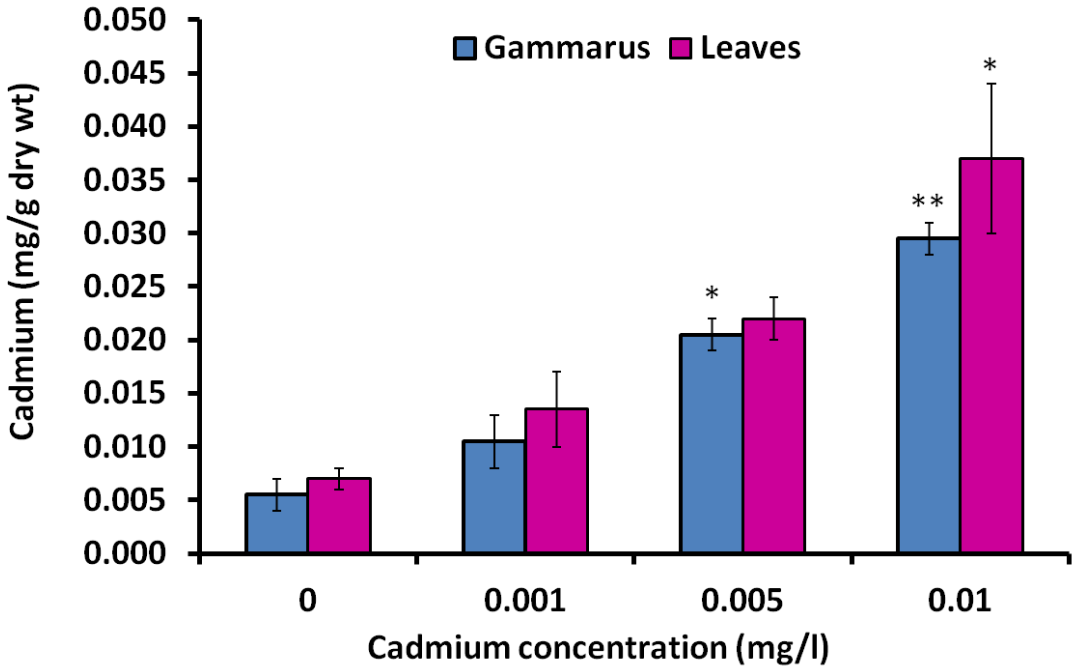

Figure 2.7 Cadmium accumulation in the whole body and leaves after 120 h exposure to different Cd concentrations. Asterisks show significance difference between each treatment and control (ANOVA and Tukey post hoc, *$p < 0.05$; **$p < 0.01$).

2.4 Discussion

2.4.1 Effect of survival

In the present study, the percentage survival of *G. pulex* gradually declined with increasing exposure time. The lethal concentration (LC_{50}) found here was 0.014 mg Cd L^{-1} after 96 h. Wright and Frain (1981) found that the lethal Cd concentration for 96 h was 0.12 mg Cd L^{-1} after exposure of intermoult *G. pulex* to 0.1–5 mg Cd L^{-1} in water containing 20 mg Ca L^{-1}, although, the concentration 0.1 mg Cd L^{-1} was more toxic after 24 h to post-moult sage. McCahon and Pascoe (1988c) found the LC_{50} after 96 h exposure to 0.018 mg Cd L^{-1} in intermoult and 0.021 mg Cd L^{-1} in immediate post-moult of *G. pulex*. Abel and Bärlocher, (1988) found that exposure of the amphipod *G. fossarum* to 1 mg Cd L^{-1} in soft water killed all the animals in less than 10 days. Vellinger et al. (2012b) found that a 28.5 µg Cd L^{-1} there was an increase in the mortality of *G. pulex* to 82.1% after 240 h exposure. From previous studies it was concluded that the difference in findings was associated with the experimental setting (temperature, salinity, and acidity/alkalinity) and animal condition (adult, juveniles, gender, life cycle stage and moult cycle stages). Vellinger et al. (2012c) found that the mortality of *G. pulex* was higher at 15^{o}C than 10^{o}C after 96 h exposure to Cd (Vellinger et al., 2012c), adult *G. pulex* were more tolerant to Cd toxicity (0.3–2 mg Cd L^{-1}) than juveniles at 15^{o}C (Alonso et al., 2010b). Low bivalent ions such as Ca^{2+} and Mg^{2+} in water was more hazardous to freshwater organisms due to Cd and Ca compete the same for transport mechanism in the channel which regulates ion permeation (Wright and Frain, 1981., Nelson, 1986., Newman, 2010., Sigel et al., 2013).

2.4.2 DNA damage

The genotoxicity of Cd is targeted at DNA bases (Devi et al., 2001). Cadmium acts by inducing ROS indirectly by displacement of Cu, Zn and Fe ions from intracellular sites, the release of Fe ions stimulates the Fenton reaction to generate ROS (Stohs and Bagchi, 1995; Waisberg et al., 2003). This results in induction of lipid peroxidation processes, DNA damage and apoptosis (Bertin and Averbeck, 2006; Liu et al., 2009; Slobodskova et al., 2010; Wang et al., 2011). The results showed that Cd at increasing concentrations gradually increased DNA damage in haemolymph cells of *G. pulex* after 48 h, while 1 mg Cd L^{-1} was lethal for all gammaridean after 48 h. DNA damage in the multifunctional haemolymph cells of *G. pulex* may weaken the immune defence system, and disrupt homeostatic processes such as transport and excretion in the animal. Isani et al. (2009) found that exposure of *Sparus aurata* (Fish) to 0.1 mg Cd L^{-1} did not cause any damage to DNA of erythrocytes after 4 and 11 days. In addition to that the exposure to 1.5 µg Cd L^{-1} for 5 days did not show any genotoxicity to the spermatozoa of the amphipod *G. fossarum,* while the concentration 15 µg Cd L^{-1} increased the mortality of gammaridean (Lacaze et al., 2010). Spermatozoa of the freshwater *G. fossarum* were shown to be more sensitive to anthropogenic pollutants than hemocytes and oocytes in field studies (Lacaze et al., 2011). In some cases, acute exposure to Cd may induce production of antioxidant enzymes as a mechanism to protect the cell contents (Chang et al., 2009; Slobodskova et al., 2010; Wang et al., 2012), or bind with metallothionein.

The present result indicates that Cd induced DNA damage significantly at sublethal concentration 0.01 mg Cd L^{-1} in adult *G. pulex* (57.30 ± 12.15%, $p < 0.001$) after 14 days. While low concentrations (0.001 and 0.005

mg Cd L^{-1}) did not show a significant effect (14.37 ± 4.34%; 15.95 ± 3.03%; $p > 0.05$, respectively). DNA damage was associated with increased oxidative stress as indicated by the change in lipid peroxidation as observed for exposure to 0.005 and 0.01 mg Cd L^{-1} in the tissues of *G. pulex* 1.22 ± 0.52 nmol/MDA/g and 2.61 ± 0.078 nmol/MDA/g wet weight the influence of Cd on freshwater *G. pulex* on DNA may need a longer period to accumulate and induce ROS before the damage can be observed. Previous studies showed that exposure to 4.25 and 8.50 µ mol L^{-1} of Cd significantly increased DNA damage to haemocytes and hepatopancreas cells of the white shrimp *Litopenaeus vannamei* (Chang et al., 2009). High concentrations of Cd ranged from 0.5 to 128 mg/k b. wt were highly genototoxic to leukocytes of Swiss Albino male mice after 24 h (Devi et al., 2001). Using concentrations ranging from 5–150 Cd µM caused DNA damage to human lymphocytes after 1 h exposure (Błasiak, 2001). Concentrations of 200 µM for 5 and 8 h induced DNA damage in the hepatoma cells of rat; whilst a lower Cd concentration (20 µM). Expand on relevance of lysosomal damage in these cells (Fotakis et al., 2005).

2.4.3 Lipid peroxidation

In this study Cd induced lipid peroxidation significantly in tissues of freshwater *G. pulex* after exposure to 0.005 and 0.01 mg Cd L^{-1}, while at the concentration 0.001 mg Cd L^{-1} it did not show any significant effect. Increase malondialdehyde (MDA) as the secondary product of polyunsaturated fatty acid peroxidation is considered a marker of tissue damage (Sevanian and Hochstein, 1985; Gutteridge, 1995; Liu et al., 2009). Cadmium is a redox-inactive metal in the Fenton reaction, although it can induce ROS

indirectly (as outlined above), leading to oxidative damage (lipid peroxidation) via disruption of the balance between antioxidant/pro oxidant processes within the organism. This allows free radicals such as hydroxyl radical ($HO^{\bullet}$) and superoxide radical ($O^{\bullet -}_2$) to attack double bonds in membrane lipids, protein and DNA, leading to membrane damage, protein dysfunction and impaired DNA repair (Ercal et al., 2001; Livingstone, 2003; Valko et al., 2005). The relationship between generation of lipid peroxidation and exposure to Cd has been clearly observed in different species. In crustacea, exposure to 300 µg Cd L^{-1} for 13 days led to a significant increase in the level of MDA in *G. pulex* (Khan et al., 2010). Concentrations ranging from 7.25 to 116 mg Cd L^{-1} for 7 days increased oxidative stress in the heart of the freshwater crab *S. yangtsekiense* and the testis of freshwater crab *S. henanense* (Lei et al., 2011; Wang et al., 2011) and alimentary system of the freshwater crab *S. henanense* 14.5 and 29 mg Cd L^{-1} (Wu et al., 2012).

In vertebrates, concentrations ranging from 50 to 100 µM of Cd for 2 h led to increase MDA in C6 rat glioma cells (Wätjen and Beyersmann, 2004), the cortical neurons of fetal rat after exposure to 100 µM Cd for 24 h (Lòpez et al., 2006), and the testes of cultured frog *Rana limnocharis* after 14 days to 10 mg Cd L^{-1} (Zhang et al., 2012). In the present study the average MDA content in *G. pulex* was significantly higher at concentrations of 0.01 mg Cd L^{-1} (2.61 ± 0.078 nmol MDA/g wet weight). In comparison, the freshwater crab *S. henanense* showed lipid peroxidation levels of 1.42 ± 0.33 nmol MDA/g wet weight at 116 mg Cd L^{-1} (Wang et al., 2011). Correia et al. (2003) found that the rate of lipid peroxidation in the amphipod *G. locusta* was higher in the males than in females and it was the lowest in juveniles. In the present study, the concentration 0.01 mg Cd L^{-1} induced lipid peroxidation production and DNA

damage in haemolymph cells of *G. pulex*, the rate of Cd accumulation in the body may lead to decline antioxidants.

2.4.4 Cadmium uptake

Cadmium accumulation in the leaves was significantly higher (0.037 mg/g dry weight) than in the amphipod *G. pulex* (0.030 mg/g dry weight) at 0.01 mg Cd L^{-1} after 120 h. The amount of Cd in gammaridean (control) in the present study is 5.5 µg Cd L^{-1} that is nearly the average allowable for drinking water 5 µg Cd L^{-1} (European Union, United States and China). Felten et al, (2008a) found the concentration of Cd in amphipod *G. pulex* exposed to 15 µg Cd L^{-1} for 168 h was 2.5 fold higher. The ability of these organisms to concentrate Cd from contaminated water than ambient water may be hazardous for secondary consumers such as amphibian and fish which depend upon amphipods as a source of food. It is more than 3-fold increased in exposed animals at 0.01 mg Cd L^{-1}. Wright and Frain, (1981) found that exposed post moult *G. pulex* to 0.01 mg Cd L^{-1} accumulated 0.0228 mg/g dry weight in artificial stream water (20 mg Ca L^{-1}). This is slightly lower than in the present study, although the post-moults are highly sensitive. The mid-gut gland (hepatopancreas) of crustacea has the ability to reduce the metals in their bodies by sequestering or detoxification in specific cells (Schultz, 1976) or binding with the sulfhydryl group (Abel and Bärlocher, 1988). The rate of Cd accumulation is faster than the elimination or detoxification in the body of organisms as it is not regulated like essential elements by normal biological processes (Rainbow and White 1989; Rainbow, 2007). Wright, (1980a) found that the process of Cd accumulation in the freshwater *G. pulex* occurs via “accidental active cadmium uptake” by competing with Ca for the same sites (e.g. Ca channels), on account of the ionic

radius of Cd being 0.92 Å and for Ca ions is 0.94 Å (Newman, 2010). The rate of Cd accumulation is higher in freshwater crustacea than marine species, because of salinity (Wright, 1980a). Uptake of Cd through contaminated food was less detrimental to the amphipod *G. fossarum* than through water (Abel and Bärlocher, 1988). The results of Schaller and his colleagues (2011a) indicated that the gut epithelium of *G. pulex* can take up 15 metals and metalloids which were significantly higher than whole body; however these elements were reduced by a series of sequestering and detoxification reactions, then excretion through the gut epithelium. Wright and Frain (1981) demonstrated that an abundance of Ca in ambient water can reduce Cd accumulation in the whole body of *G. pulex.* Abel and Bärlocher (1988) found that the freshwater crustacean *G. fossarum* accumulated more Cd in soft water than hard water. Absorption of Ca after moulting may contribute to increased Cd accumulation (Rainbow and Dallinger, 1993). In many crustaceans, gills and hepatopancreas accumulate higher amounts of Cd than other organs (Rainbow, 2007). The exoskeleton is utilised to deposit and eliminate metals during moulting (Pellet et al., 2009). Hardness of water and the Ca status during the moulting reflected the amount of Cd uptake in the freshwater *G. pulex* (Wright and Frain, 1981). Increased Cd accumulation in the leaves raises the risk to organisms, due to the freshwater *G. pulex* depending upon leaf litter as a food source. If leaves are contaminated with Cd, this may reduce the growth rate and viability. Duddridge and Wainwright, (1980) found the growth of mycelium (fungi) as a food source at Cd concentrations of 150–170 μg g^{-1} had been reduced, leading to reduced survival of *G. pulex* to 15% after 8 days.

In conclusion, exposure to low, environmentally realistic concentrations of Cd increases oxidative stress which leads to tissue damage. It is also evident from the literature that Cd accumulation could influence the behaviour of gammaridean via reductions in feeding, ventilation rate and physiological process such as ion regulation. This will be explored and discussed in the next Chapters.

Chapter 3 Effect of sublethal concentrations of cadmium chloride on ventilation rate and movement behaviours of *Gammarus pulex*

3.1 Introduction

Discharge of pollutants (heavy metals, pesticides, radioactive materials, pharmaceutical, and oil dispersants) extensively in aquatic environments may lead to changes in behavioural and physiological responses of aquatic organisms (Blaxter and Ten Hallers-Tjabbes, 1992, De Lange et al., 2006). Behavioural studies have been developed in toxicology as early warning responses to pollutants in short-term exposure, because many of them are rapid, uncomplicated, inexpensive, and sensitive (Gerhardt, 1996; Wallace and Estephan, 2004; Felten et al., 2008a; Sornom et al., 2010; Vellinger et al., 2012c). Moreover, behavioural studies can provide a link between the influence of contaminants at a biochemical and cellular level in the laboratory and effects on communities and population level in the field (Newman, 2010; Sornom et al., 2010; Vellinger et al., 2012c).

The detritivore *G. pulex* feeds on different varieties of food such as algae, deciduous leaves, and animal materials, and decomposing leaf litter in streams and rivers (Willoughby and Sutcliffe, 1976; Welton and Clark, 1980). They prefer feeding on leaves which are colonised with fungi and bacteria (so-called conditioned leaves). The action of microorganisms is to transfer deciduous leaves into a more digestible form by softening the ultrastructure and raising the bioavailable protein content (Marchant and Hynes, 1981; Graça et al., 1993a & b; Chamier and Willoughby, 2006; Bloor, 2010). The time taken for leaves to be conditioned also leaves them vulnerable to interaction with any toxicants

present in the water column, thus potentially allowing for the transfer of toxicants to *G. pulex* during feeding.

The detrimental effects of various toxicants on the behaviour of the freshwater *G. pulex* has been documented extensively on locomotion, swimming, feeding rate, ventilation, reproduction and avoiding predators as a sensitive bioindicator in aquatic environments for monitoring pollutants and water quality (Ward, 1986; Pascoe et al., 1994; Mills et al., 2006; Felten et al., 2008a; Sornom et al., 2010; Vellinger et al., 2012c).

The feeding rate of *G. pulex* has been developed by researchers as a bioindicator to assess water quality and the impacts of contaminants in the aquatic environments. This includes for example, metalliferous discharges, acid mine drainage, the pesticide Lindane, and metals. Feeding rate assays typically utilise leaf, therefore, the majority of food consumed depends on decomposing leaf litters as a source of food (Naylor et al., 1989; Maltby and Crane, 1994; Malbouisson et al., 1995; Maltby et al., 2002; Macedo-Sousa et al., 2007; Dedourge-Geffard et al., 2009) or use the eggs of *Artemia salina* (Taylor et al., 1993; Blockwell et al., 1998) as a food source. The multispecies freshwater biomonitor (MFB) is a new technique to estimate several kinds of behaviour (feeding, swimming and ventilation) under the same conditions and at the same time as accurate and automatic technique (Alonso et al. 2009).

Deleterious effects of pollutants on the feeding behaviour may influence the rate of energy available for growth and reproduction of the population (Dedourge-Geffard et al., 2009). The capability of Cd to accumulate in the aquatic organisms such as molluscs and crustacean raises the rate of hazard, as they are an essential food for primary and secondary consumers in aquatic systems (Felten et al., 2008a; Vellinger et al., 2012a). Food is a source of

energy for many biological activities in living organisms and the pathway of contaminants to the alimentary canal. Reduction of feeding activity has been reported previously in *G. pulex* after 168 h exposure to sublethal concentrations of Cd (Felten et al, 2008a), in the freshwater *Echinogammarus meridionalis* (Amphipoda) and in the decapods *Atyaephyra desmarestii* (Decapoda) after 96 h exposure to sublethal concentration of Cd and Zn (Pestana et al, 2007). However, the feeding rate of *G. pulex* is not only influenced by contaminants in aquatic, but also environmental factors such as temperature, daytime, salinity, acidity/alkalinity of water and digestive enzymes activities (Willoughby, 1983; Felten and Guerold, 2001; Maltby et al., 2002; Felten et al., 2008b; Dedourge-Geffard et al., 2009; Sornom et al., 2010; Vellinger et al., 2012a).

The jointed legs of amphipods have been modified to allow for multiple functions including for grooming and feeding, swimming, walking and copulation, as well as generating currents of water on the gills to provide enough oxygen. Locomotor activity helps many amphipods to mate, avoid predators, find food and shelter (Gerhadt, 1995; Mills et al., 2006; Felten et al., 2008b; Morillo-Velarde, et al., 2011). Increase Cd concentrations in aquatic environments have an inhibitory effect on locomotor activities of crustacea if it increased than allowed level 1 µg Cd L^{-1}. Cadmium impaired horizontal and vertical swimming activity of the costal amphipod *G. lawrencianus* at concentrations excess than 62 µg Cd L^{-1} (Wallace and Estephan, 2004), and mobility of *G. pulex* at different degrees of temperature for 96 h (Vellinger et al., 2012c). In marine crustacea, Cd at 0.5 µg Cd L^{-1} inhibited the swimming ability of *Neomysis integer* against the current (Roast et al., 2001) and moving velocity of shrimp *Hippolyte inermi* at 1 ppm (Untersteiner et al., 2005).

There are a few studies examining the effect of contamination on gender (Sornom et al., 2010) and age (Taylor et al., 1993; Wright and Frain, 1981; Alonso et al., 2010b) in *G. pulex*. The majority of studies used only males (Felten et al., 2008a & b; Vellinger et al., 2012a & c; Vellinger et al., 2013), due to differences in sensitivity between males and females. However, using both males and females is common in the laboratory studies (Gerhardt et al 1995; Pellet et al., 2009). Therefore, the males were less demanding to energy for spermatogenesis, and better physiological status than female during oogenesis and eggs incubation which is demanding increase energy (Sornom et al., 2010). The variation in size between male and female of these species could be a reason to prefer the males.

The aim of this study was to investigate the effects of sublethal Cd concentrations on the behaviour of *G. pulex* including: ventilation rate, feeding rate and horizontal movement in adult male and female animals.

3.2 Material and methods

3.2.1 Feeding rate

A binocular microscope (Wild Heebrugg) was used to discriminate between males and females, the second antenna of the male is longer than in the female, on the male antenna there are 4 and 5 segments of peduncle with a short setae, which are arranged in 3 longitudinal rows (Pinkster, 1970). The length of adult animals was between 10.02 to 16.7 mm; this was measured to the nearest 0.01 mm by using a dissecting microscope via ocular micrometer (from the top of the head to the telson).

Leaf discs were prepared from fallen oak leaves (*Q. cerris*), collected from the field. The leaves were washed and left to air dry under laboratory conditions, at a temperature of 20 ± 2^{o}C. Before starting the experiment, each leaf was cut to form a disc (28 mm in diameter), and then dried in an oven at 60^{o}C for 48 h, weighed to the nearest 0.01 mg, and rehydrated in dechlorinated tap water for 24 h prior to the experiment.

The amphipods *G. pulex* were placed separately in wall cell culture dishes (Ø3 cm in diameter, 1.5 cm high), containing 10 ml of dechlorinated tap water (73 ppm as $CaCO_3$) which was changed every 24 h to avoid accumulation of excreted waste and re-exposed (Fig. 3.1). At each concentration of Cd 12 adult males and 12 adult females were exposed, at 12 ± 1^{o}C. No juveniles or gravid females were used in the experiments.

Feeding rate (FR) for the preliminary experiment was expressed as mg consumed leaf weight per mg dry weight of *G. pulex* per day.

To measure the feeding rate equations 1 and 2 were used

$$FR = \frac{Lb * k - Le}{g * t} \quad (1)$$

Where **Lb** is the initial dry weight of the leaf disc in mg, **Le** is the final dry weight of the leaf disc in mg, **g** is the dry weight of *G. pulex* in mg, and **t** is the feeding time in days.

K is the leaf change correction factor given by

$$K = \frac{((Le/Lb)}{n} \quad (2)$$

Where, **Lb** is the initial dry weight of leaf discs and **Le** is the final dry weight of the leaf disc (mg) both measured in the replicates without the animal and **n** is

the number of replicates (Naylor et al, 1989; Maltby and Crane, 1994; Maltby et al, 2002., Bloor and Banks, 2006; Wilding and Maltby, 2006; Felten et al., 2008a).

An exposure period of 120 h was decided based on the time taken for ingestion of food matter and the optimum survival of the animals. At the end of the exposure, leaves and animals were washed twice in deionised water, dried in an oven at 60^{o}C for 24 h, and weighed to the nearest 0.01 mg.

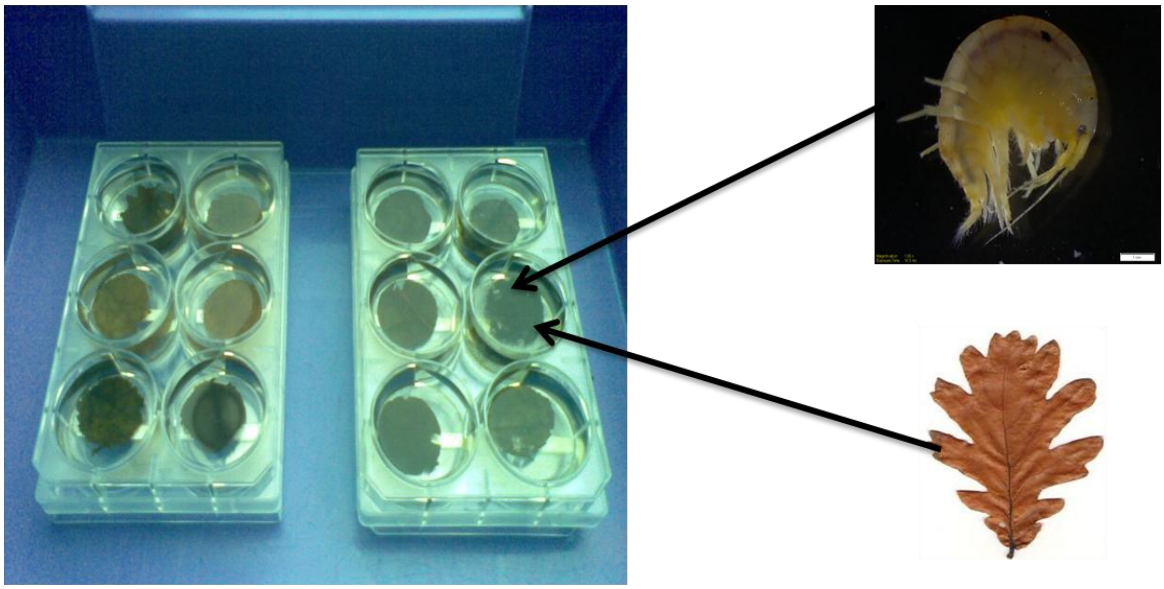

Figure 3.1 Oak leaf discs used for estimating the feeding rate of males and females *G. pulex* exposed to Cd.

3.2.2 Ventilation rate

The amphipods were placed in cell culture dishes aforementioned (section 3.2.1), with 10 ml of dechlorinated tap water, dissolved oxygen (> 7 mg/l) at 12 ± 1^{o}C. The ventilation rate was measured as the number of pleopod beats per minute for each animal (n = 12 per treatment) after a 30 min acclimation to the test solution. A videotape from above with low light was made by using a

video camera (Ikegami, Japan) and manual counting undertaken. The ventilation rate was counted after 60 min and 24 h after exposure to different concentrations of Cd. Counting was performed on the same day to avoid circadian rhythm of respiration (Rosas et al., 1992; Felten et al., 2008a). The animals were not fed throughout the experiment.

3.2.3 Horizontal movement

The horizontal movement activity of animals (clockwise or counter clockwise) in each cell culture plate (ø3 cm in diameter, 1.5 cm high) was counted on the same animal used to measure the ventilation rate after 60 min and 24 h (Fig 3.2).

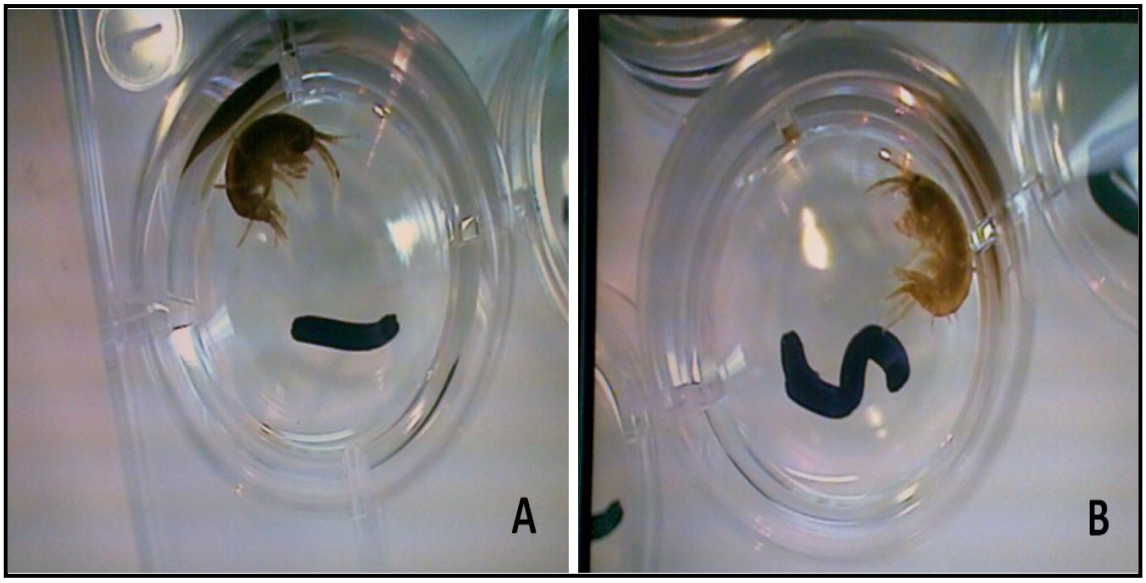

Figure 3.2 Horizontal swimming of *G. pulex* (males and females), clockwise (A), counter clockwise (B) in wall cell culture dishes.

3.2.4 Statistical analysis

The results expressed as mean ± standard error for feeding, ventilation rate and horizontal movements of males and females. Box plots represent median

values for dry weight of males and females. Median and mean are used ventilation and horizontal movement of juveniles, males and females. One-way ANOVA, Post Hoc tests (Scheffe) to assess any significant differences between means.

3.3 Results

3.3.1 Feeding rate

The feeding rate decreased sharply in females exposed to 0.005 mg Cd L^{-1} from 0.15 ± 0.04 mg/mg/day by 2% to 0.08 ± 0.04 mg/mg/day by 1% at 0.01 mg Cd L^{-1} after 120 h. It also decreased in males to 1.25 ± 0.96 mg/mg/day by 17% at 0.005 mg Cd L^{-1} to 0.51 ± 0.33 mg/mg/day, 7% at 0.01 mg Cd L^{-1} (Fig 3.3), while, the concentration 0.001 mg Cd L^{-1} has no effect on the feeding rate of males. The results show that the feeding rate was not significantly different compared with control.

The dry weight of females for 120 h varied from 4.33 ± 0.96 mg d.wt in control group to 4.67 ± 0.7 and 4.92 ± 0.62 mg d. wt at 0.005 and 0.01 mg Cd L^{-1} in females. The dry weight of males in the control group was 10.17 ± 0.44 mg d. wt, while in males exposed to 0.005 and 0.01 mg Cd L^{-1} this was reduced to 7.75 ± 1.02 mg d. wt and 8.25 ± 1.11 mg d. wt (Fig 3.4).

3.3.2 Ventilation rate

The number of pleopod beat per min (pbm) was variable in both sexes. The mean ventilation rate of males was higher (206.8 ± 9.96 pbm) than females (182 ± 9.43 pbm), while the average ventilation rate in juveniles was higher than in adult females 188.4 ± 8.4 pbm after 30 min acclimation (Fig 3.5). The

ventilation rate in males after 60 min acclimation to the test solution declined to 138.4 ± 19.8 pbm by 23% at 0.001 mg Cd L^{-1} and remaining constant at 0.005 mg Cd L^{-1} 138.5 ± 26.5 pbm by 23% and the declined to 123.2 ± 17.8 pbm by 21% receptively. Ventilation rate declined in males and females after 24 h in control to 109.8 ± 27.5 pbm, 30%, and 91.67 ± 33, 31%, respectively, it decreased sharply to 93.3 ± 12.4 by 25%; and 60.5 ± 12.4 pbm, 17% in males exposed to 0.005 and 0.01 mg Cd L^{-1}, respectively after 24 h (Fig 3.6A).

In females exposed to 0.001 and 0.005 mg Cd L^{-1} reduced gradually from 150 ± 18.7 by 26%; to 146 ± 11.7 by 25%, respectively, after 60 min. While the females exposed to 0.01 mg Cd L^{-1} decreased significantly to 107.7 ± 12.6 pbm by 18%, $p < 0.05$ after 60 min (Fig 3.6B). Hypoventilation was also observed in female *G. pulex* exposed to 0.005 and 0.01 mg Cd L^{-1} (67.8 ± 32.1, 23%; and 52.8 ± 25.6 pbm, 18%, respectively) after 24 h.

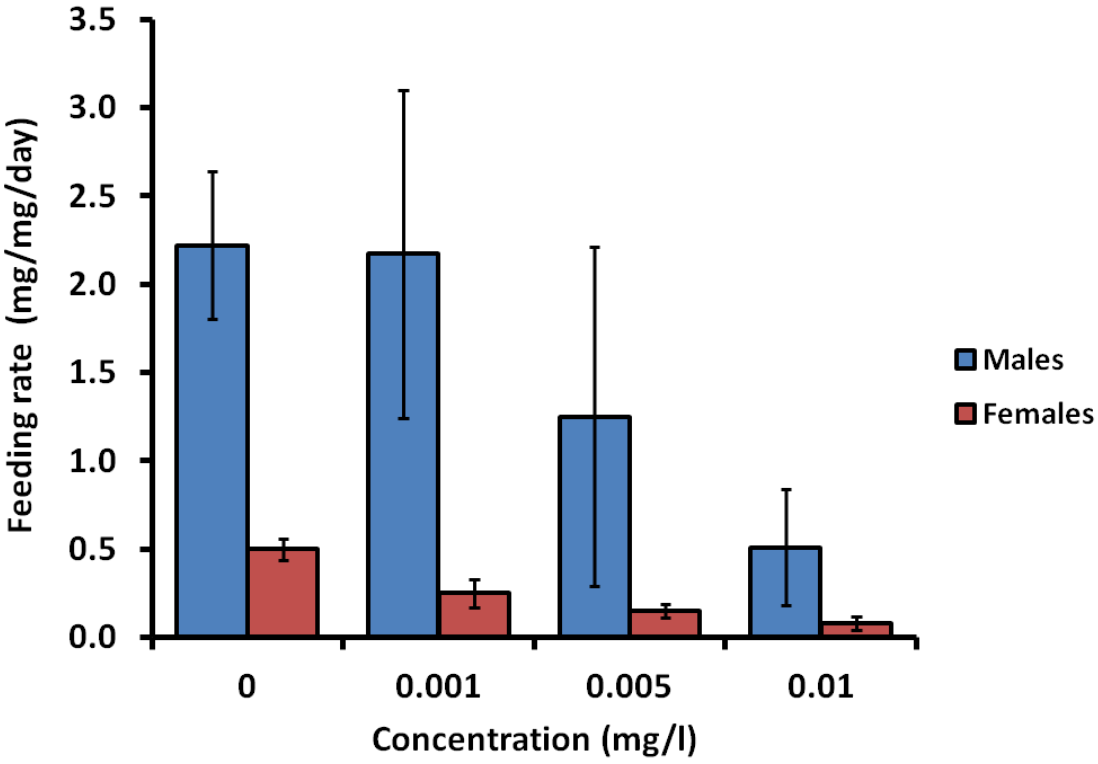

Figure 3.3 Feeding rate (mg/mg/day) after exposure males and females *G. pulex* to Cd for 120 h in comparison to non-exposed. (each point represent the mean ± SEM, ANOVA and Scheffe post hoc, $p > 0.05$; $n = 12$ each treatment).

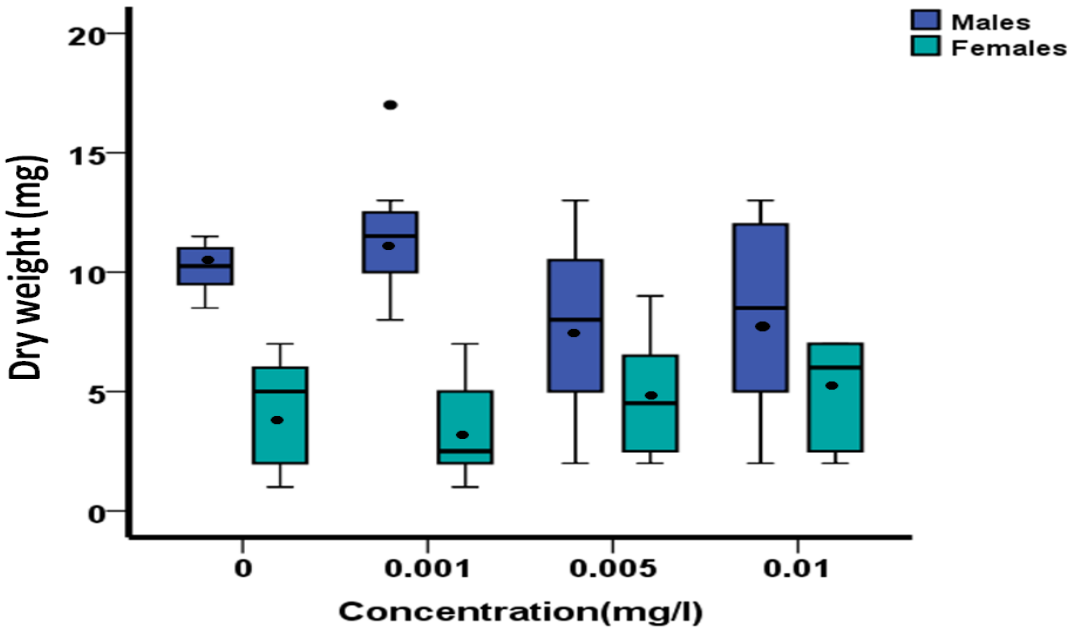

Figure 3.4 Box plots represent median values with the percentiles 25% and 75% maximum and minimum dry weight (mg) of males and females after feeding on leaf discs during exposure to Cd concentrations. • Represents mean of dry weight, n = 12 each treatment.

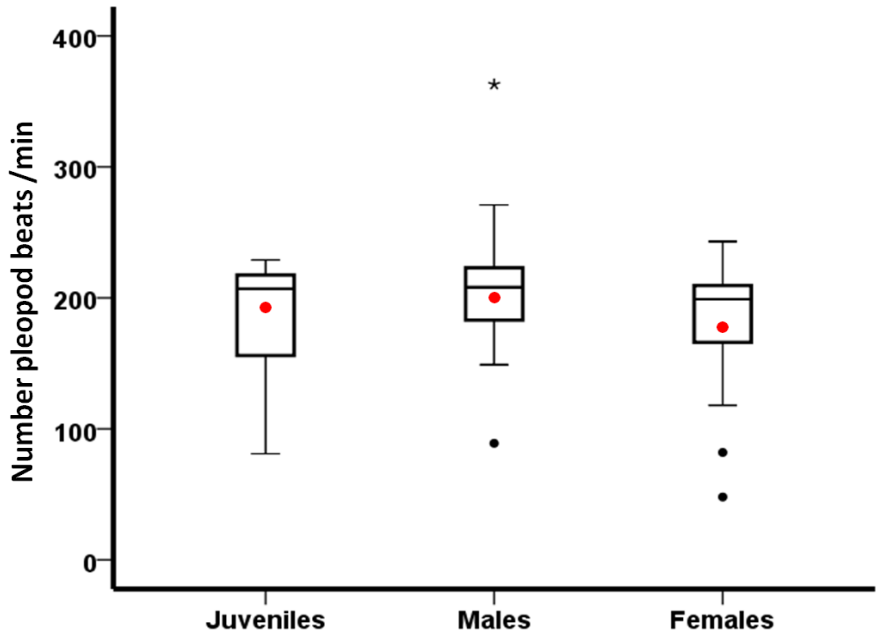

Figure 3.5 Box plots represent median values with the percentiles 25% and 75% maximum and minimum of ventilation rate of juveniles, adult males and females after 30 min accumulation without exposure to cadmium. • Represents mean of ventilation rate (n = 23 each treatment).

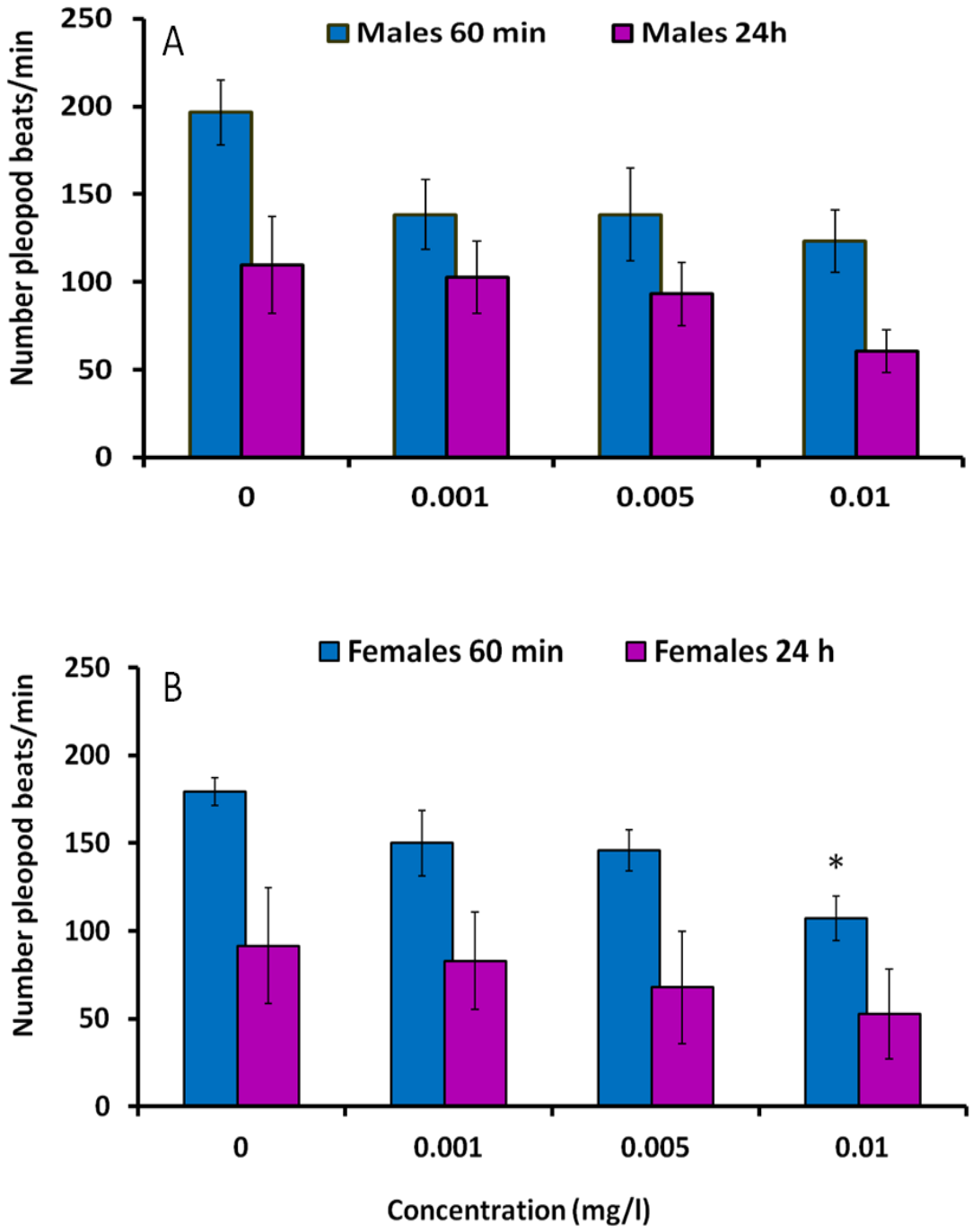

Figure 3.6 Number of pleopod beats/min throughout exposure to Cd in males *G. pulex* for 60 min and 24h (A), in females 60 min and 24 h (B). Asterisks show significance difference between each treatment and control (each point represent the mean ± SEM, ANOVA and Scheffe post hoc, $p < 0.05$, $n = 12$ each treatment).

3.3.3 Horizontal movement

The average locomotor activity of each individual animal per min (clockwise and counter clockwise) after 30 min exposure to Cd was higher in juveniles and males 5.04 ± 1.5 and 5.1 ± 0.7 movement per min, $p > 0.05$ respectively. While, in adult females the horizontal movement was the lowest 3.7 ± 0.7 movement per min (Fig 3.7). After 60 min, horizontal movement in the control group of females and males was slightly decreased 2.57 ± 0.67; 38% and 2.5 ± 0.56; 32%, respectively. It was reduced in males to 1.67 ± 0.55; 21% per min after 60 min exposure to 0.01 mg Cd L^{-1} to 1.45 ± 0.62; 20% after 24 h (Fig 3.8A). Cadmium concentrations had a more detrimental effect on the females than males, it was 1.18 ± 0.43; 18% after 60 min exposure to 0.005 mg Cd L^{-1}. While, the horizontal movement in females exposed to 0.01 mg Cd L^{-1} was completely stopped 0.9 ± 0.43 after 60 min and 0.8 ± 0.42 after 24 h (Fig 3.8B), all of the animals exposed to 0.01 mg Cd L^{-1} were tended to reduce their locomotion and curved.

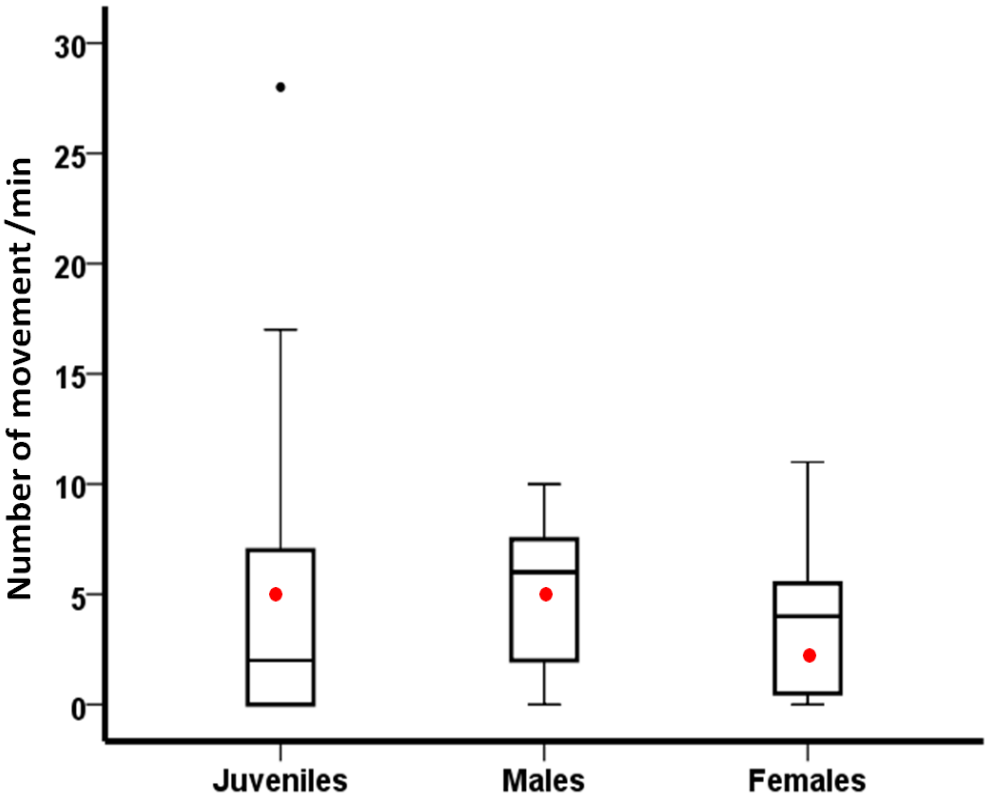

Figure 3.7 Box plots represent median values with the percentiles 25% and 75% maximum and minimum of horizontal movement per min to juveniles, adult males and females after 30 min accumulation with exposure to cadmium. • It represents mean movement each group, n = 23 each treatment.

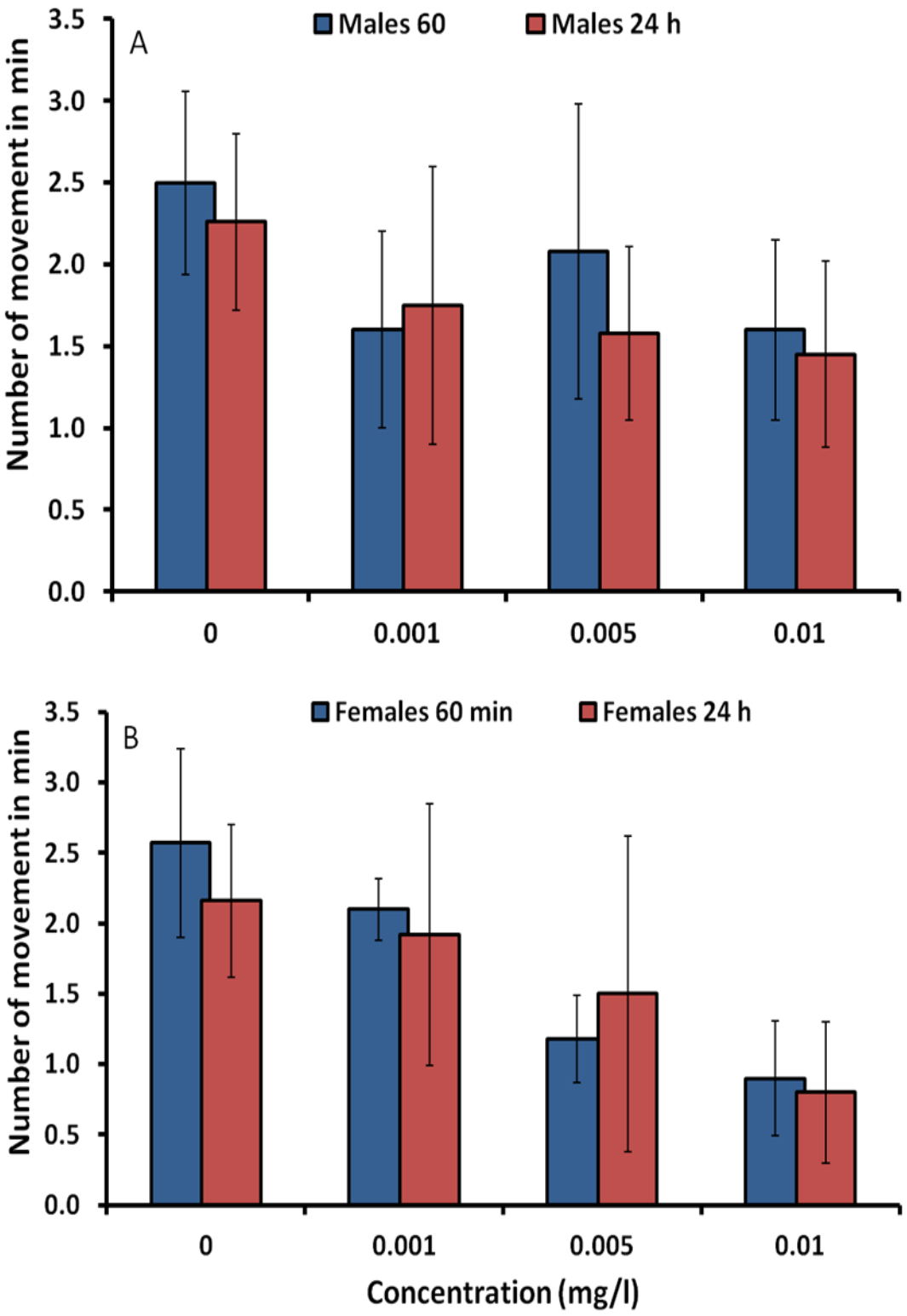

Figure 3.8 Horizontal movement of males (A) and females (B) *G. pulex* non-exposed and exposed to Cd for 60 mins and 24 h (each point represent the mean ± SEM; n = 12 each treatment; ANOVA and Scheffe post hoc, $p > 0.05$).

3.4 Discussion

3.4.1 Feeding rate

In the present study, the feeding rate of males on oak leaves was higher than in females. Cadmium concentration (0.01 mg Cd L^{-1}) reduced the feeding rate of males to 0.51 mg/mg/day by 7% and 0.08 ± 0.041 mg/mg/day by 1% in females after 120 h. Reduction of feeding rate in the female animals exposed to Cd may affect the abundance of females and their ability to produce a new generation in contaminated water, leading to impairment of the population and equilibrium of aquatic ecosystems. Especially, the amphipods represent a main source of food for many macroinvertebrates, fish, amphibians and birds (Felten et al., 2008a). Using unconditioned alder leaf discs reduced feeding rate of males *G. pulex* from 0.04 mg/mg/day at 7.5 µg Cd L^{-1} to 0.037 mg/mg/day at 15 µg Cd L^{-1} after 168 h (Felten et al, 2008a). Increase Cd concentration to 0.05 mg Cd L^{-1} after 48 h reduced the percentage of feeding activity of *G. pulex* on conditioned leaves discs to 33.4% (Alonso et al., 2009). Sublethal Cd concentration 6 µg Cd L^{-1} reduced feeding rate of the amphipods *Echinogammarus meridionalis* after 96 h on condition chestnut leaves (Pestana et al., 2007). Feeding activity of juveniles *G. pulex* on eggs *Artemia salina* reduced after 96 h exposure to copper and Lindane (Blockwell et al., 1998).

There are many factors which can lead to reduce the feeding rate in the natural environment such as metalliferous effluents, acid mine drainage, industrial waste, and water quality in the environment; impairment of feeding rate may reflect on reproduction and growth rate of population in aquatic environments (Crane and Maltby, 1991; Blockwell et al., 1998; Wilding and Maltby, 2006; Macedo-Sousa et al., 2007; Pestana et al., 2007).

Previous studies have been reported that conditioned leaves are a better quality food for the detritivore *G. pulex* than unconditioned leaves (Taylor et al., 1993; Alonso et al., 2009). The microorganisms such as fungi and bacteria transferred deciduous leaves to a more digestible form by softening and raising the bioavailable protein content (Marchant and Hynes, 1981; Willoughby, 1983; Graça et al., 1993a & b; Chamier and Willoughby, 2006; Bloor, 2010). Foucreau et al. (2013) found that *G. pulex* consumption rate on the soft leaves was higher than tough leaves. If the rate of contaminates increased in aquatic environments, it could have a direct effected on growth and abundance of microorganisms, which are necessary for modifying leaf materials to the freshwater *G. pulex* (Graça et al., 1993a & b). Nevertheless, the microorganisms play a vital role to decompose leaf litter, they have ability to accumulate a high amounts of contaminates (Schaller et al., 2011b).

Although, feeding rate is a good indicator to contaminants, there are limitations to the assay. For example, it is very difficult to obtain organism from the field which are all from the same life stage and as the experiment was undertaken ex-site, it may have an effect on feeding rate. In the present study, there was an inverse dose-dependent relationship between increasing Cd levels and decreasing feeding rate. During the moulting, amphipods are more sensitive to Cd toxicity especially in post-moult stage. Furthermore, they avoid feeding until the mouthparts and exoskeleton are hardened (Wright and Frain, 1981; Willoughby and Earnshow, 1982). Reduction of digestive enzyme activity and feeding rate of *G. fossarum* was related directly with elemental pollutants such as arsenic, cadmium, copper, nickel, and zinc (Dedourge-Geffard et al., 2009). Increased accumulation of toxicants from food or water (Chapter 2) damages tissues and cell organelles which are responsible for absorption and

synthesis of enzymes. Examples include the hepatopancreas which is crucial for the digestion of food as a source of energy for many physiological activities. Histological study confirmed that exposure to 0.005 and 0.01 mg Cd L^{-1} damaged microvilli of the hepatopancreas (Chapter 4) which are responsible for absorbing digested food. Damaged cells of the hepatopancreas lead to reduce the feeding rate.

3.4.2 Ventilation rate

Crustaceans utilise pleopods beat as an approach to enhance the flow of water over the gills to provide enough oxygen for aerobic metabolism via ATP production (Vellinger et al., 2013), particularly in crustacea having a hard exoskeleton which can prevent respiration through the skin. The number of pleopods beats/min clearly decreased in this study with increasing concentrations of Cd in both sexes during 60 mins and 24 h of exposure to 0.005 and 0.01 mg Cd L^{-1}. Felten et al. (2008a) found the ventilation rate of the freshwater *G. pulex* exposed to 15 and 7.5 µg Cd L^{-1} for 120 h reduced significantly, as a result of the gills are the first target for toxicants in ambient water. The data presented here show that the males and juveniles had higher ventilation rates than females. The juveniles require oxygen for energy, growth and movement. The size of males is modified to carry the females during copulation that requires energy. Hypoventilation occurs when the animal needs to compensate ion lost or close to death. This may happen depending on the intensity of the exposure (Felten et al., 2008b). Reduction of the ventilation and locomotion rate is the pathway to preserve the energy within the body to be utilised in detoxification, elimination and ion/osmo regulation during metallic

stress (Vellinger et al., 2012a & c; Sornom et al., 2010). In the present result, the ventilation rate of male *G. pulex* exposed to varying Cd concentrations after 60 min and 24 h was lower than observed on male *G. pulex* exposed to 7.5 and 15 µg Cd L^{-1} for 120 and 168 h (Felten et al, 2008a). Increasing the Cd concentration to 0.05 mg Cd L^{-1} impaired ventilation rate of *G. pulex* to 50.4% after 48 h (Alonso et al., 2009). Hypoventilation in *G. pulex* during exposure to Cd is associated with increase temperature. The latter acts on reducing oxygen in water and enhances Cd accumulation (Vellinger et al. 2012a & c).

Hypoventilation is also a route to redirection of energy in detoxification mechanism or inducing/ activating of Na^+/K^+-ATPase mechanism instead of losing in ventilation and locomotion activities (Lawrence and Poulter, 1998; Felten et al., 2008a; Sornom et al., 2010).

Pollutants have been associated not only with reduced ventilation rates but also disturbance in the respiratory chain reaction and ion regulation mechanism in the gills. Excess Cd accumulation resulted in damaged tissue and cell organelles of the gills of *G. pulex* especially the mitochondria which are responsible for the respiratory chain reaction (Chapter 4).

3.4.3 Horizontal movements

In the present study, horizontal movement of gammarids decreased after 60 min following 24 h exposure to 0.005 and 0.01 mg Cd L^{-1} by 13% and 20% in females and males, respectively after 24 h, associated with a reduction in ventilation rate. It may approach to reduce the amount of energy consumed in the movement activity and ventilation, redirect for osmoregulation (Vellinger et al. 2013). Disorder and asymmetrical movement (clockwise and counter

clockwise) were observed during exposure to Cd; the majority of animals exposed to Cd tended to stop moving all jointed legs except the pleopods and curved shape (personal observation). Cadmium accumulation induced indirectly ROS via damaged tissue and cell organelles such as mitochondria which are responsible for the respiratory chain reaction by impairing respiration enzyme activities (Wallace and Estephan, 2004). Cadmium reduced locomotion activity, in *G. pulex* by 39% to 15 µg Cd L^{-1} after 168 h (Felten et al., 2008a) and horizontal swimming activity during daytime in *G. lawrencianus* exposed to more than 500 µg Cd L^{-1} after 72 h (Wallace and Estephan, 2004), to save energy and reduce oxygen consumption (Vellinger et al., 2012). Roast et al., (2001) pointed out that exposure to 0.5 µg Cd L^{-1} impaired swimming ability of *Neomysis integer* against the current and increased oxygen consumption which led to exhaustion of exposed animal. Furthermore, the moving velocity of marine shrimp *Hippolyte inermis* declined after 3 h exposure to 1 mg Cd L^{-1} (Untersteiner et al., 2005). Morillo-Velarde, et al. (2011) found that swimming activity of *G. aequicauda* during the night for 24 h at 0.24 and 0.28 mg Cd L^{-1}. Increasing the water temperature to 15^{o}C during exposure to 51.1 µg Cd L^{-1} increased Cd toxicity and reduced locomotion activity of *G. pulex* (Vellinger et al., 2012b). Cadmium may have an influence on the nervous system which is responsible for regulation of locomotion behaviour (Roast et al., 2001). Reducing locomotor activity could result in gammarids vulnerable to predation and starvation (Vellinger et al., 2013). In fact, the histological sections (light microscope) showed that Cd damaged the epithelial layer under the cuticle of legs the amphipod *G. pulex* after exposure to 0.01 mg Cd L^{-1} (Chapter 4).

Impairment of locomotor activity in the freshwater *G. pulex* was observed by researchers in the laboratories during exposure to copper and lead (Gerhardt, 1995; Mills et al., 2006), increase acidity of water (Felten et al., 2008b) and release pharmaceuticals such as fluoxetine and ibuprofen (De Lange et al., 2006).

In conclusion, Cd has a significant effect on feeding, ventilation rate and horizontal movement on both genders during exposure to sublethal concentrations; the females *G. pulex* are more sensitive to Cd toxicity than males. Decrease locomotion activities in the natural environment could lead to a reduced ability of an organism to find shelter, food, avoid predators, and mate.

Chapter 4 Ultrastructural observations on the midgut gland (hepatopancreas) and gills of the freshwater *Gammarus pulex* exposed to sublethal concentrations of cadmium chloride.

4.1 Introduction

Anthropogenic activities in recent years have increased the release of Cd to aquatic environments, causing potential impacts on the life of aquatic organisms with increasing accumulation in aquatic bodies (Engel and Fowler, 1979; Cuypers et al., 2010; Valavanidis and Vlachogianni, 2010; Chiarelli and Roccher, 2012). Industrial activities, weathering, leaching from soil, mining and domestic waste water are also contributing factors to increase heavy metals in aquatic environments (Babich and Stotzky, 1978; Landis and Yu, 2004; Tulonen, et al., 2006).

Cadmium is a non-essential element in biological activities in animal kingdom. It is a highly toxic priority pollutant and is often associated with zinc and lead ores, as well as smelting and agricultural activities (Stohs and Bagchi, 1995; Martelli et al., 2006). Reviews of Cd concentrations in freshwater systems across Europe place typical concentrations in the range of 10–500 ng/L Cd in rivers (Jensen and BorRasmussen, 1992), the median Cd concentration around the world recorded from unpolluted natural water was less than 0.1 µg Cd L^{-1} (USEPA, 2001), criteria to protect aquatic life were assessed, is 0.017 µg Cd L^{-1} for freshwater and 0.12 µg Cd L^{-1} for estuarine and marine environment, based on lethal concentrations to high sensitive species in aquatic environments (Marine, 1999). Local hotspots of pollution mostly related to industrial and mining activities where concentrations may exceed these limits, with effects reported within the concentration range that may be encountered by

organisms in freshwater systems (Messner et al., 2012). However, its biological role in impacting aquatic organisms at these sublethal concentrations has been relatively understudied.

The effects of Cd on the tissues and cells of a range of species in the aquatic environments have been established in several studies. Couch (1977) on the gills of marine shrimp *Penaeus duorarum,* Griffiths (1980) on the gut diverticula of *Daphnia magna,* Papathanassiou and King (1983, 1986) on the gills and hepatopancreas of the prawn *Palaemon seerratus*; Papathanassiou (1985) on the gill cells of the brown shrimp *Crangon crangon*, Soegianto et al. (1999) on the epithelial cells of gills, hepatopancreas and epipodites of the late juveniles *Penaeus japonicas,* Wu et al. (2009) on the gills of a white shrimp *Litopenaeus vannamei,* and Wang et al. (2012) on the gills of the freshwater crab *Sinopotamon henanense*. Collectively, these studies suggest that Cd concentrations more than unpolluted aquatic environments alter the cell organelles. The function and processes of these organelles are responsible for the synthesis of enzymes and the release of energy such as mitochondria (Papathanassiou, 1985; Papathanassiou and King., 1986; Cereghetti and Scorrano, 2006; Yamuna et al., 2009).

In aquatic environments, the risk of Cd toxicity is considered greater for freshwater organisms than marine organisms. It seems likely that salinity of water and hardness play an essential role to protect living species, in particular sea water (Engel and Fowler, 1979; Wright and Frain, 1981). In freshwater species, Cd has the ability to accumulate in the bodies of crustaceans and fish in higher amounts than ambient water, transported to food chain (Cuypers et al., 2010). A greater understanding of the sublethal effects of Cd on freshwater crustacean species is therefore a high priority for research.

Increase pollutants in aquatic environments have histopathological effects on aquatic organisms. Both the respiratory organs and mid-gut gland appear vulnerable to a range of toxic substances. Mercury causes adverse effects on the gill and hepatopancreas of the prawn *Macrobrachium malolmsonii* (Yamuna et al., 2009), zinc and cadmium caused damage to the hepatopancreas cells of the terrestrial isopod *Porcellio scaber* at sublethal concentrations (Žndaršič, et al., 2003). Petroleum hydrocarbons at high sublethal concentrations caused intense damage to the hepatopancreas cells (B-cells) of the penaeid shrimp *Metapenaeus dobsoni* (Sreeram and Meron, 2005). A mixture of heavy metals had a similar impact on the gills, hepatopancreas and epipodites of the juveniles the white shrimp *Litopenaeus vannamei* (Frías-Espericueta et al., 2008).

Cadmium induces programmed cell death in most animal cells (Hinkle, et al., 1987; Abel and Bărlocher, 1988; IARC, 1993; Bertin, and Averbeck, 2006; Messner et al., 2012; Wang et al., 2012). It can accumulate in many cells and tissues of organisms such as kidney, liver, lung, gills, pancreas, placenta and bone in vertebrates, hepatopancreas and gills in invertebrates, it leads to damage cell structure through inducing ROS (Soegianto et al., 1999; Cuypers et al., 2010), resulting in dysfunction of the osmoregulation and gas exchange in aquatic organisms and leading at high doses to the destruction of a cell's contents and tissues (Thurberg et al., 1973; Papathanassiou, 1985).

In crustaceans, gills have a multi-functional role and are responsible for ion and osmotic regulation, respiration, acid-base balance and excretion. In addition, they are the first targets for pollutants (Soegianto et al., 1999; Henry et al., 2012). Histopathological alterations in crustacean gill structures have been observed in the epithelial cells as well as cell organelles of crustacea exposed

to Cd (Papathanassiou, 1985; Wang et al., 2012). This has the potential to impair the ventilation rate and gas-exchange (Felten et al., 2004).

The mid-gut gland of crustaceans is primarily responsible for synthesis and secretion of digestive enzymes (glycogen, lipids and trace metals). It plays an essential role in regulating, storing and detoxifying heavy metals (Schultz, 1976; Icely and Nott, 1980; Muskò, 1988; Correia et al., 2002b; Frías-Espericueta et al., 2008), and has been used as “a monitoring organ” in toxicological studies (Icely and Nott, 1980; Lyon and Simkiss, 1984; Correia et al., 2002b). The previous studies pointed out the role and function of this organ in toxicological studies on *G. lacustris* (Schmitz, 1967), *G. minus* (Schultz, 1976; Carlton and Schmitz 1989), *G. roeselii* Gervialis (Muskò, 1988) and *G. locusta* (Correia, et al., 2002a & b).

The freshwater *G. pulex* was chosen in the present study because it is widespread, is a sensitive species to pollutants, a source of food for many organisms (macroinvertebrates, fish, birds and amphibians), and is easily cultured in the aquarium. It plays an important role in decomposing leaf litter (Felten et al., 2008a) and therefore is of high ecological importance. The mid-gut gland of the freshwater amphipod *G. pulex* was shown to be a particularly sensitive organ to toxicants such as lead acetate (Kutlu et al., 2002) and thallium acetate (Ozalp, et al., 2011) which caused damage to fine structure, whilst compounds including: Lindane, copper and 3,4-dichloroaniline have previously been shown to affect the external structure of the hepatopancreas (Blockwell et al., 1996).

The study aims to investigate the effect of Cd at sublethal concentrations for 120 h on the tissues and cells of the hepatopancreas and gills of adult *G. pulex* at different environmentally relevant concentrations. These target organs were

chosen because of their documented sensitivity and because the main pathway for penetration of many pollutants and toxic metals to aquatic organisms is through food or water to the alimentary canal and through the gills to the circulatory system.

4.2 Materials and methods

4.2.1 Test organisms

The adult freshwater *G. pulex* were collected from the lower Hoopern valley stream (50^{o} 43`N; $3^{o}$ 31`W), Exeter, transported to the lab, and placed in glass beakers (2 L) continuing dechlorinated tap water, each aerated constantly via glass pipette, the photoperiod cycle was 12 light: 12 dark, at 12 ± 1^{o}C for a week. The amphipods were fed fallen oak leaves.

4.2.2 Experimental design

Prior to the experiment, all glassware was pre-soaked in an acid wash (10% HCl). The average length of amphipod *G. pulex* was 13.76 ± 0.38 mm (adult males). The animals were acclimated to lab conditions, fed unconditionally on oak leaves, and were exposed to sublethal concentrations of $CdCl_2$ (Fluka) (0, 0.005 and 0.01mg Cd L^{-1}) for 120 h. Animals were placed in crystallised dishes (5 in each dish), with 150 ml of dechlorinated tap water. All concentrations were done in triplicate, and beakers were covered by parafilm to avoid evaporation, and aerated constantly via a glass pipette.

4.2.3 Tissue preparation

Dissection was conducted under a light microscope (Wild Haardage), by a fine needle and forceps. Samples for light microscopy were fixed in Bouin solution for 24 h, and rinsed in 70% alcohol and dehydrated in a series of ethanol from 70% to 100%. Clearing in xylene, and embedding in paraffin. Finally, the serial sections of 5 µm thickness were cut by microtome (AS 325, Shandon), and stained with Hematoxylin and Eosin (H&E). The slides were studied under a binocular microscope (Carl Zeiss). Analysis software was used to take images.

Samples were prepared for the electron microscope, fixed in 3% glutaraldehyde (buffered to a pH 7.2 with an 0.1 M phosphate buffer) for 24 h, washed three times for 10 min in buffer, embedded in osmium tetroxide (OsO_4) for 24 h, washed three times for 10 min in deionised water. Following a series of dehydration in ethanol, the samples were dried by CO_2 at a critical point (33^oC and 1200 mb), coating with gold and polonium via Argon, for examination under SEM (JEOL JSM 5200 scanning electron microscope). Samples were additionally prepared for transmission electron microscopy (TEM), fixed in primary fixation 3% glutaraldehyde with 0.1 M phosphate buffer pH 7.2 for 3 hours, washed three times per 10 minutes in 0.1 M phosphate buffer pH 7.2. Following secondary fixation in 1% osmium tetroxide (OsO_4) for an hour, the samples are kept in deionised water overnight in the fridge, washed in deionised water three times per 10 minutes, and dehydrated in a gradual series of ethanol at room temperature (30%, 50%, 75%, 90%, and two 100%) every 10 mins. The dehydration solution was replaced to complete the penetration process in the resin. The samples were embedded in propylene oxide twice for 15 min and then in (1:1 mixture) propylene oxide: TAAB low viscosity hard resin

for 2 h, after that (1:2 mixture) in propylene oxide: resin (TAAB) for one and half hour, the fresh resin (TAAB) 100% was added for one and half an hour, renewed and kept in overnight on rotary mixer to accelerate penetration of resin in the tissue. Embedding was completed by polymerisation in an oven at 60^{o}C for 24 hours. The sections were cut by a diamond knife (80–90 nm), mounted on 200 mesh Formvar coated copper grids, and stained in Uranyl acetate 35 µl for 30 mins, washed in filtered deionised water twice for 5 mins, and counterstained with lead citrate 35 µl is used for each sample for 4 mins with covering the samples to prevent any reaction with CO_2 (sodium hydroxide is added), washed twice every 5 mins and dried to diagnose with an electron microscope (JEOL 1200 EX).

4.3 Results

4.3.1 Effect of Cadmium on epithelial layer and cell organelles of the gills

The gills or lamellae of *G. pulex* are oval plates, transparent, and compressed on both sides into a flattened shape (Fig 4.1A). They are covered with cuticle, and the gills are attached to the posterior part of the thoracic leg of thoracic segments (pereionites).

The pillar cells (PC) were clearly observed in cross-sectioned in the control animals. Haemolymphatic lacuna (HL) was wide and the nucleus is seen in both sides (Fig 4.1B). For the animals exposed to 0.01 mg Cd L^{-1}, it was shown that the pillar cells had thickened and shrunken HL, with redistribution of the chromatin of the nucleus in the marginal parts (Fig 4.1C).

The fine structure of the gill was then examined. The gill epithelial cells under the cuticular layer consisted of two parts: an apical infolding system (AIS) was

directly under the cuticle (Cu), whilst the basal infolding system (BIS) is located close to the hemolymph sinus. The microtubule bundles are clearly visible between AIS and BIS (Fig 4.2A). The apical microvilli under the cuticle layer were associated with numerous rod-shaped mitochondria (Fig 4.3A). The animals exposed to 0.005 and 0.01 mg Cd L^{-1} showed degeneration of the apical microvilli under the cuticle layer, with destruction of some parts of the epithelial layer between AIS and BIS (Fig 4.2B). Mitochondria in the gill cells exposed to 0.005 mg Cd L^{-1} lost their regular shape, and were swollen. The matrix content inside the inner membrane became opaque. In addition, the microtubules between AIS and BIS disappeared completely (Fig 4.2B & 4.3B). At the exposure concentration of 0.01 mg Cd L^{-1}, there was a more pronounced effect on the epithelial layer of *G. pulex.* The apical microvilli were absent from the apical infolding system completely, microtubule bundles had disappeared, with many spaces between the epithelial tissues that meant the potential for exchange gases or ion regulation in the gills have been reduced. The damage to epithelial tissues and cell organelles would be expected to be associated with a reduced ventilation rate. Mitochondria in the gill cells exposed to 0.01 mg Cd L^{-1} lost some parts of outer and inner member with apparent leaking of matrix contains (Fig 4.2C & 4.3C).

4.3.2 Effect of cadmium on hepatopancreas.

4.3.2.1 Muscular fibres

The mid-gut gland of *G. pulex* consists of two paired caeca, which are blind-ended and run in parallel along the thorax (Fig 4.4A). In the controls, the external surface of the mid-gut gland was covered by a network of longitudinal

and circular muscle fibres, well organized along each tubule (Fig 4.5A). The morphological structure of the mid-gut gland after 120 h exposure to 0.005 mg Cd L^{-1}, showed muscular fibre expansion, this could cause disorder in the flow of fluids through the mid-gut gland (Fig 4.5B) in comparison with the control. Increase Cd concentration to 0.01 mg Cd L^{-1} resulted in further retraction of the muscular fibres (Fig 4.5C).

4.3.2.2 Microvilli

The microvilli were well-developed in the controls (Fig 4.4B and 4.6A). Cadmium had an effect on the microvilli of hepatopancreas in animals exposed to 0.005 mg Cd L^{-1} (Fig 4.6B), causing lysis of the base of the microvilli within cells, whereas exposure of animals to 0.01 mg Cd L^{-1} for 120 h caused a more pronounced effect on the whole structure and the microvilli were broken down (Fig 4.6C).

4.3.2.3 Mitochondria

The effect of Cd includes also the cell organelles in the hepatopancreas, in the controls, the mitochondria were rod-shaped with well developed cristae (Fig 4.7A), whilst after 120 h exposed to 0.005 and 0.01 mg Cd L^{-1} exhibited collapsed inner mitochondrial membrane and lysis of the cristae (Fig 4.7B & 4.7C).

4.3.2.4 Nucleus

The nucleus in the hepatopancreas is elongated with visible nucleolus in the controls (Fig 4.8A). After 120 h exposure to 0.005 mg Cd L^{-1}, the contents of

the nucleolus tend to move to the nuclear envelope of the nucleus, the nuclear envelope of the nucleus became irregular (Fig. 4.8B). At the concentration 0.01 mg Cd L^{-1}, fragmentation of nucleolus and lysis of the nuclear membrane was apparent, indicating that Cd induces programmed cell death (PCD) and this was observed in concentration 0.005 and 0.01 mg Cd L^{-1} in all sections were examined by TEM, each section represents a small part of whole organ (Fig 4.8C).

4.4 Discussion

4.4.1 Gills of adult *G. pulex*

In crustaceans, gills play an essential role in ion and osmoregulation, acid-base balance, ammonia excretion, respiration and large surface area to pass large amounts of water and oxygen (Meyer et al., 1991). These organs are directly exposed to toxicants in aquatic environments, and have been considered as target organs to assess health conditions for organisms in toxicological studies (Papathanassiou and King, 1983; Dhavale et al., 1988).

In this study, the pillar cells seen in light microscopy section were wide, to allow the passage of ions to the haemolymph and gas exchange in control. In animals exposed to 0.01 mg Cd L^{-1}, there were shown to be changes in chromatin in the nucleuos, associated with a shrunken appearance of hemolymphatic canals (HC) and thickening the pillar cells. This may be a response to reduce the flow of toxic elements. Pillar cells are also contributing agents to gas exchange and ion regulation from water to haemolymph, in particular, Na^+ and K^+, also as a cytoskeletal component to support haemolymph flow (Lawson et al., 1994). A similar observation was shown in

pillar cells of *G. fossarum,* exposed to 15 µg Cd L^{-1} for 72 and 168 h, after 72 and 168 h, which was associated with collapse and disappearance of the hemolymphatic canal, resulting in reduction of exchange surface to oxygen diffusion (Issartel et al., 2010). Acute exposure to ammonia (16.5 and 27.5 mM) for 96 h led to thickening pillar cells in gill lamellae of the estuarine crab *Chasmagnathus granulata* which decrease of haemolymph space and gas exchange (de Freitas Rebelo et al., 2000).

In transmission electron sections, the apical infolding system (AIS) as it appeared in the control animals is composed of numerous mitochondria under the cuticular layer. The abundance of these organelles provides a lot of energy for respiration and osmoregulation activities. It has been estimated that the freshwater *G. pulex* spends about 11% of energy in osmoregulation (Sutcliffe, 1984). Microtubules extending between AIS and BIS could contribute to ion regulation, gas exchange and cytoskeletal support (Papathanassiou and King, 1983; Kikuchi et al, 1993; Kikuchi and Matsumasa, 1997). The epithelial cells of the gills act as cellular boundaries against changes in osmotic concentrations in crustaceans (Shires et al., 1994; McNamara and Lima, 1997).

Exposure to 0.005 and 0.01 mg Cd L^{-1} resulted in reduction in the number of mitochondria, corresponding to changes in their appearance as well as changes in tissue structure of the gills after 120 h. The effect was more intense at 0.01 mg Cd L^{-1}, that means the rate of energy production in the gill is likely to be decreased, which is necessary for respiratory and active transport.

Generally, the number of cell organelles decreased in the gill of exposed organisms in contrast with the control. The damage to microvilli is also observed in AIS and vacuolation in this part of the tissue in animals exposed to 0.005 mg Cd L^{-1} increased with increasing Cd concentrations. While, after

120 h exposure to 0.01 mg Cd L^{-1}, the microvilli and the bundles of microtubules disappeared completely from AIS as a consequence of lysis. In addition to that increase of the empty spaces in the whole tissue of the gill. This is likely to lead to respiration dysfunction and the mechanism of osmoregulation may be affected. Silvestre et al. (2005) demonstrated that exposure to 0.5 mg Cd L^{-1} caused alteration in subcuticular, associated with form vacuoles in the epithelial cells of the anterior gill the Chinese crab *Eriocheir sinensis* after 3 days.

Mitochondria in most animal cells play an essential role in producing energy and regulating cellular metabolism from ATP through the respiratory chain. In the present study, swelling of mitochondria was observed in the epithelial layer of the gills of *G. pulex,* exposed to 0.005 and 0.01 mg Cd L^{-1}, with lysis of the outer and inner mitochondria membrane. This may lead to release matrix contents and change to opaque after exposure to 0.005 mg Cd L^{-1}. Those alterations attribute to disorder enzymes activities in those organelles (Papathanassiou and King, 1983; Lei et al., 2011). This could be a contributing factor in reducing the supply of energy in the form of ATP from the respiratory chain, which is important for metabolic haemostasis and ion regulation (Papathanassiou, 1985; Martel et al., 1990; Qin et al., 2012). With relevance to the present study, the ventilation rate was reduced with increase time exposure and concentration in males and females *G. pulex* exposed to the same concentrations of Cd (Chapter 2). Alteration of cell organelles after exposure to Cd was observed in many aquatic species. Couch (1977) found that exposure to 763 µg Cd L^{-1} caused apoptosis and necrosis to gill tissues of the pink shrimp *Penaeus duorarum* after 15 days exposure (Couch, 1977). Bioaccumulation of Cd in the gills of the prawn *Palamon serratus* more than ambient water (5–25

mg Cd L^{-1}) led to loss mitochondria regular shape, associated with fragmentation of Golgi complexes and reduce a number cell organelles after 44 h (Papathanassiou and King, 1983), the similar concentrations utilised on the brown shrimp *Crangon crangon* caused reducing the number of cell organelles, alternation in the structure of mitochondria and rough endoplasmic reticulum (Papathanassiou, 1985). Alterations in cell organelles after exposure to Cd were also observed in, the heart cells of the freshwater crab *Sinopotamon yangtsekiense* (Lei, et al., 2011), and in the renal cortex cells of the rat (Early et al., 1992; Tang and Sheikh, 2001).

Cadmium induces generation of free radicals and indirectly increases ROS in particular in mitochondria to stimulate apoptosis through replacement metals in Fenton reaction (Kowaltowski et al., 2009; Qin et al., 2012; Wang et al., 2012). It also inhibits antioxidants via binding with the sulfhydryl group of mitochondrial enzymes (Ercal, et al., 2001; Wang et al., 2004; Bertin and Averbeck, 2006; Valko et al., 2005), which causes increased opening of the membrane permeability pore in mitochondria via the Ca^{2+} uniporter in the inner membrane of the mitochondria, resulting in a loss of balance between the intermembrane space and mitochondria matrix. This breaks down the outer membrane, straightening cristae and causing swelling which leads to release of matrix contents (Martel et al., 1990; Koike et al., 1991; Miccadei and Floridi, 1993; Skulachev, 1996; Li et al., 2003; Mignotte and Vayssiere, 1998; Wang et al., 2004; Cannino et al., 2009; Issartel et al., 2010). These effects are likely to have contributed to the effects seen here.

Cadmium may also induce PCD in cells by alternation of the structure appearance, in mitochondrial membranes, chromatin condensation and DNA fragmentation in the nucleus (Cereghetti, and Scorrano, 2006; Bertin and

Averbeck, 2006; Lei et al., 2011). Such effects were also clearly observed in the gills and hepatopancreas cells in the present study, which started losing the regular shape of the cell membrane, with margination of nuclear material. Exposure to 0.01 mg Cd L^{-1} led to appearance alteration in the gill tissue which including: shrinkage of cells, fragmentation of nuclear material, swelling and degenerating of the outer and inner membrane of mitochondria. Several studies have reported that Cd induces programmed cell death, in the hemocytes of oyster *Crassostrea virginica* (Sokalova et al., 2004), and the gill cells, hemocytes and testis of the freshwater crab *Sinopotaman henanense* (Qin et al., 2012; Wang et al., 2011, and 2012).

4.4.2 Hepatopancreas

Mid-gut gland (hepatopancreas) of crustacea has multifunction: including, secretion of digestive enzymes, storage (lipids, glycogen and trace elements), assimilation of nutrients, and a site of accumulation and detoxification of metals. It absorbs directly from contaminated food with Cd or indirectly through via the gills to haemolymph. Cadmium induces alteration in the appearance of features of the mid-gut gland of *G. pulex*, as shown by SEM expanding of external muscular layer of hepatopancreas in animals exposed to 0.005 mg Cd L^{-1} after 120 h, while the external muscular fibres of the hepatopancreas were shrunken in exposed animal to 0.01 mg Cd L^{-1} for 120 h.

The muscle fibres in the hepatopancreas of crustacea act to produce waves of contractions to allow the transport of fluids to mix lumen contents which are filtered from the gut (Sheader and Evans, 1975; Moore and Rainbow, 1984; Correia et al., 2002). Toxicants can alter the peripheral muscle fibres and impair

the feeding rate of the amphipod *Parathemisto gaudichaudi* (Sheader and Evans, 1975). Copper, Lindane and 3,4-dichloroaniline caused disorganization of the myoepithelium of hepatopancreas cells of *G. pulex* after 24 h (Blockwell et al., 1996). SEM showed that gut diverticula of *Daphnia magena* shrinkage after 2 h exposed to 52 and 12 µg Cd L^{-1} of Cd and attributed this to interference from Cd in calcium metabolism as an essential element for muscle contraction and regulation of osmosis (Griffiths, 1980; Stohs and Bagchi, 1995; Valko et al., 2005; Cannino et al., 2009). It might be a reason for the change seen in the muscular fibres of hepatopancreas after exposure to Cd, attributed to changes in Ca homeostasis.

In the present study, Cd started degenerating the apical part of microvilli then the base of attachment with the cell, and finally the whole parts of microvilli after 120 h exposure to 0.005 and 0.01 mg Cd L^{-1}. Damage to these essential components of the cell might disturb the secretion of enzymes and absorption of nutrients between the lumen and hepatopancreas cells with increasing Cd accumulation in whole body associated with time exposure. The impact of Cd toxicity on the hepatopancreas might be a reason to reduce the feeding rate (Chapter 3), or the rate of assimilation capacity and reproduction (Blockwell et al., 1998).

Papathanassiou and King, (1986) found that apical part (microvilli) of R-cell in hepatopancreas of the prawn *P. serratus*, lost organisation and accumulated high percent of Cd in hepatopancreas (800%) after 44 h exposure to 50 ppm. Bioaccumulation of Cd in the freshwater crayfish *Procambarus clarkii* after 10 days exposure caused damage to hepatopancreas cell structure and increasing vacuolisation (Al Kaddissi et al., 2012). It seems to be the heavy metals have similar effects on the tissues of *G. pulex*, lethal concentration of lead acetate

was decreased the length of microvilli of the hepatopancreas associated with alterations in cell organelles such as Golgi bodies and degenerating mitochondria after 96 h (Kutlu et al., 2002), and exposure to lethal concentration of thallium acetate decreased microvilli of hepatopancreas, associated with swollen mitochondria cristae. Increase lipid droplets and vocalisation in the tissue (Ozalp et al., 2011).

Mitochondria in hepatopancreas cells damaged after 120 h exposure to 0.005 and 0.01 mg Cd L^{-1}, suggest that Cd could disrupt the energy production and inhibit ATPase synthesis and the Kerb's cycle in these organelles (Yamuna, et al., 2009). The present result corresponds with the study Papathanassiou and King (1986) where Cd also changed the mitochondria in R-cells of the hepatopancreas in the prawn *P. serratus*. Cadmium exposure induced oxidative stress, resulting in the destruction of cellular components which stimulated mitochondria to apoptosis (Bertin and Averbeck, 2006; Cuypers et al., 2010) and injured hepatopancreas cells in the freshwater crab *S. yangtsekiense* when exposed to high Cd concentrations ranged from 7.25 to 116 mg Cd L^{-1} (Bo et al., 2008). It is clearly observed in the present study, after mitochondria changes, the chromatin of nucleus condense, DNA fragmentation, and the cells shrink in hepatopancreas and gill cells after 120 h exposed to 0.005 and 0.01 mg Cd L^{-1}. Liu et al. (2013) found that Cd caused swelling and rupture in mitochondria and nucleus associated with shorten or disappearance of the cristae in the hepatopancreas of the freshwater crab *S. henanense*. The result suggests that Cd induced production of malondialdehyde (MDA) significantly in the whole tissues of freshwater *G. pulex* exposed to 0.005 and 0.01 mg Cd L^{-1} after 14 days. Furthermore, DNA damage is more significant at 0.01 mg Cd L^{-1} (Chapter 2). In this case, the authors suggested that the toxic effects were not

free radical mediated but were due to Cd replacing copper or iron in the cytoplasm and membrane proteins of the cell, causing dysfunction to lipids, proteins and DNA (Ercal et al., 2001; Valko et al., 2005; Wang et al., 2011).

Nevertheless of alternation in the fine structure of gills and hepatopancreas, the percent of survival was higher than 70% in *G. pulex* exposed to 0.005 and 0.01 mg Cd L^{-1} after 120 h. It might be other agents acted on resistant Cd toxicity such as antioxidant and water quality will be conducted in next experiments.

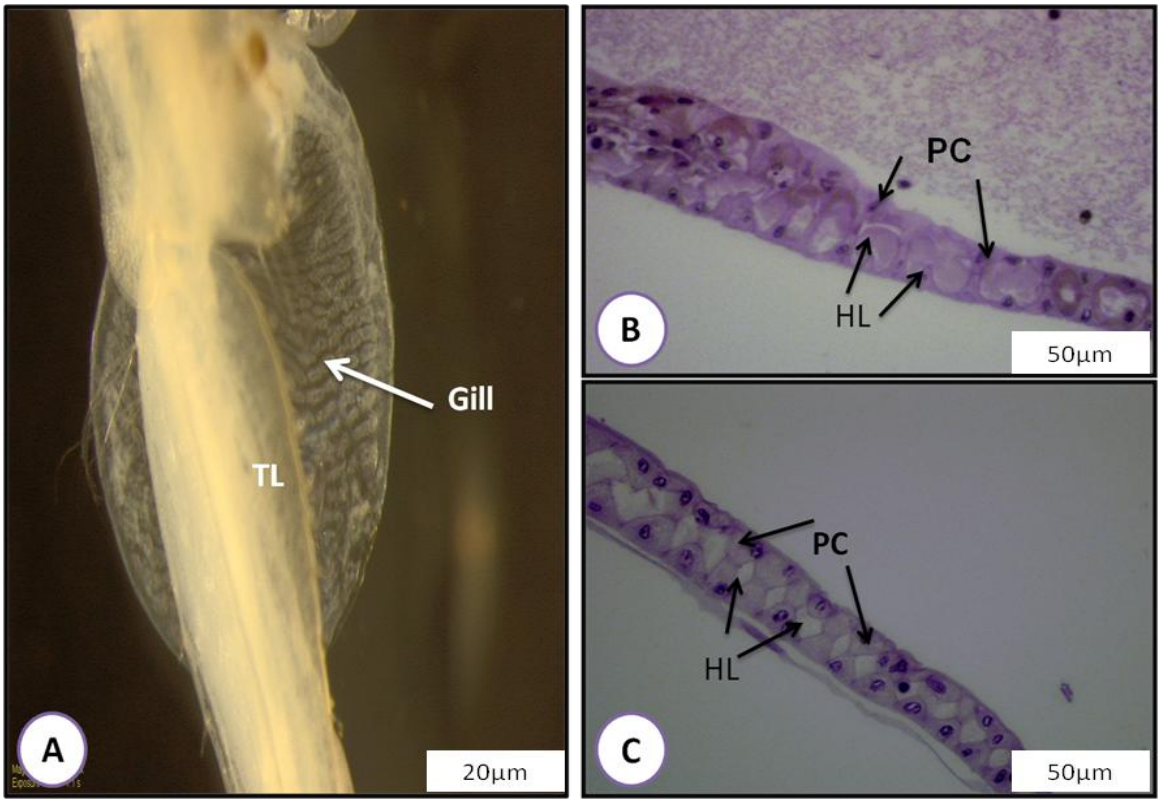

Figure 4.1 Light microscope. (A) Gill attached the thoracic leg (TL) of the freshwater *G. pulex.* (B) Cross-section of the gill, pillar cells (PC), haemolymphatic lacuna (HL), control animal. (C) The cross- section of the gill exposed to 0.01 mg Cd L^{-1}, pillar cells (PC), haemolymphatic lacuna (HL).

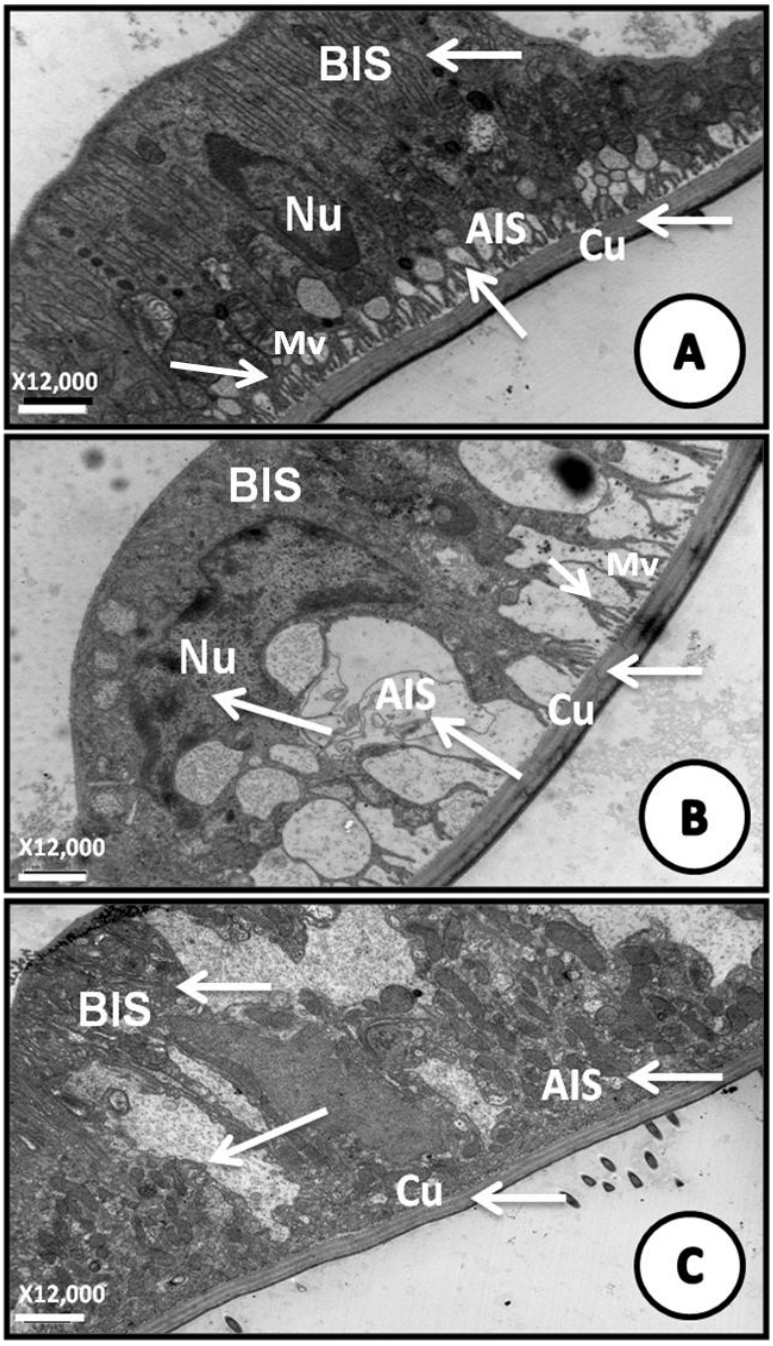

Figure 4.2 Transmission electron micrographs of ultrastructure of gill the freshwater *G. pulex*. (A) Cross-section of the gill, mitochondria (M), apical infolding system (AIS) and basal infolding system (BIS), control animal. (B) Cross-section of the gill, vacuolation and lysis of microvilli (Mv) in AIS exposed to 0.005 mg Cd L^{-1}. (C) Cross-section of the gill, vacuolation in epithelial layer (AIS & BIS) exposed to 0.01 mg Cd L^{-1}. (n=3, 3 replicates).

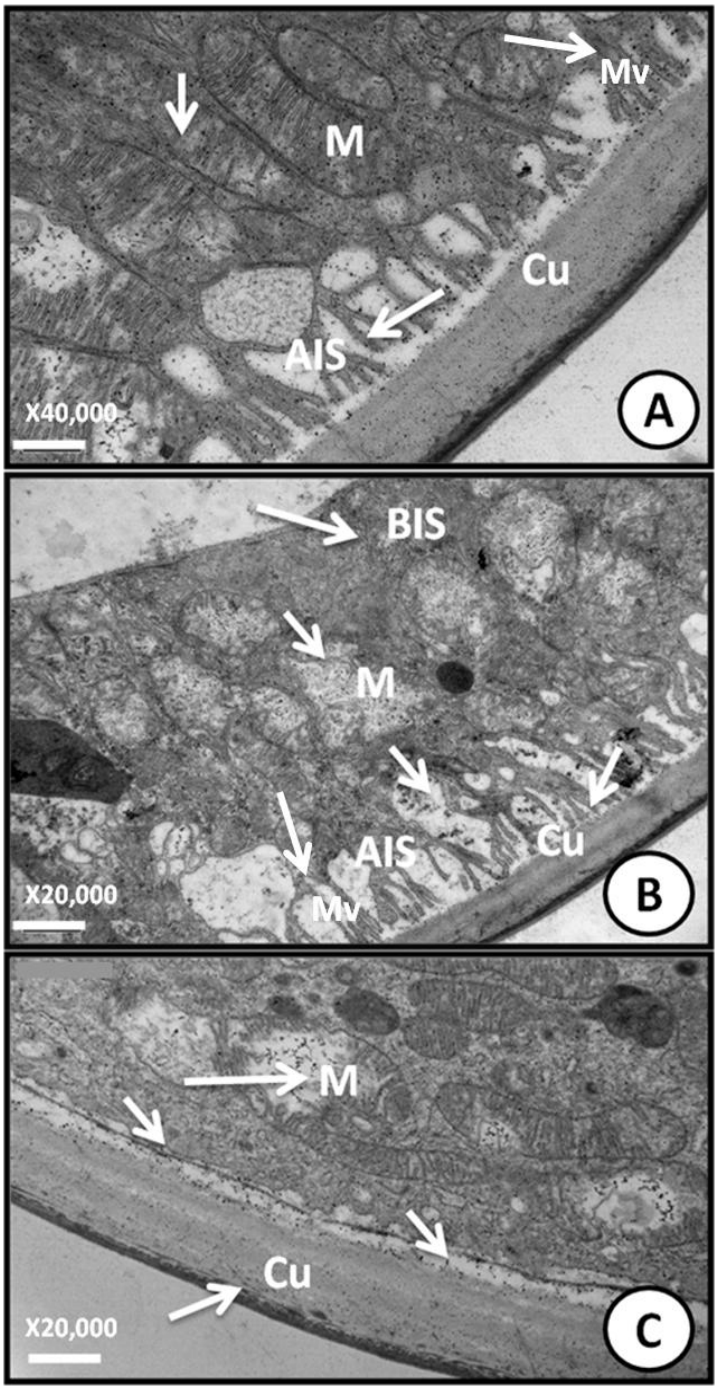

Figure 4.3 Transmission electron micrographs of ultrastructure of gill the freshwater *G. pulex.* (A) cross- section of the gill, mitochondria (M), cuticle (Cu), apical infolding system (AIS), microvilli (Mv) in control animal. (B) cross- section of the gill, exposed to 0.005 mg Cd L^{-1} for 120 h, mitochondria (M), microvilli (Mv), apical infolding system (AIS) and Basal infolding system (BIS). (C) Cross-section of the gill exposed to 0.01 mg Cd L^{-1} for 120 h, arrows under cuticle represents disappearance microvilli (Mv), mitochondria (M). (n=3, 3 replicates).

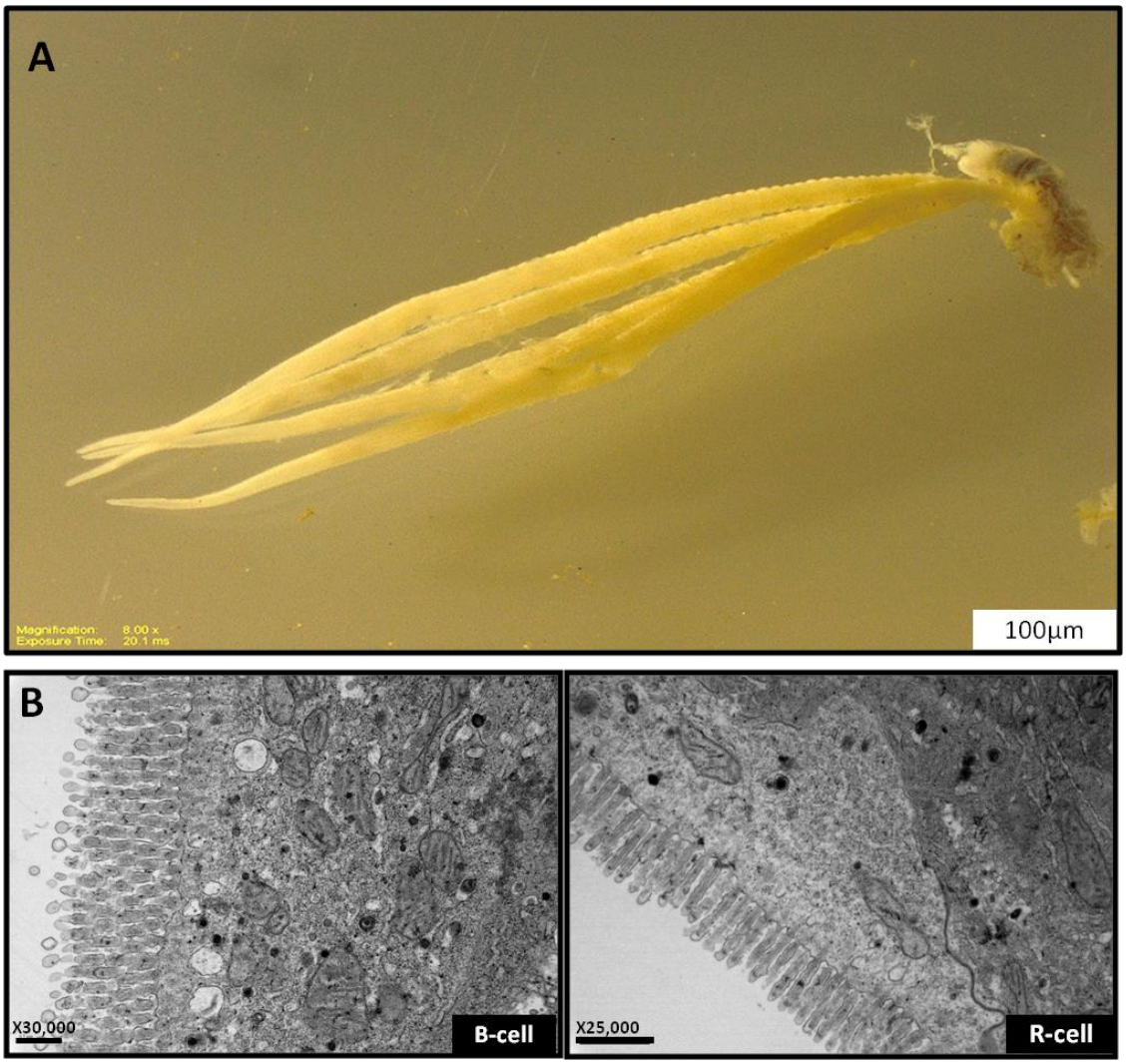

Figure 4.4 Light microscope of two pairs of the hepatopancreas (A). Transmission electron micrographs of the hepatopancreas cells, B-cell and R-cell (B).

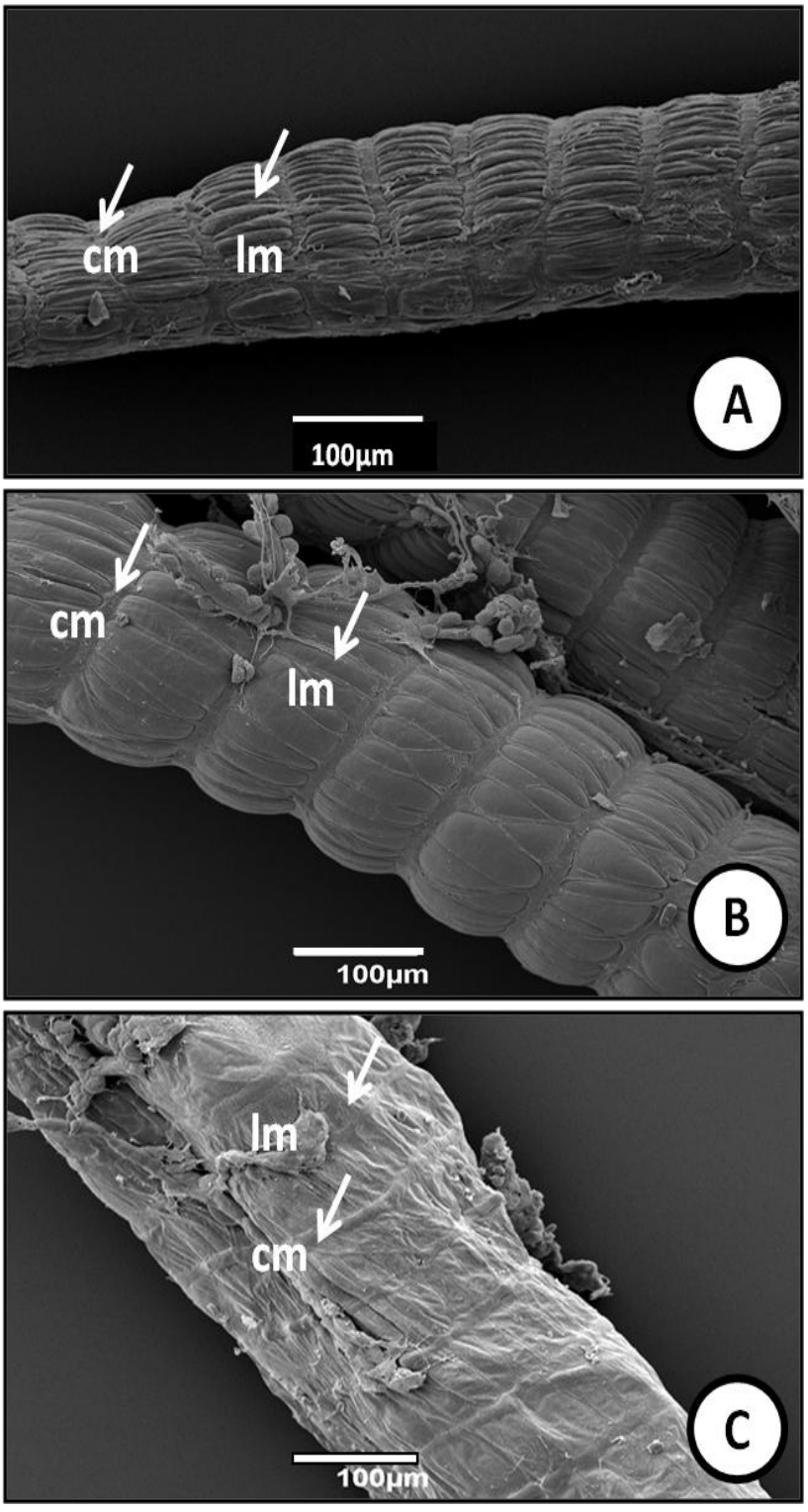

Figure 4.5 Scanning electron micrograph, hepatopancreas the freshwater *G. pulex.* (A) Longitudinal muscle (lm) and circular muscle (cm) of hepatopancreas control. (B) Muscular fibres (lm & cm) of hepatopancreas exposed to 0.005 mg Cd L^{-1} for 120 h. (C) Muscular fibres (lm & cm) of hepatopancreas exposed to 0.01 mg Cd L^{-1} for 120 h. (n=3, 2 replicates).

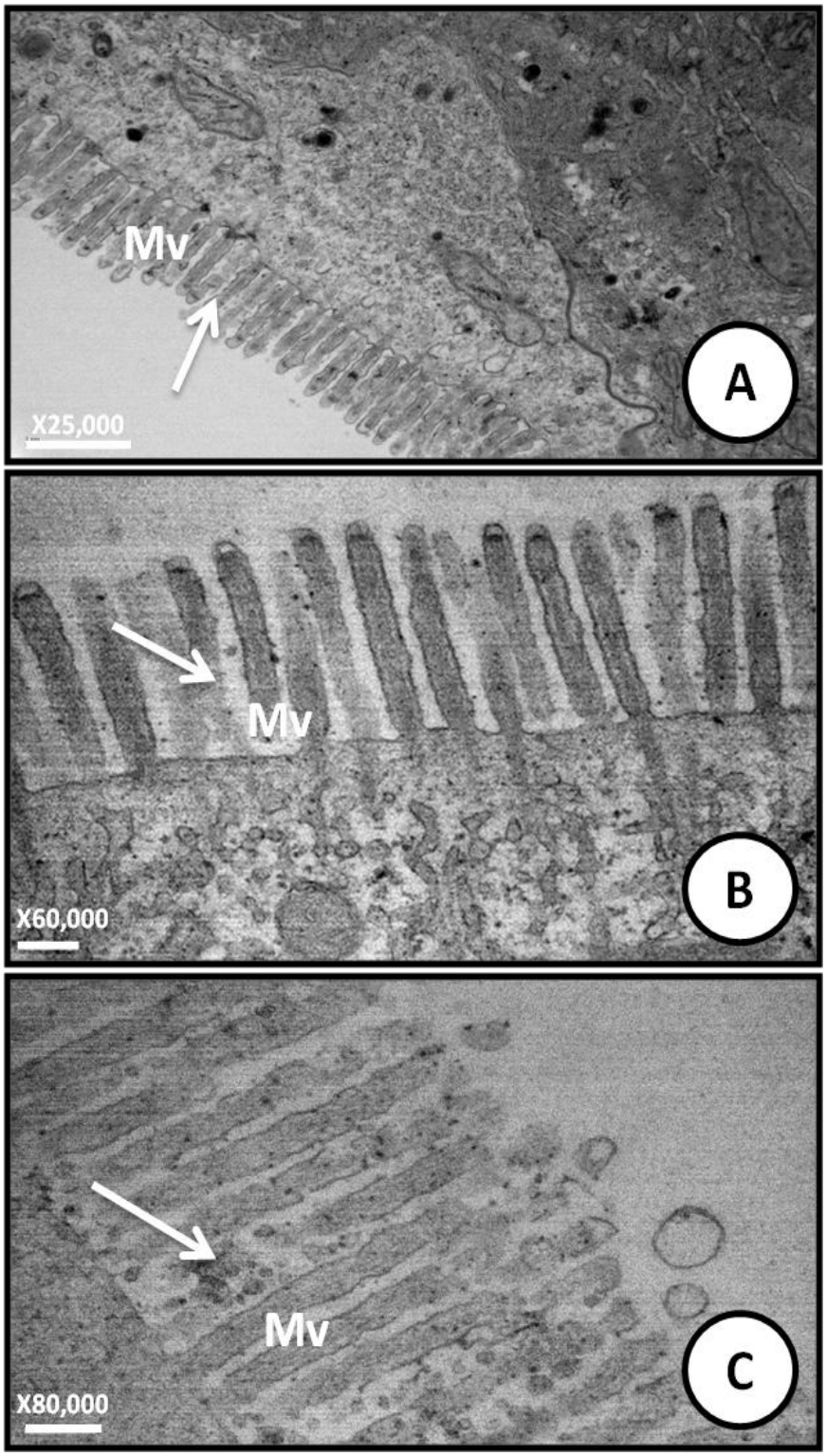

Figure 4.6 Transmission electron micrographs sections of hepatopancreas. (A) Microvilli of hepatopancreas (Mv), control. (B) Microvilli (Mv) of hepatopancreas exposed to 0.005 mg Cd $L^{-1}$for 120 h. (C) Microvilli (Mv) of hepatopancreas exposed to 0.01 mg Cd L^{-1} for 120 h. (n=3, 3 replicates).

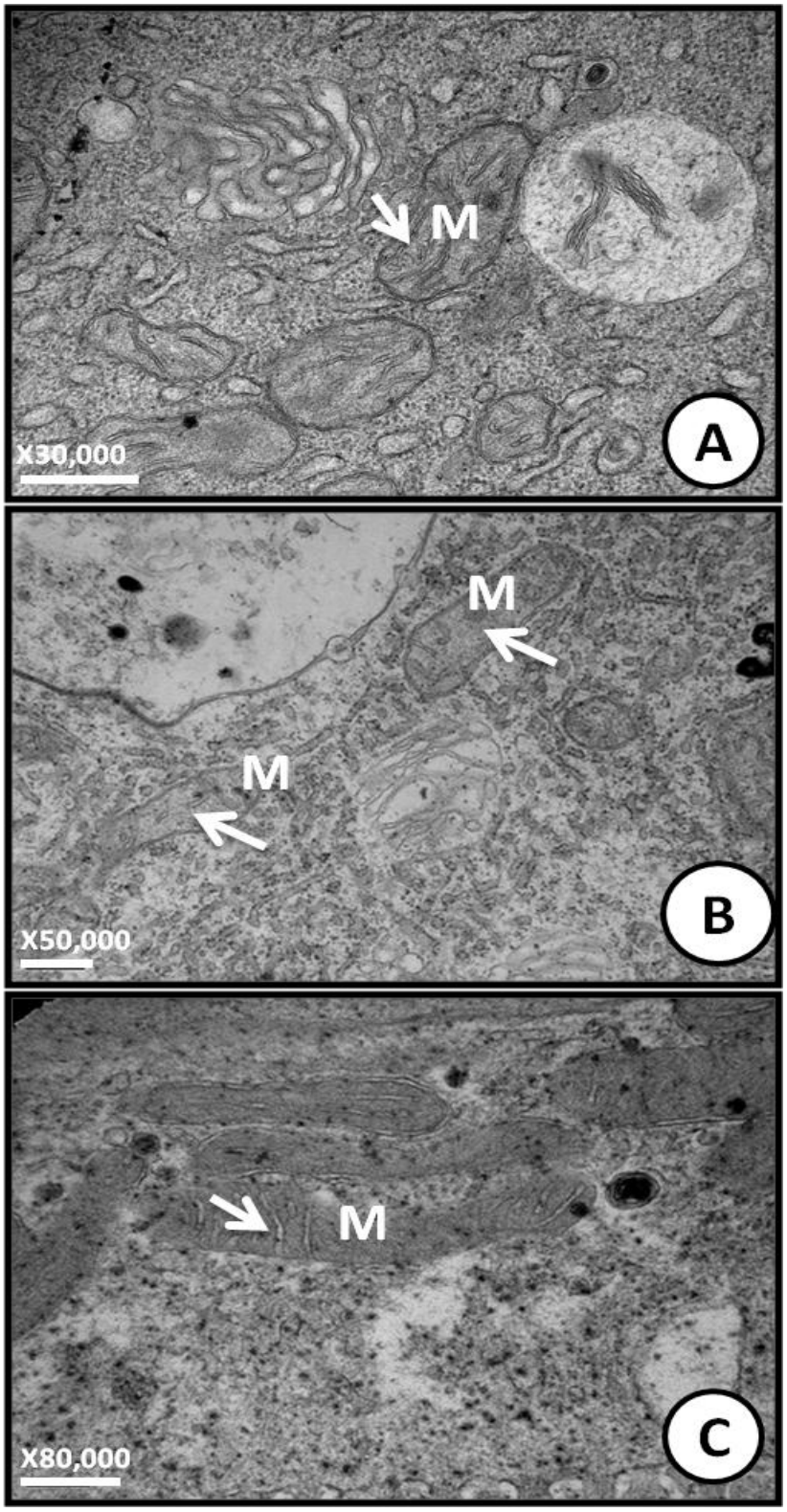

Figure 4.7 Transmission electron microscope sections of hepatopancreas. (A) Mitochondria (M) of hepatopancreas cell, control animal. (B) Mitochondria (M) of hepatopancreas cell exposed to 0.005 mg Cd L^{-1} for 120 h. (C) Mitochondria (M) of hepatopancreas cell exposed to 0.01 mg Cd L^{-1} for 120 h. (n=3, 3 replicates).

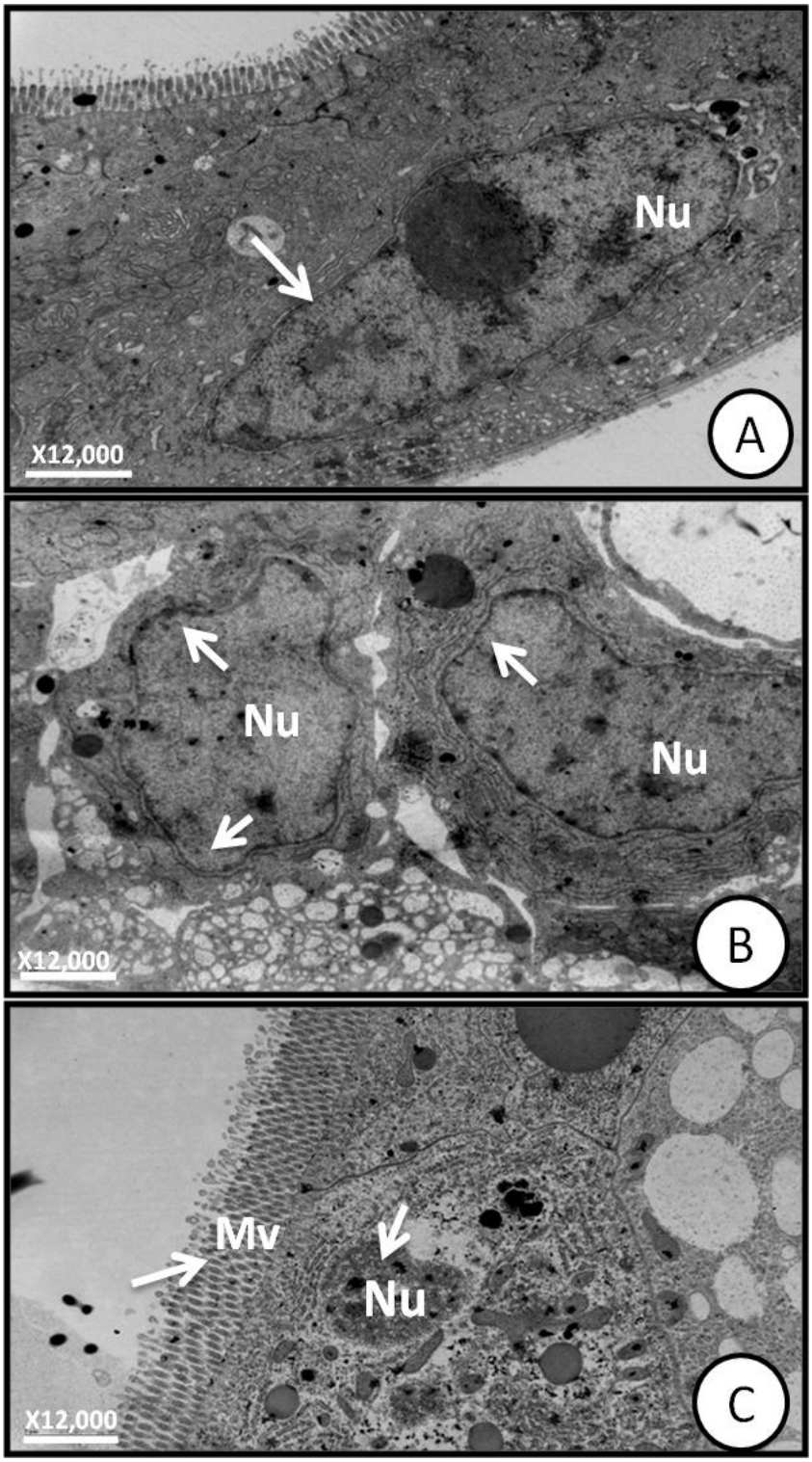

Figure 4.8 Transmission electron microscope sections of hepatopancreas. (A) Control animal, the nucleus (Nu). (B) Margin chromatin in the nucleus of *G. pulex* exposed to 0.005 mg Cd L^{-1}. (C) The stage of apoptosis at concentration 0.01 mg Cd L^{-1}, the nucleus (Nu). (n=3, 3 replicates).

Chapter 5 Effect of soft and hard water on juvenile *Gammarus pulex* chronically exposed to cadmium chloride.

5.1 Introduction

Water chemistry for instance, alkalinity, hardness, salinity, dissolved organic carbon and pH can all influence directly on heavy metal toxicity by reducing the free metal ion concentration or indirectly through antagonistic effects (Jackson et al., 2000). Increased calcium and magnesium salts as bicarbonate or sulfate in freshwater of 61 ppm or above as $CaCO_3$ are classed as 'hard' waters. Hardness originates from water flowing over on sedimentary rocks and runoffs from soil which contain limestone. Although hardness of water of more than 500 ppm is considered unacceptable for domestic purposes, hard water acts to protect aquatic organisms against heavy metal toxicity (EPA, 1980; Saglam et al., 2013).

Several previous studies have demonstrated that freshwater species were more sensitive to Cd toxicity than marine species, and more resistant to Cd toxicity in hard water (HW). For example, water hardness reduced Cd toxicity, in saprophytic and nitrifying bacteria (Aimel and Khan, 1984), *Daphnia magna* (Winner and Gauss, 1986), rainbow trout and brook trout (Calamari et al., 1980; Pascoe et al., 1986; Hollis, et al., 2000), the amphipod *Hyalella azteca* (Jackson et al., 2000), and the freshwater tubificid worm sludge worm *Tubifex tubifex* (Rathore and Khangarot, 2003). It was found that the cladocera *D. magna* accumulated less Cd in a high calcium (Ca) environment during long term exposure (Tan and Wang, 2008). Calcium also protected the brook trout *Salvelinus fontinalis* and the amphipod *H. azteca* more against Cd toxicity than

other ions such as sodium, sulphate and magnesium (Carroll et al., 1979; Jackson et al., 2000).

In soft water, heavy metals can dissolve easily and become more toxic to aquatic organisms than in hard water (Rathore and Khangarot, 2003). Cadmium accumulation was increased in *D. magna*, *T. tubifex* (Tubificid worm), *Vilosa vibex* (mussel), chinook salmon, goldfish, green sunfish, striped bass (EPA, 1980), the gills and liver of *Tilapia sparranii* (Van Aardt and Booysen, 2004) and reached to tenfold higher in juvenile rainbow trout exposed to sublethal Cd compared with soft water (Hollis et al., 2000). Copper was more toxic to the freshwater *G. pulex* in soft water than hard water (Stephenson, 1983). In addition to that SW increased the mortality of freshwater species of algae (*Nitella flexlis*), rooted plants (*Elodea canadensis*), infusoria snails (*Ampullaria paludosa*), catfish (*Corydoros punctatus*), and guppies (*Lebistes reticulates*), when exposed to Cd concentrations (Kinkade and Erdman, 1975).

Several studies reported that the early stage of aquatic organisms was less tolerant to Cd toxicity than adults. For example, the amphipods *G. pulex* and *G. fossarum* (Alonso et al., 2010b), the amphipod *Hyalella curispina* (Garíca et al., 2010) and the juveniles amphipod *H. azteca* (Giusto et al., 2012). Six-week old juvenile striped bass were more resistant to Cd toxicity in low Ca than larvae stages (Wright et al., 1985).

In freshwater, heavy metal ions non static and can combine with organic (amino acid, carboxylic acid, fulvic acid, humic acid), inorganic ligands (CO_3^{2-}, HCO_3^{2-}, HO^-, Cl^-, F^-, SO_4^{2-}) and cations such as Ca^{2+}, Mg^{2+} and H^+ in water to form metal speciation and complexes. The bioavailability and toxicity of free ion metals depend on water chemistry (Newman, 2010). Competition between Cd

and hardness cations (Ca^{2+} and Mg^{2+}) is also the pathways to decrese bioavailability and toxicity of heavy metals in water column (Korfali and Davies., 2004., Newman, 2010).

The aim of this experiment was to provide a first investigation of the effect of Cd on juvenile amphipods (*G. pulex*) in hard (150 ppm as $CaCO_3$) and soft water (15 ppm as $CaCO_3$) measured as the percent of survival over a long term chronic exposure for 4 and 8 weeks. Concentrations of Mg and Ca in the body parts of juveniles (haemolymph, hepatopancreas, and remaining tissues) were measured after 4 and 8 weeks and the effect of Cd compared in hard water and soft water. In addition to that the study aims to investigate the effect of Cd in both media on food consumption and the growth rate of juveniles for 4 and 8 weeks.

5.2 Materials and methods

5.2.1 Test animals

The freshwater *G. pulex* were collected from the same place as in previous chapters (lower Hoopern valley of Exeter), in May 2011, transported immediately to the lab, each male and female in precopula was placed in a 250 ml beaker with dechlorinated tap water until the release of the juveniles, Newly hatched juveniles were transferred by a wide plastic pipette to many beakers (50 ml), avoiding cannibalism by adults of the same species. They were fed on the same food as adults, with leaves provided as surfaces on which to rest (Welton and Clarke, 1980). The juveniles were reared for two months in dechlorinated tap water from the Aquatic Resource centre laboratories (75 ppm as $CaCO_3$), at 12^{o}C, 12 light: 12 dark with constant aeration (Fig 5.1).

The lengths of body was measured via dissecting microscope (MOTIC) with the eye-piece micrometer to the nearest millimetre (from the anterior part of the head to the end of telson), the number of segments counted also via a microscope. The weight of juveniles was measured to the nearest 0.01 mg. The average length of juveniles used was 4.28 ± 0.09 mm, weight (1.64 ± 0.07 mg) and the number of segments on the primary antenna was 12–13 segments.

5.2.2 Experimental design

The juveniles were divided into two groups according to water quality (SW or HW) and time exposure (4 and 8 weeks); cadmium concentrations ($CdCl_2$) prepared (0.5 and 1 µg Cd L^{-1}). The experiment was done with nine replicates per treatment and 5 juveniles per dish. The juveniles transferred from acclimated glassware to Pyrex glass crystallising dishes with 150 ml, to SW (15 ppm as $CaCO_3$) and HW (150 ppm as $CaCO_3$) via wide plastic pipettes to acclimate for a week, and fed unconditional oak leaves, aerated constantly via a glass pipette. The rate of dissolved oxygen did not drop below 70% saturation in the experiment, photoperiod 12 light: 12 dark, at temperature 12 ± 1^{o}C. A static water and food were renewed every 48 h (Fig 5.2).

Daily food consumption was measured by the amount of leaves consumed each 48 h divided by the number of surviving animals (Truhlar et al., 2013). The deciduous oak leaves of trees were selected as food, washed, dried until used, re-dried in an oven for 48 h at 60^{o}C, weighed to the nearest 0.1 g, the amount of food is used 0.29 g per 5 juveniles, immersed in ultrapure water for 24 h, dried between tissue paper prior to transfer to crystallised dishes, the remaining leaves rinsed, dried in the oven at 60^{o}C for 48 h and re-weighed.

5.2.3 Preparing hard and soft water

Samples of water were taken from the aquarium, to measure Ca and Mg by Atomic absorption spectrophotometer (AAS), according to the results, HW and SW prepared. In order to increase hardness of water (150 ppm as $CaCO_3$), 0.087g/L of calcium nitrate tetrahydrate ($CaNa_2O_6.4H_2O$, Sigma-Aldrich) was added and 0.0198 g/L of magnesium suflate Anhydrous ($MgSO_4.H_2O$) (Fisher-chemical). The elements such as Na, Cl, K, Ca and Mg in both media in addition to a sample were taken from Hoopern valley to measure by Dionex (Table 5.1).

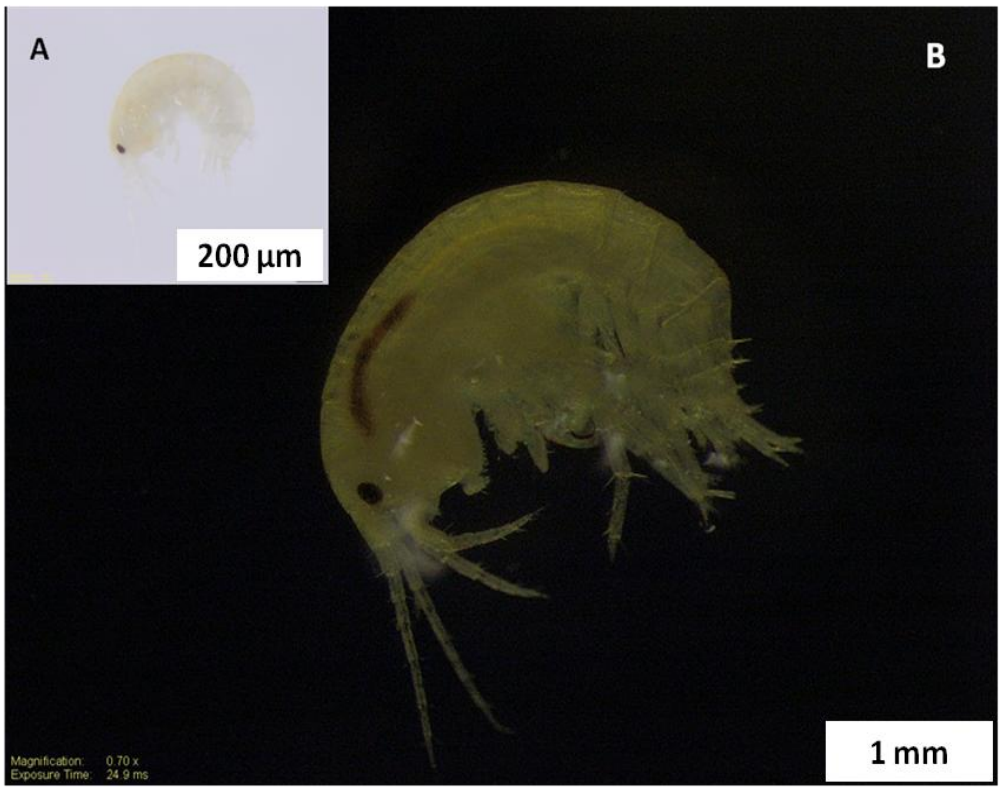

Figure 5.1 Juveniles *G. pulex*, new hatched (200 µm) length (A), more than two weeks (1 mm) length (B).

Figure 5.2 The juveniles *G. pulex* divided into two groups in hard water (150 ppm as $CaCO_3$) and another group in soft water (15 ppm as $CaCO_3$), 9 replicates, (n = 5 per dish), in Aquatic Resource centre laboratories.

Volumetric complex was utilised to determine the total hardness of water (TH) every time. In brief: 50 ml of water was added to a beaker with 3 ml of buffer ammonia ammonium chloride solution (Fisher), adjusted to pH 10, and 3 ml of Erochrome black T was added as an indicator, the mixture titrate against Ethylenediaminetetraacetic acid disodium (EDTA) 0.01 M (Sigma-Aldrich), the endpoint of titration recognized, when the colour change from sky blue to a red wine. The average of titration was taken (Harris, 2003).

Soft water (15 ppm as $CaCO_3$) was prepared via the dilution amount of dechlorinated tap water with ultrapure water. Temperature of water (^oC), dissolved oxygen (ppm), conductivity (µS/cm), total dissolved solid (ppm), and

pH was measured by portable meter (Multi-parameter water quality, HANNA HI 9828).

5.2.4 Dissecting animals and chemical analysis

Samples were taken from three parts of the body including: haemolymph (HL), hepatopancreas (HP), and the remaining tissue (RT). Each animal was dried gently between tissues, a sample of haemolymph was taken from the posterior segments of the telson from each individual via 0.3 ml syringe, the hepatopancreas was withdrawn from each individual animal under a dissecting microscope (MOTIC) by fine forceps. The samples were weighed and digested in 3 ml ultrapure nitric acid 65% (Fluka) and 0.5 ml hydrochloric acid (Sigma) at $80^{o}C$ for minutes and diluted to 25 ml of ultrapure water. Cadmium was measured by Atomic absorption spectrometry (UNICAM 939 AA spectrometer) Ca and Mg were also measured by Atomic absorption spectrometry (Sol AAR-Thermo Elemental).

5.2.5 Statistical analysis

Percent of survival, Ca, Mg and Cd are represented as mean ± standard error. Box and whisker plots were utilised to illustrate daily food consumption. After checking data for normality. The mean values were calculated. Significant difference between the control and exposed group were calculated by One-way ANOVA (SPSS, 19) assuming a significance value of $p = 0.05$.

5.3 Results

5.3.1 Survival in hard and soft water for 4 and 8 weeks

Water parameters were stable throughout the experiment in HW and SW (temperature, pH, dissolved oxygen, conductivity and total dissolved solid) (Table 5.2). In hard water, the percentage of survival throughout four weeks remained high in control animals and those exposed to 0.5 µg Cd L^{-1} 100%, while the percentage of survival decreased slightly by 97.78% at 1 µg Cd L^{-1}. After 8 weeks, the percentage of survival decreased by 93.3% in controls. While, the juveniles exposed to 0.5 and 1 µg Cd L^{-1} were similar in both concentrations, at 86.7%. In soft water, the percentage of survival was 87.78% and 85.56% at 0.5 and 1 µg Cd L^{-1} respectively after 4 weeks. While, the rate of survival declined to 40% in control and 50% in animals exposed to Cd after 8 weeks. The soft exoskeleton after moulting and delays in the period of calcification led to animals being vulnerable to cannibalism resulting in decreased numbers of individuals (Fig 5.3).

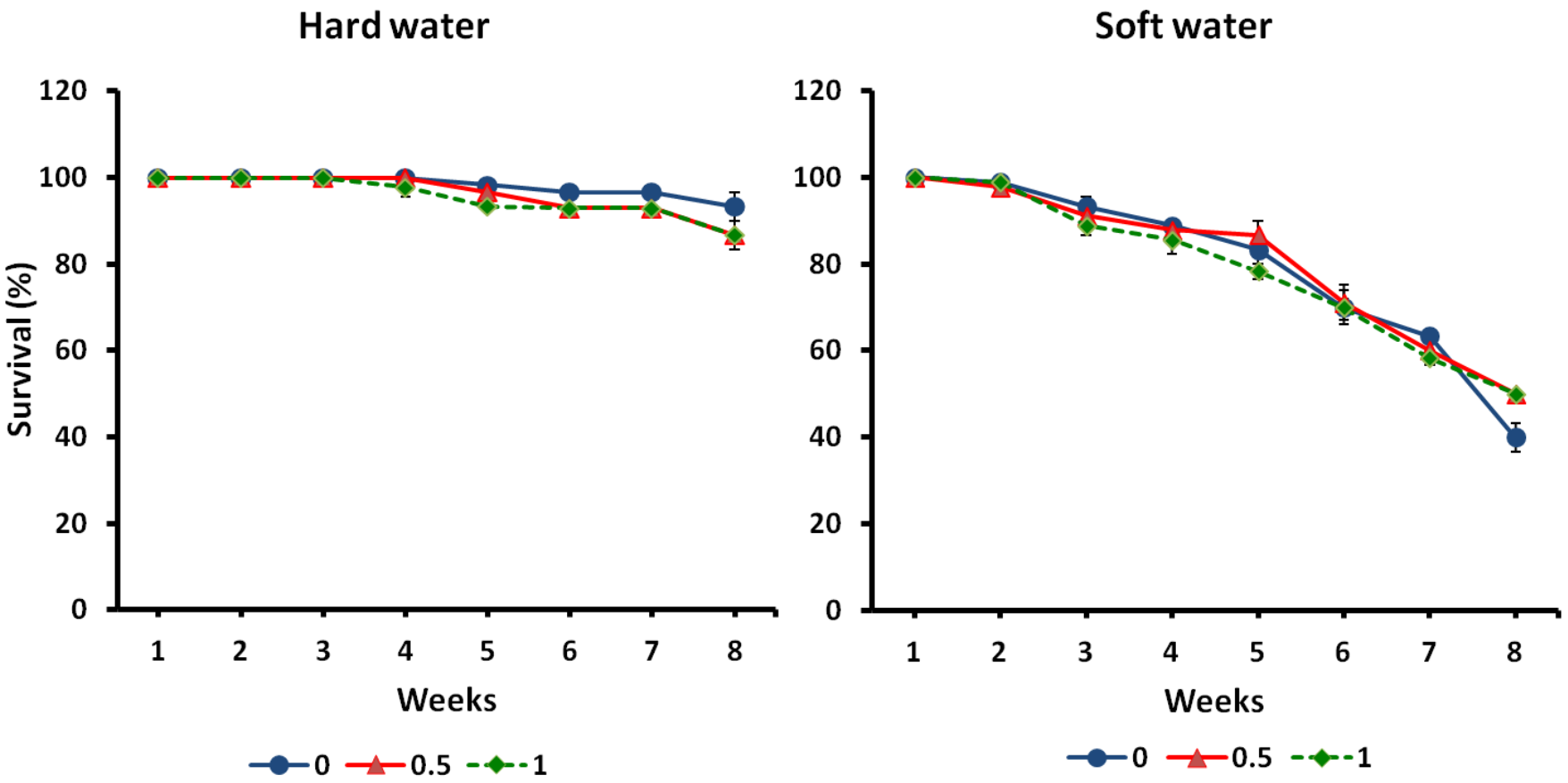

Figure 5.3 Mean (± SEM) the percentage of survival juvenile *G. pulex* exposed to Cd (0.5 and 1 µg Cd L^{-1}) in hard water and soft water for 8 weeks (n=45 per treatment).

Table 5.1 Mean and standard error for the elements in water was used in the experiment in comparison to fresh water from the stream Hoopern valley.

	Cl^-	Na^+	K^+	Mg^{2+}	Ca^{2+}
Hard water	12.62 ± 0.0006	8.44 ± 0.003	1.76 ± 0.0006	6.71 ± 0.001	35.23 ± 0.01
Soft water	1.84 ± 0.0004	1.38 ± 0.0002	0.23 ± 0.0001	0.66 ± 0.0005	3.33 ± 0.002
Hoopern valley	29.07 ± 0.004	20.67 ± 0.002	5.12 ± 0.0003	12.62 ± 0002	40.44 ± 0.004

The values were expressed as mg/l

Table 5.2 Water quality measured for 4 and 8 weeks in hard water (150 ppm as $CaCO_3$) and soft water (15 ppm as $CaCO_3$), mean ± SEM.

	Hard water	Soft water
Dissolved oxygen (ppm)	8.34 ± 0.33	8.54 ± 0.36
pH	7.30 ± 0.12	7.18 ± 0.12
Temperature ^{o}C	12.95 ± 0.43	12.94 ± 0.40
Conductivity (µS/cm)	330.5 ± 6.71	37.54 ± 2.59
Total dissolved solid (ppm)	147.23 ± 4.43	18.38 ± 1.36

5.3.2 Cations in the body parts of juveniles *G. pulex*

5.3.2.1 Calcium in the body parts in hard water

The results show that the haemolymph Ca was 5.52 ± 0.14 mg/mg w wt in control, decreasing gradually at 0.5 µg Cd L^{-1} (5.25 ± 0.07 mg/mg w wt) and 1 µg Cd L^{-1} (4.95 ± 0.21 mg/mg w wt, $p < 0.05$). In hepatopancreas, Ca was 5.41 ± 0.93 mg/mg w wt in the control group, and then decreased significantly at 0.5 µg Cd L^{-1} (5.13 ± 0.26 mg/mg w wt, $p < 0.05$) and (5.01 ± 0.08 mg/mg w wt, $p < 0.001$) at 1 µg Cd L^{-1}. Calcium concentration in remaining tissues was 7.39 ± 0.64 mg/mg w wt higher than the juveniles exposed to 0.5 and 1 µg Cd L^{-1} 6.18 ± 0.35 and 6.52 ± 0.86 mg/mg w wt respectively (Fig 5.4A).

After 8 weeks of exposure, Ca increased in all the body parts, exposure to Cd led to increase haemolymph Ca (5.75 ± 0.12 mg/mg w wt) at 0.5 µg Cd L^{-1} and 6 ± 0.23 mg/mg w wt at 1 µg Cd L^{-1} (Fig 5.4B). It also increased in hepatopancreas of animals exposed to 0.5 µg Cd L^{-1} (5.73 ± 0.056 mg/mg w wt) and 1 µg Cd L^{-1} (6.34 ± 0.16 mg /mg w wt, $p < 0.001$). High Ca concentration

was 9.12 ± 0.88 and 9.46 ± 0.75 mg/mg w wt respectively in the remaining tissues of control and exposed to 0.5 µg Cd L^{-1} but it tended to decrease at 1 µg Cd L^{-1} (7.39 ± 0.37 mg/mg w wt).

5.3.2.2 Calcium in the body parts in soft water

The mean Ca concentrations in the haemolymph and hepatopancreas was 0.73 ± 0.24 mg/mg w wt and 1.22 ± 0.13 mg/mg w wt after 4 weeks in SW control animals (Fig 5.5A). Exposure to Cd led to significantly increased Ca in the haemolymph (2.55 ± 0.025 mg/mg w wt; $p < 0.001$) at 0.5 µg Cd L^{-1} to 3 ± 0.039 mg/mg w wt; $p < 0.001$ at 1 µg Cd L^{-1}. It was also increased significantly in hepatopancreas at 0.5 µg Cd L^{-1} (2.69 ± 0.001 mg/mg w wt; $p < 0.01$) and 1 µg Cd L^{-1} (3.85 ± 0.29 mg/mg w wt; $p < 0.001$). The maximum of Ca accumulated in the remaining tissues of control 12.76 ± 1.8 mg/mg w wt, the animals exposure to 0.5 and 1 µg Cd L^{-1} were 7.35 ± 0.5 mg/mg w wt; $p < 0.001$ and 7.71 ± 0.25; $p < 0.05$ respectively.

After 8 weeks, Ca increased significantly in the haemolymph of juveniles 3.92 ± 0.12 mg/mg w wt, $p < 0.001$ and 4.17 ± 0.044 mg/mg w wt; $p < 0.01$ at 0.5 and 1 µg Cd L^{-1}. Exposure to 1 µg Cd L^{-1} resulted in significantly increased Ca in the hepatopancreas (4.75 ± 0.2 mg/mg w wt, $p < 0.001$). In the remaining tissues, Ca was reduced significantly after exposure to 0.5 and 1 µg Cd L^{-1} (6.82 ± 0.64, $p < 0.001$ and 7.58 ± 0.23 mg/mg w wt; $p < 0.05$) in comparison to remaining tissues of juveniles after 4 weeks (Fig 5.5B).

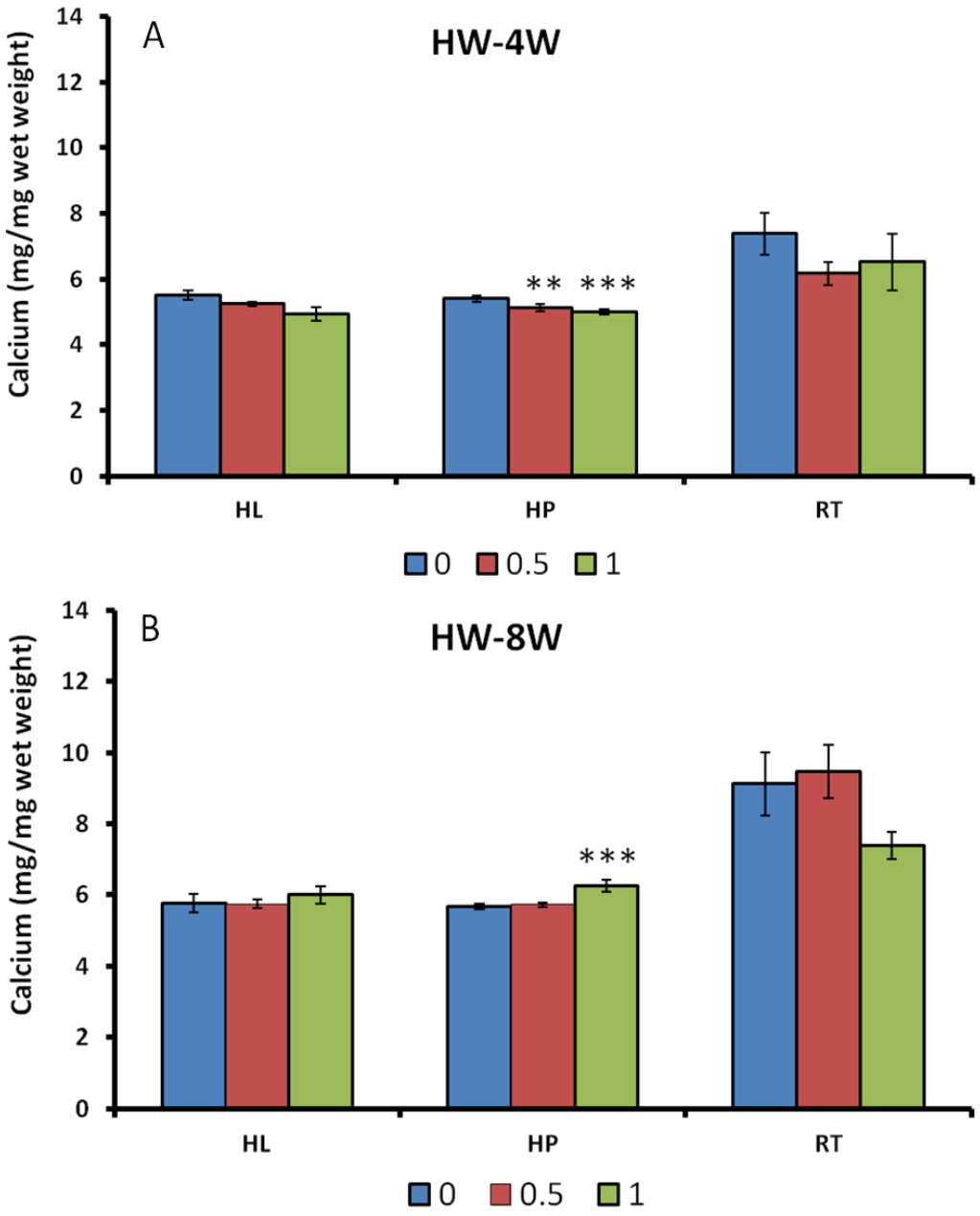

Figure 5.4 Mean calcium concentrations in the body parts (mg/mg w wt) of *G. pulex* exposed to Cd (0.5 and 1 µg Cd L^{-1}) for 4 (A) and 8 (B) weeks in hard water. Error bars represent SEM. HL, haemolymph; HP, hepatopancreas; RT, remaining tissues. *Asterisks represent a significant difference compared to the control (n= 8, ANOVA and Scheffe post hoc, *p < 0.05; **p < 0.01; ***p < 0.001).

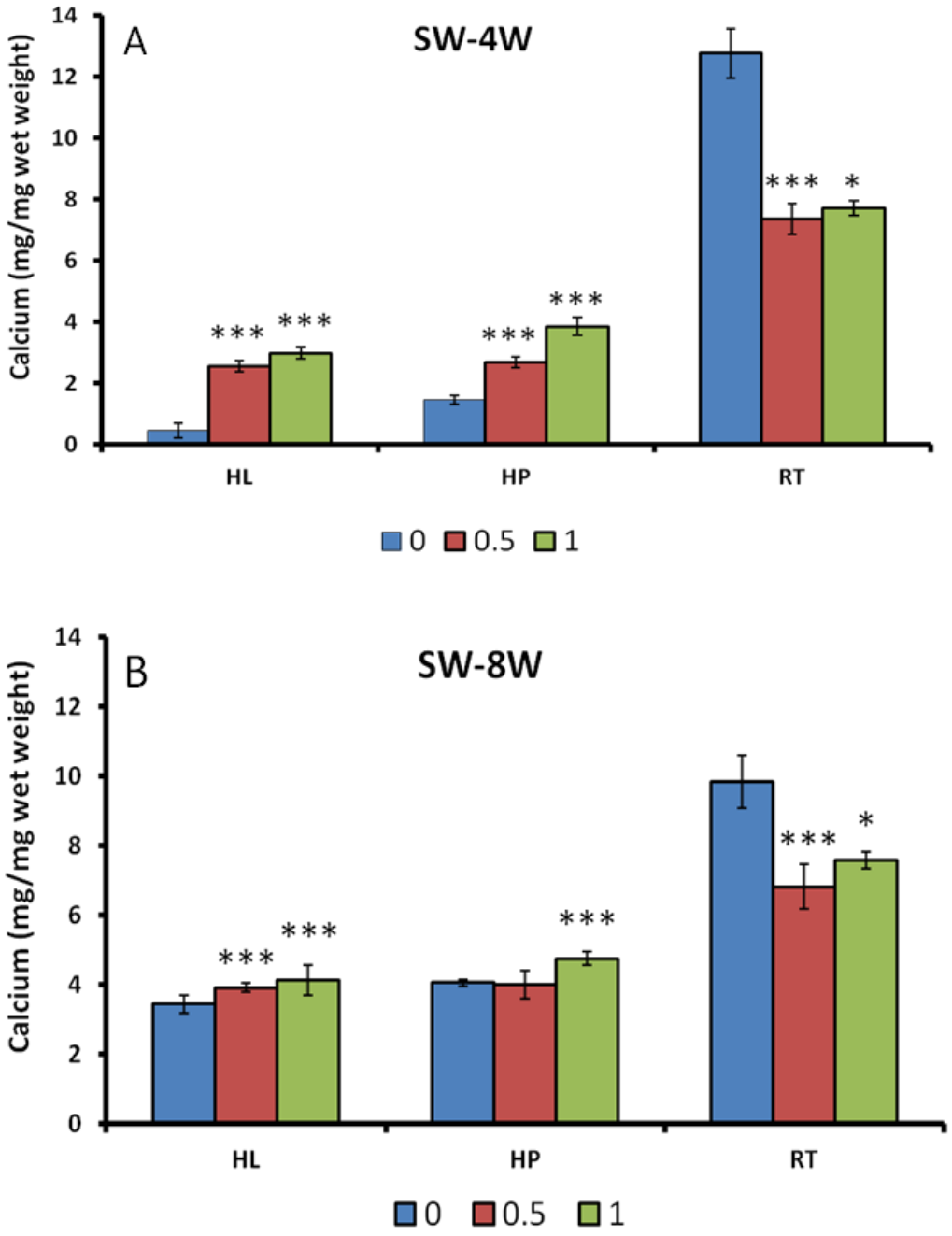

Figure 5.5 Mean (± SEM) calcium concentrations in the body parts (mg/mg w wt) of *G. pulex* exposed to Cd (0.5 and 1 µg Cd L^{-1}) for 4 (A) and 8 (B) weeks in soft water. Error bars represent SEM. HL, haemolymph; HP, hepatopancreas; RT, remaining tissues. *Asterisks represent a significant difference compared to the control (n=8, ANOVA and Scheffe post hoc, *$p < 0.05$; ***$p < 0.001$).

5.3.2.3 Magnesium in the body parts in hard water

After 4 weeks, the average of Mg in haemolymph was higher in control (0.11 ± 0.026 mg/mg w wt) than in animals exposed to 0.5 and 1 µg Cd L^{-1} (0.068 ± 0.22; 0.084 ± 0.023 mg/mg w wt, respectively). In hepatopancreas, Mg was 0.081 ± 0.032 mg/mg w wt in control, while, it decreased at 0.5 and 1 µg Cd L^{-1} (0.071 ± 0.014; 0.046 ± 0.01 mg/mg w wt respectively). In the remaining tissues, Mg increased in control (0.16 ± 0.04 mg/mg w wt), then decreased sharply 0.08 ± 0.05 mg/mg w wt at 1 µg Cd L^{-1} (Fig. 5.6A).

After 8 weeks, it increased in the haemolymph of control and animals exposed to 0.5 µg Cd L^{-1} (0.15 ± 0.01; 0.12 ± 0.02 mg/mg w wt), and was the lowest at 1 µg Cd L^{-1} (0.08 ± 0.03 mg/mg w wt). In hepatopancreas, Mg rose slightly in 0.5 (0.076 ± 0.004 mg/mg w wt, $p < 0.05$); associated with declining in control (0.049 ± 0.006 mg/mg w wt). Magnesium increased to more than double in the remaining tissues of control (0.37 ± 0.06 mg/mg w wt) and exposed to 0.5 and 1 µg Cd L^{-1} (0.26 ± 0.05 and 0.14 ± 0.06 mg/mg w wt respectively) (Fig 5.6B).

5.3.2.4 Magnesium in the body parts in soft water

After 4 weeks, Mg in the haemolymph and hepatopancreas was lower than in the group of animals in HW. It was 0.0038 ± 0.004 mg/mg w wt in control then increased in haemolymph at 0.5 and 1 µg Cd L^{-1} (0.045 ± 0.004; 0.06 ± 0.06 mg/mg w wt respectively). In hepatopancreas, increased significantly at 0.5 µg Cd L^{-1} (0.058 ± 0.004 mg/mg w wt, $p < 0.001$), and then declined slightly at 1 µg Cd L^{-1} (0.051 ± 0.005 mg/mg w wt, $p < 0.001$). The majority of Mg concentrated in the remaining tissues of control 0.36 ± 0.01 mg/mg w wt as well as exposed

to 0.5 and 1 µg Cd L^{-1} (0.25 ± 0.04, $p < 0.05$ and 0.28 ± 0.02 mg/mg w wt) (Fig 5.7A).

After 8 weeks, Mg increased in hepatopancreas and the remaining tissues of the control group (0.12 ± 0.007 mg/mg w wt and 0.41 ± 0.03 mg/mg w wt). While in the animals exposed to Cd, Mg decreased significantly in hepatopancreas (0.08 ± 0.004, $p < 0.01$ and 0.05 ± 0.003 mg/mg w wt, $p < 0.01$) at 0.5 and 1 µg Cd L^{-1} respectively. It was also reduced in the remaining tissues of animals exposed 0.05 µg Cd L^{-1} (0.36 ± 0.06 mg/mg w wt) and 1 µg Cd L^{-1} (0.23 ± 0.04 mg/mg w wt, $p < 0.05$).

5.3.2.5 Cadmium in the body parts after 4 and 8 weeks in hard and soft water

Exposure to 0.5 and 1 µg Cd L^{-1} did not show any accumulation in the body parts for 4 weeks in HW and SW. Cadmium was observed in the body parts after 8 weeks exposure in soft water. The results demonstrated that HW protected the juvenile *G. pulex* with prolonged exposure, coinciding with a high percent of survival in HW. Juveniles exposed to 0.5 and 1 µg Cd L^{-1} showed that Cd accumulated in the body parts was more than ambient Cd after 8 weeks exposure in SW, it increased twofold in haemolymph 0.006 ± 0.001 mg/mg w wt $p < 0.05$ at 0.5 µg Cd L^{-1} and 0.0065 ± 0.001 mg/mg w wt at 1 µg Cd L^{-1} in comparison to control was 0.003 ± 0.001 mg/mg w wt.

In hepatopancreas, Cd increased 0.0065 ± 0.001 mg/mg w wt (0.5 µg Cd L^{-1}), then declined slightly 0.0055 ± 0.001 mg/mg w wt at 1 µg Cd L^{-1}. In the remaining tissues, Cd was 0.0075 ± 0.001 mg/mg w wt, $p < 0.05$) at

0.5 µg Cd L^{-1} then descended slightly to 0.0065 ± 0.005 mg/mg w wt, $p < 0.05$ at 1 µg Cd L^{-1} (Fig 5.8B).

Hard water inhibited Cd in all body parts to 0.001 mg/mg w wt in hepatopancreas and less than 0.003 mg/mg w wt in the remaining tissues.

5.3.3 Growth

5.3.3.1 Length

In hard water, the average length of juveniles was 5.44 ± 0.26 mm in control animals after 4 weeks. While, the length of exposed animals to Cd was 5.47 ± 0.14 mm at 0.5 µg Cd L^{-1} and 5.57 ± 0.44 mm at 1 µg Cd L^{-1}, after 8 weeks, the results demonstrate that the length of tested animals increased in control 6.41 ± 0.34 mm, while the animals exposed to Cd increased 6.72 ± 0.22 mm at 0.5 µg Cd L^{-1} and 6.58 ± 0.27 mm at 1 µg Cd L^{-1} (Fig 5.9A)

In soft water, after 4 weeks, the length of juveniles was 6.22 ± 0.2 mm, in control. While, the animals exposed to Cd were 5.54 ± 0.2 mm and 5.83 ± 0.23 mm (0.5 and 1 µg Cd L^{-1} respectively). After 8 weeks, the length of juveniles ascended in control 6.80 ± 0.3 mm, whereas, the length of juveniles exposed to Cd was 6.31 ± 0.23 mm and 6.11 ± 0.22 mm at 0.5 and 1 µg Cd L^{-1} respectively (Fig 5.9B).

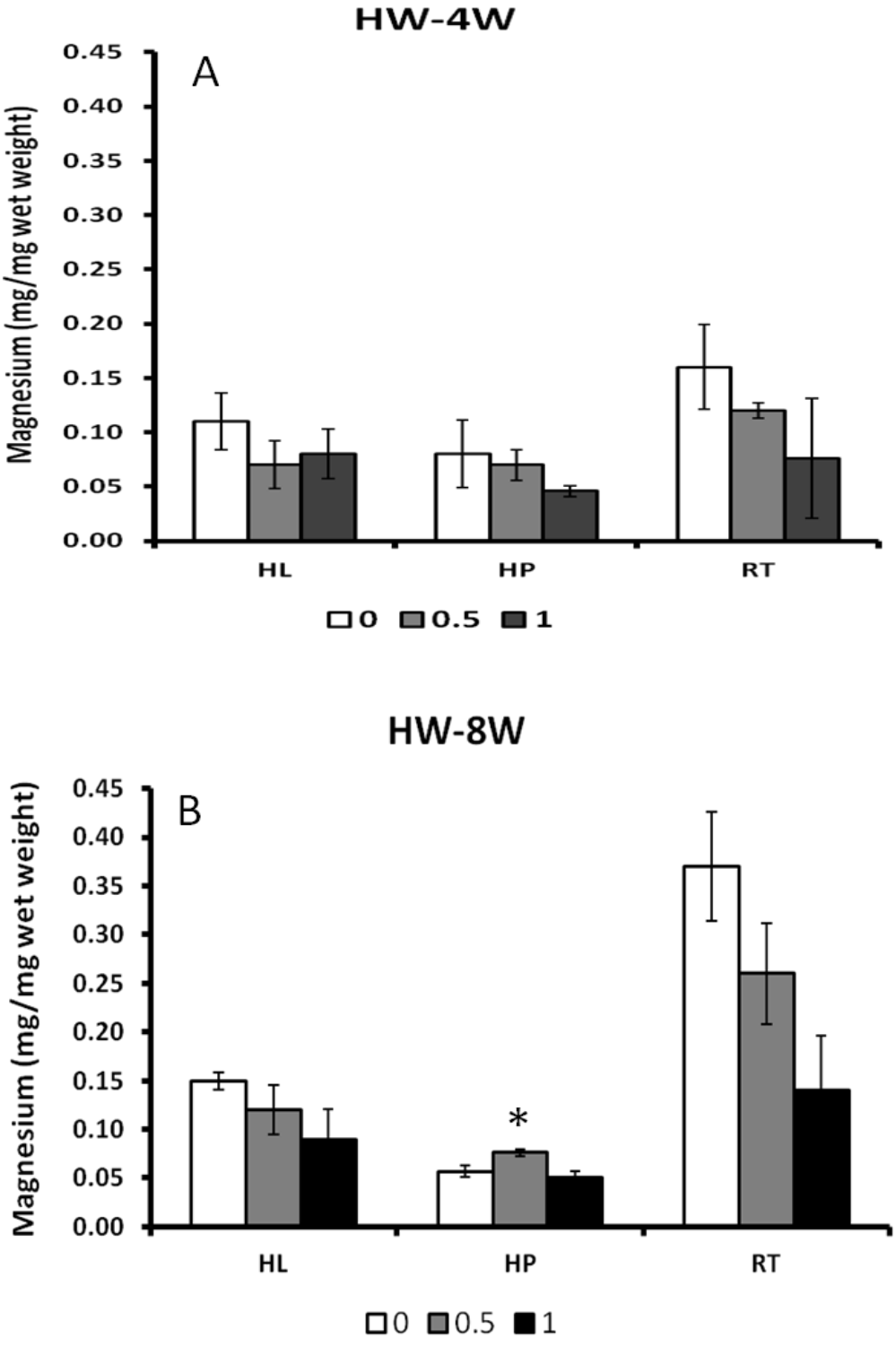

Figure 5.6 Mean magnesium concentrations in the body parts (mg/mg w wt) of juveniles exposed to Cd (0.5 and 1 µg Cd L^{-1}) for 4 (A) and 8 (B) weeks in hard water. Error bars represent SEM. HL, haemolymph; HP, hepatopancreas; RT, remaining tissues. * Asterisks represent a significant difference compared to the control (n=8, ANOVA and Scheffe post hoc, *$p < 0.05$).

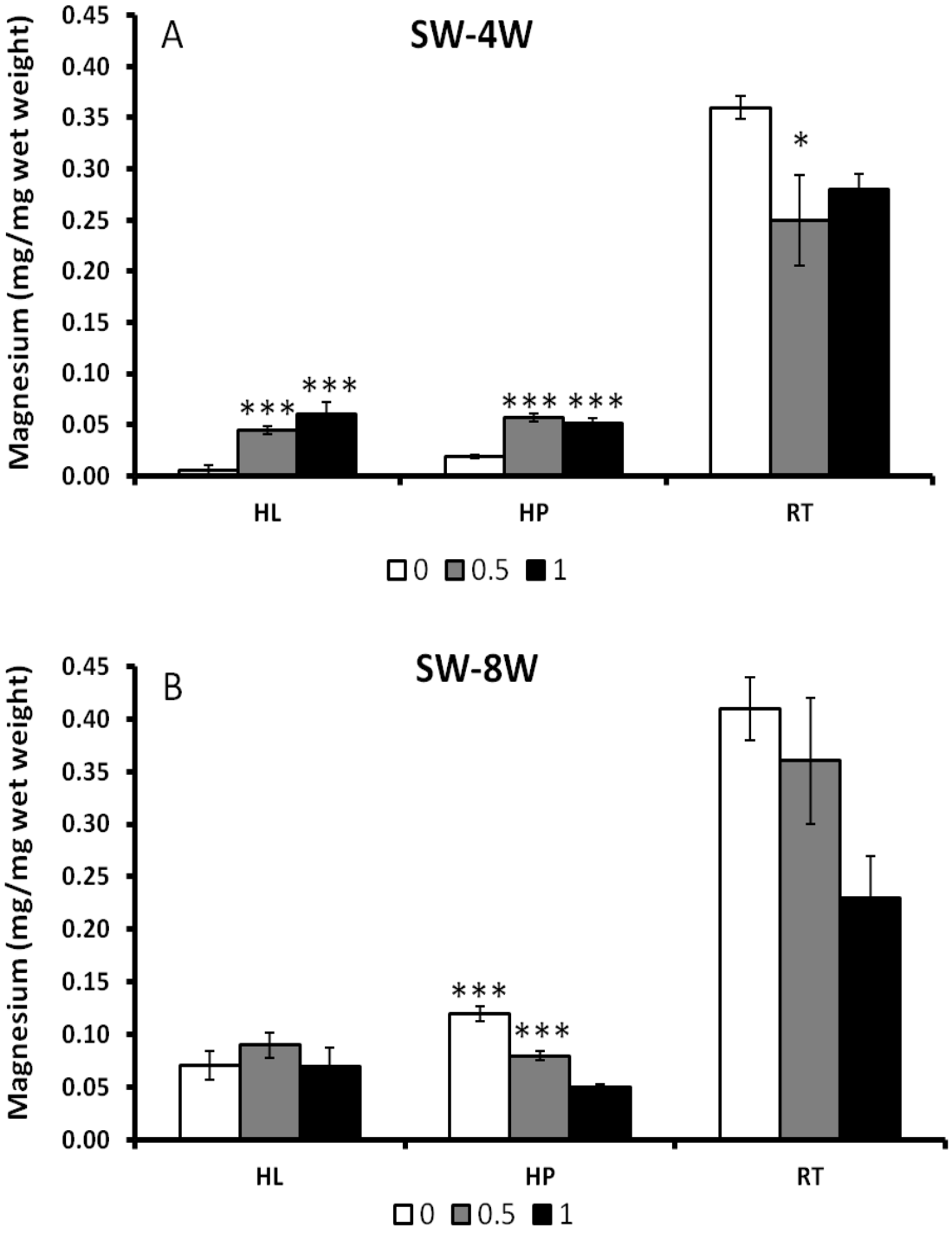

Figure 5.7 Mean magnesium concentrations in the body parts (mg/mg w wt) of *G. pulex* exposed to Cd (0.5 and 1 µg Cd L^{-1} for 4 (A) and 8 (B) weeks in soft water. Error bars represent SEM. HL, haemolymph; HP, hepatopancreas; RT, remaining tissues. * Asterisks represent a significant difference compared to the control (n=8, ANOVA and Scheffe post hoc, *p < 0.05; ***p < 0.001).

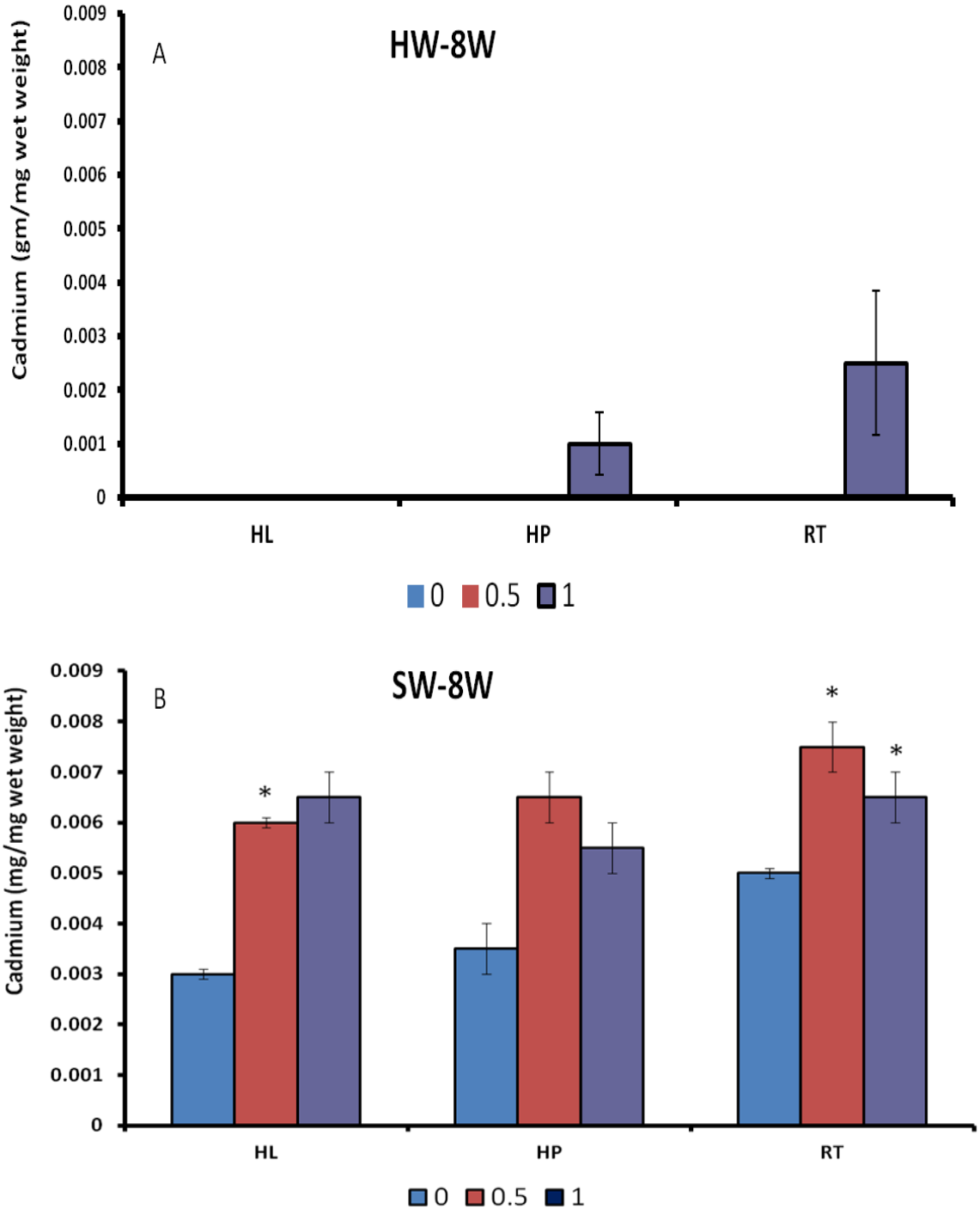

Figure 5.8 Mean Cd accumulation in the body parts for 8 weeks in hard water (A) and soft water (B). Error bars represent SEM. HL, haemolymph; HP, hepatopancreas; RT, remaining tissues. *Asterisks represent a significant difference compared to the control (n=8, ANOVA and Scheffe post hoc, *$p < 0.05$).

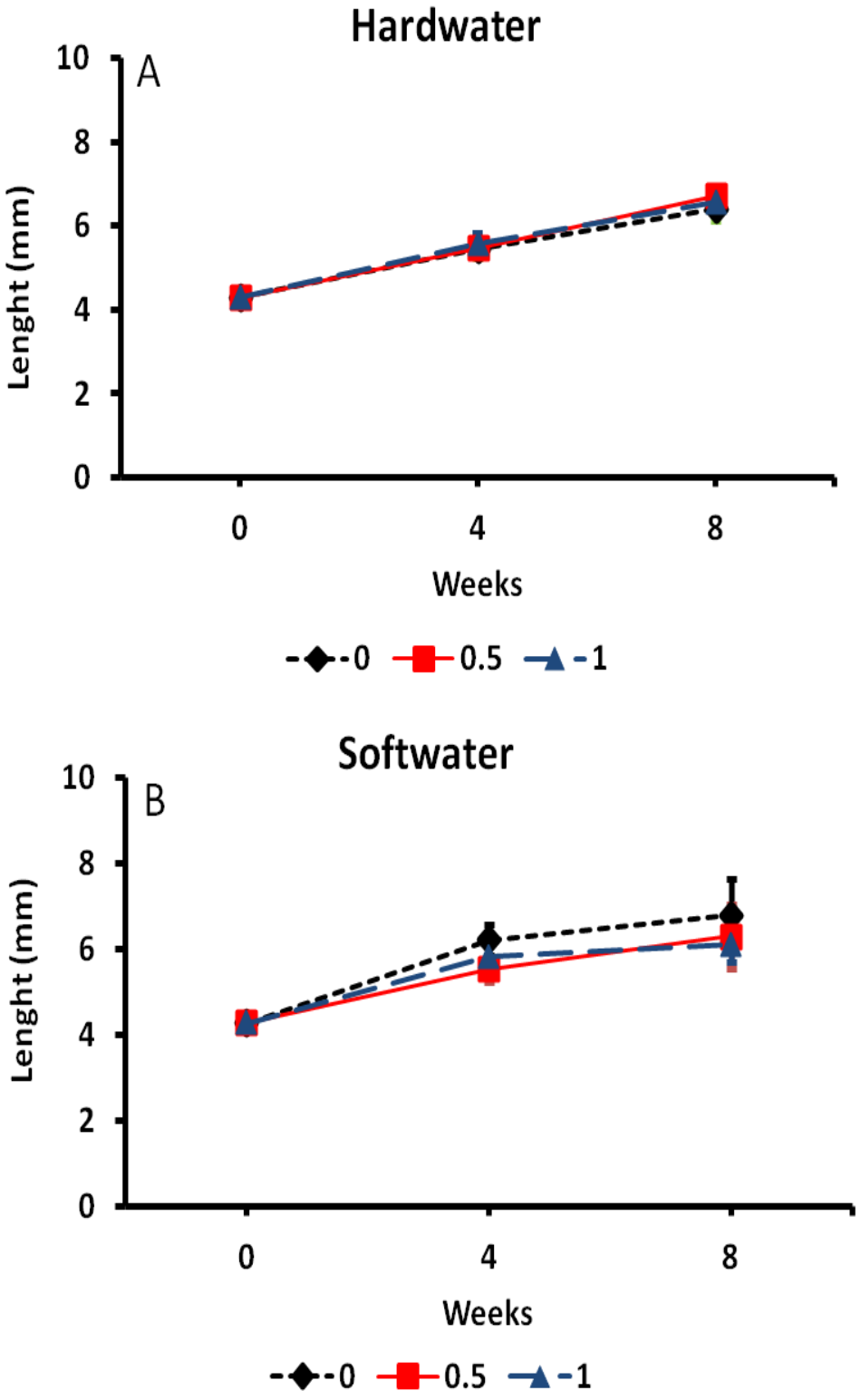

Figure 5.9 Length of juveniles *G. pulex* exposed to 0.5 and 1 µg Cd L^{-1} in hard water (A) and in soft water (B) for 4 and 8 weeks compared to time zero. Each point represents the mean ± SEM, n = 12.

5.3.3.2 Number of segments of 1st antenna

In hard water, the number of segments, the primary antenna increased 16.08 ± 0.26, while, the number of segments in animals exposed to Cd was 15.83 ± 0.17 at 0.5 µg Cd L^{-1} and 15.83 ± 0.21 at 1 µg Cd L^{-1}. After 8 weeks, the number of segments increased in the control (17.25 ± 0.7) as well as exposed to Cd 17.33 ± 1.3 and 17.17 at 0.5 and 1 µg Cd L^{-1} respectively.

In soft water, the number of segments was less than the animals in HW there is no differences between the numbers of segments in control (15.50 ± 0.72) and exposed to Cd 15.42 ± 0.54 and 15.50 ± 0.7 at 0.5 and 1 µg Cd L^{-1} respectively. After 8 weeks, the number of segments tended to raise 17.67 ± 0.92 at 0.5 µg Cd L^{-1}, while the average number of segments in control was similar to animal exposed to 1 µg Cd L^{-1} (16.92 ± 0.7 and 16.92 ± 0.47 at 0.5 and 1 µg Cd L^{-1} (Fig 5.10).

5.3.3.3 Weight

In hard water, the mean weight of juveniles was 2.35 ± 0.3 mg w wt, in control, while the average weight of juveniles exposed to 0.5 µg Cd L^{-1} was 2.07 ± 0.03 and 2.95 ± 0.17 mg w wt at 1 µg Cd L^{-1}. After 8 weeks, the average weight of juveniles increased 6.50 ± 0.5 mg w wt in control as well as the juveniles exposed to 0.5 and 1 µg Cd L^{-1} (6.53 ± 0.59 and 6.47 ± 0.6 mg w wt, respectively).

In soft water, the average weight of juveniles after 4 weeks was 3.45 ± 0.36 mg w wt in control, declined at 0.5 µg Cd L^{-1} (2.35 ± 0.3 mg w wt) and slightly increased 2.57 ± 0.31 mg w wt at 1 µg Cd L^{-1}. After 8 weeks, the average w wt of juveniles increased in control and exposed to 0.5 µg Cd L^{-1}

(5.94 ± 0.86 and 5.82 ± 0.76 mg w wt, respectively), but decreased in animals exposed to 1 µg Cd L^{-1} (4.35 ± 0.4 mg w wt) (Fig 5.11B).

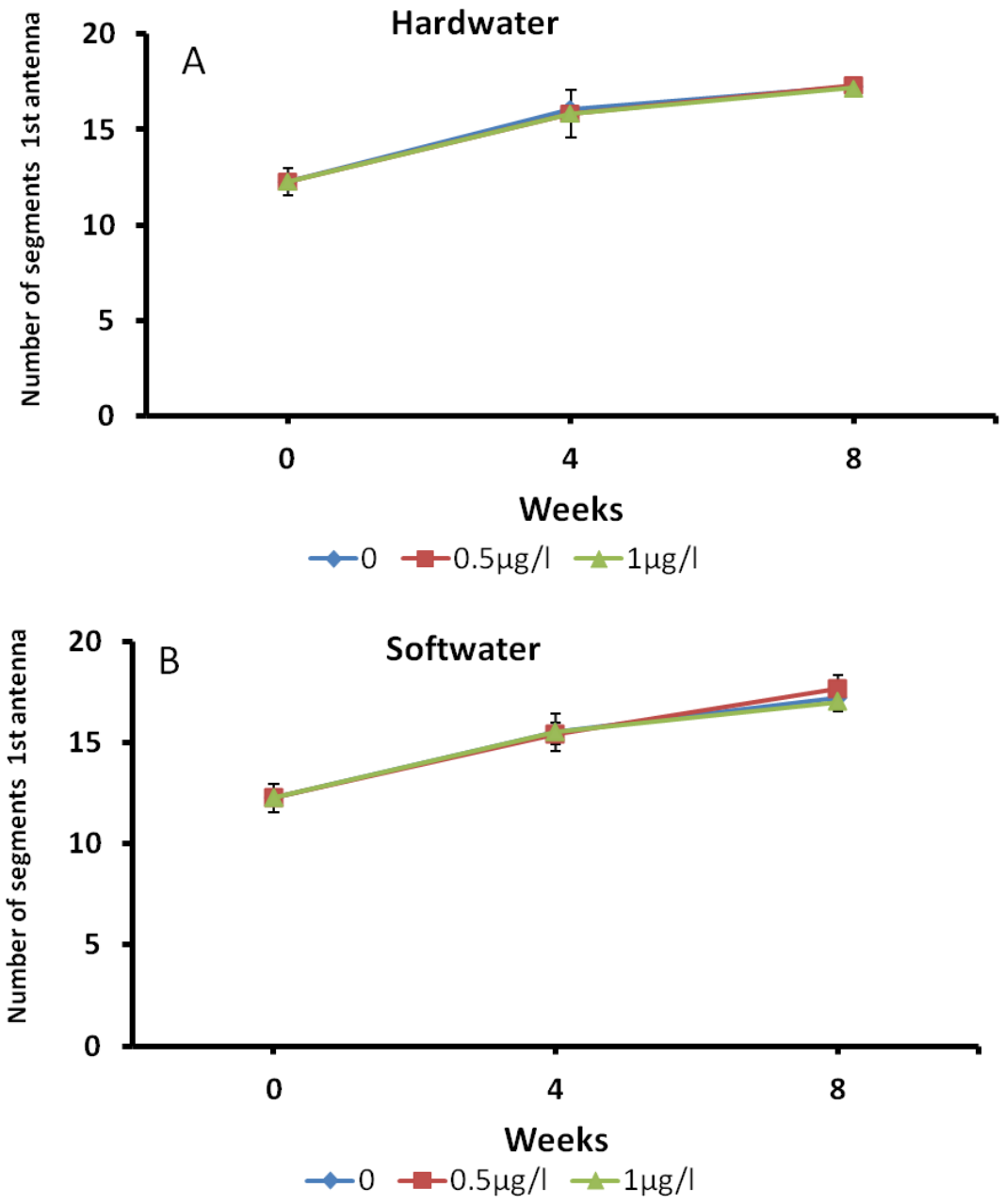

Figure 5.10 Number of segments (1st antenna) juveniles *G. pulex* exposed to Cd (0, 0.5 and 1 µg Cd L^{-1} in hard (A) and in soft water (B) for 4 and 8 weeks compared to time zero. Each point represents the mean ± SEM, n = 12.

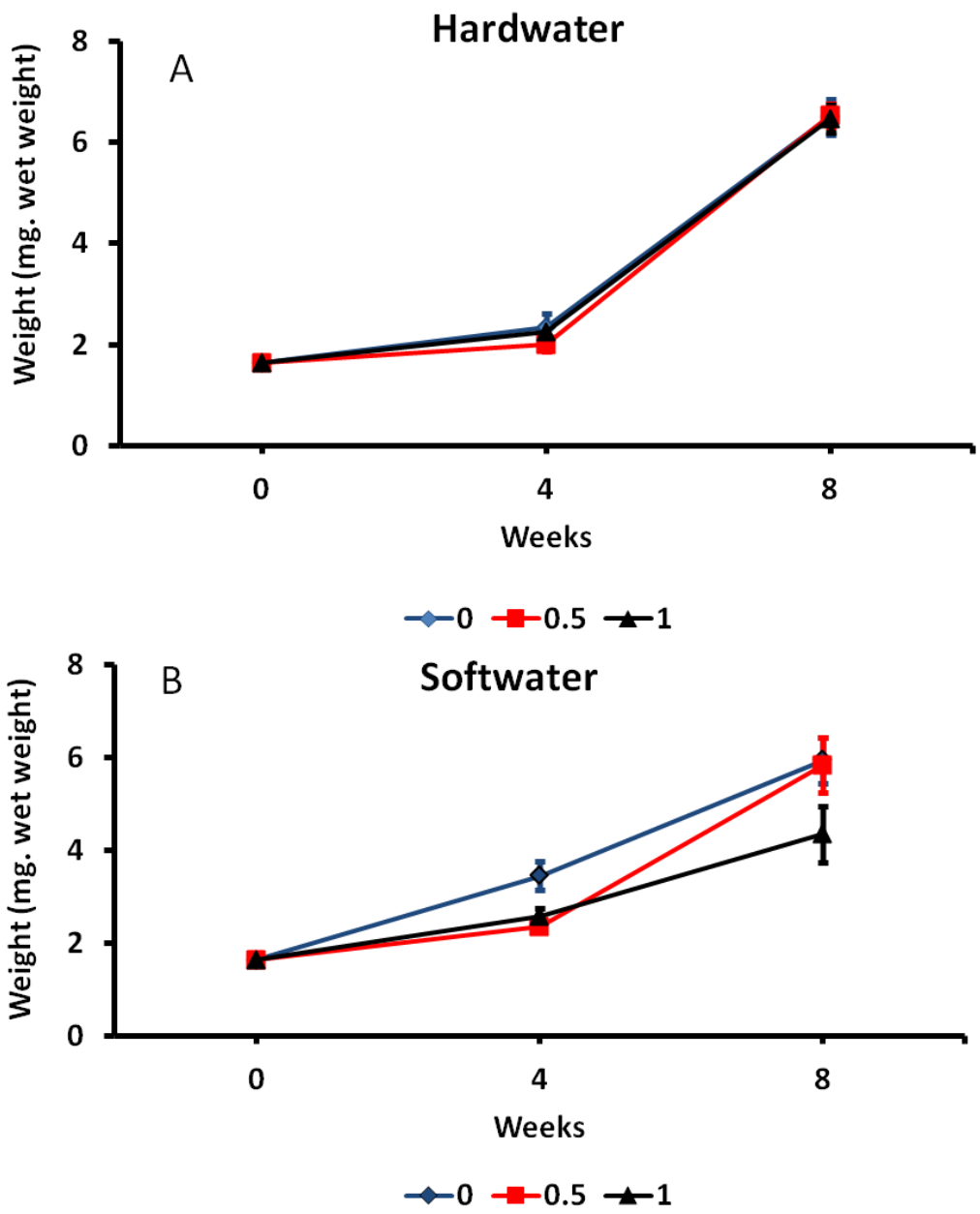

Figure 5.11 Wet weight of juvenile's *G. pulex* exposed to Cd (0.5 and 1 µg Cd L^{-1}) for 4 and 8 weeks in hard water (A) and soft water (B) for 4 and 8 weeks compared to time zero. Each point represents the mean ± SEM, n = 12.

5.3.4 Food consumption

In hard water, the animals consumed 3.71 ± 0.48 mg/day in control after 4 weeks (Fig 5.12A). While, the juveniles exposed to 0.5 µg Cd L^{-1} the rate of food consumption decreased to 3.32 ± 0.66 mg/day and returned to ascend

at 1 µg Cd L^{-1} (3.79 ± 0.59 mg/day). After 8 weeks, the rate food consumption rose in control 4.63 ± 0.33 mg/day and animal exposed to 1 µg Cd L^{-1} (4.98 ± 0.41 mg/day), while decreasing in animal exposed to 0.5 µg Cd L^{-1} (4.06 ± 0.65 mg/day).

In soft water, the results demonstrated that food consumption in control and exposed to Cd was slightly higher than the animals in HW after 4 weeks. Food consumed by animals throughout 4 week in control was 3.78 ± 0.48 mg/day. Exposing animals to 0.5 µg Cd L^{-1} tend to decrease the rate of food consumed to 3.59 ± 0.55 mg/day. However, food consumption in animals exposed to 1 µg Cd L^{-1} increased 3.86 ± 0.32 mg/day, in control. After 8 weeks, the food consumed did not increase in control and exposed to Cd as animals in HW. The food consumption of animals in control was 3.45 ± 0.9, and 3.54 ± 0.30 mg/day, 3.38 ± 0.49 mg/day at 0.5 and 1 µg Cd L^{-1} (Fig 5. 12B).

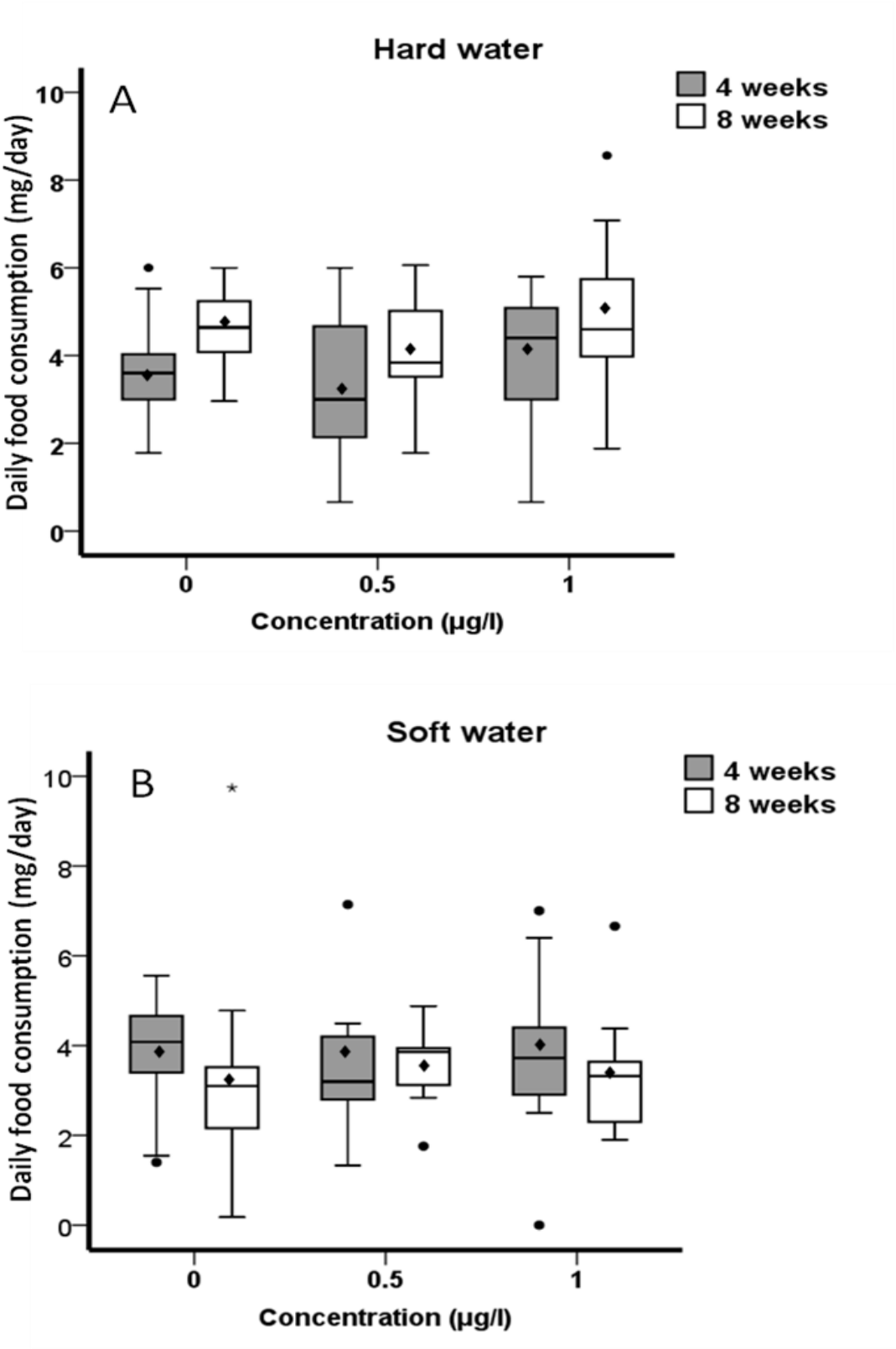

Figure 5.12 Box and whisker plot of leaf consumed throughout the experiment by juveniles *G. pulex* exposed to Cd (0.5 and 1 µg Cd L^{-1}) for 4 and 8 weeks in hard water (A) and soft water (B). ♦ It represents means of daily food consumption.

5.4 Discussion

5.4.1 Survival

The limit of Cd concentrations allowed in water bodies to protect the health of freshwater organisms and human health is 1 µg Cd L^{-1} (EPA, 1980; Eisler, 1985). The level is exceeded in Argentina to 2 µg Cd L^{-1} where the hardness of water lies between 60–120 ppm (García et al., 2010). In this current study, the percentage of survival decreased with increasing time exposure in SW after 8 weeks of exposure to 0.5 and 1 µg Cd L^{-1} by 50% and was lower than 50% of the control. Cannibalism as a behaviour was dominant in the juveniles reared in SW especially in the control and resulted in a decreased percent of survival. Water dilution led to a reduction in the elements in SW (15 ppm) to survive such as Na, Cl, Mg and Ca and induced Cd accumulation. While the juveniles reared in HW were able to survive in hardness of 150 ppm with elemental compositions of less than half of the lower Hoopern valley successively.

Heavy metals are more soluble and actively toxic agents in SW than HW (Rathore and Khangarot, 2003). The freshwater fish *Oreochromis niloticus* accumulated higher Cu and Cd in the tissues from SW than the fish from HW, all the fish were killed after exposure to Cu in SW by 12 days (Saglam et al., 2013). When Cd accumulation is more than the storage capacity in the body parts, this will lead to the animals dying (de Nicola et al., 1993). In addition to that the amount of Ca (3.33 ppm) was insufficient in SW, to allow for the accelerating rate of growth in juveniles which demands enough Ca to calcify the cuticle. It was observed that the juveniles were unable to calcify the exoskeleton and mouth parts after moulting rapidly for moving and looking for food. Soft exoskeleton and incomplete calcification lead to hazard cannibalism, predation

and mechanical damage (Rukke, 2002). Cannibalism was an alternative source of Ca particularly in the control group, where most of the animals return to eat the old exoskeleton after moulting (personal observation). The percent of survival, growth and calcification in *G. lacustris* and *A. astacus* reduced in low Ca environment (5 ppm). While, the animals reared at 10 ppm completed the calcification process successively (Rukke, 2002). The juveniles were able to live in hard water (150 ppm) containing fewer elements than in the field (low Hoopern valley stream). For example, Cl and Na were 29.07 mg/l and 20.67 mg/l in the field while hard water prepared in the lab contained 12.62 mg/l (Cl) and 8.44 mg/l (Na).

Increase of heavy metals in the water column and sediments may lead to reductions in the number of new hatched animals and incomplete life cycles for many organisms in aquatic environments. Specially, the species which are playing a vital role in the food chain and decomposing leaf litters such as the amphipod *G. pulex*. Alonso et al. (2010b) and Adam et al. (2010) investigated the juvenile *G. pulex* and *G. fossarum* and found that they were less tolerant to Cd, ivermectin and a pyrethroid insecticide (deltamethrin) than adults. It was also observed that the juveniles of *G. pulex* one day old were 250 times more sensitive to Cd (0.019 mg Cd L^{-1}) toxicity after 48 h than old age 220 days exposed to 4.7 mg Cd L^{-1} (McCahon and Pascoe, 1988a). Neonates of the amphipod *H. curvispina* were more sensitive than juveniles and adults after 72 h of exposure to Cd ranging from 0.6–22.7 µg Cd L^{-1} (Garcìa. et al., 2010). Susceptibility of juvenile invertebrates to toxicants was attributed to; they have a high surface area to volume ratio, which provides high capacity to exchange with a toxicant, and a high metabolic rate that facilitates uptake toxicant from

the environment (Buikema and Benfield, 1979; Wright, 1980b; Alonso et al., 2010b; Mohammed, 2013).

5.4.2 Elements in body parts

The results of this study show that the majority of Ca and Mg accumulated in the remaining tissues of juveniles which contain the majority of exoskeleton after dissecting. The exoskeleton of crustacea represents 50% of dry weight mineralised with Ca and Mg Carbonate (Wheatly and Gannon, 1995). The exoskeleton of *G. pulex* represents more than 96% of Ca, and is shed regularly (Willoughby and Sutcliffe, 1976; Wright, 1979), during the moulting *G. pulex* lost about 42% of the body Ca in two to three days and reabsorbed 46% from the old exoskeleton (Wright, 1980a, b; Greenaway, 1985; Neuffled and Cameron, 1993).

Low Ca in water acts to limit the distribution of crustaceans in aquatic environments (Rukke, 2002; Zehmer et al., 2002). It was observed that crustaceans in sea water and water flowing over limestone can compensate rapidly all Ca lost during moulting and possess a larger size than crustaceans from SW (Wright, 1979; Greenway, 1985; Ahearn and Zhuang, 1996; Wheatly, 1996; Meyran, 1998; Rukke, 2002).

In the present study, Ca reduced with increasing Cd concentration in the haemolymph and hepatopancreas in the group of juveniles in HW, maybe the ratio of Ca: Cd was adequate to inhibit Cd for 4 weeks or was reduced as a result of Cd's high affinity to sulfhydryl groups (–SH) in MT, leaving it in a less toxic form in the cells (Stuhlbacher, and Maltby, 1992; Rainbow and Dallinger, 1993). The level of Ca increased at high concentrations to more than 5 mg/mg

wet weight after 8 weeks as a mechanism to inhibit Cd by supplying more Ca to the hepatopancreas via the haemolymph. In soft water, Ca reduced to less than 5 mg/mg w wt in the haemolymph and the hepatopancreas may be insufficient to resist Cd toxicity.

In fact, the amount of Ca in haemolymph and other body parts in the body of crustaceans is related to moulting cycle. Post-moult there is a high Ca flux into the new carapace in comparison to premoult and intermoult. It is regulated by organs such as haemolymph, gills, gut, exoskeleton and antennal glands; the posterior caeca of amphipods is also a site to store Ca in postmoult stage for maintaining Ca stable in the blood and cuticle (Greenaway, 1985; Neuffld and Cameron, 1993). It was also found that the body Ca of crayfish was higher from SW than from HW (Wheatly and Gannon, 1992). Ca concentrations of less than 5 ppm declined the growth of *G. lacustris* and *A. astacus* (Rukke, 2002). All these studies were conducted on adults, tracking the elements in the juveniles was limited, because, the amount of samples available from each organ is small, and these animals are so fragile at this age to dissect.

Most of species living in the SW have a higher affinity to Ca than the species in HW (Greenaway, 1985; Rukke et al., 2002), and have the potential to take up Ca via active transport or permeability to increase Ca uptake from water via a change of electrochemical gradient direction (Neuffled and Cameron, 1993). Vincent (1963, 1969) found that population *G. pulex* from SW (1.5–1.8 mg Ca L^{-1}) have a high affinity to Ca and can live in an environment less than 1 mg Ca L^{-1}, While the population from HW (100–110 mg Ca L^{-1}) was unable to live in less than 3 mg Ca L^{-1}, with a long period of calcification. Vincent (1969)

found also the amphipods *G. pulex* to have higher haemolymph Ca (5.85 mM/l) than ambient water 0.038–0.045 mM/l.

In the present study, the highest Mg accumulation was in the remaining tissues of juveniles than haemolymph and hepatopancreas in particular, the animals in SW after 8 weeks. Magnesium plays an essential role for growth, osmoregulation of enzymes such as Na^+/K^+-ATPase (Péqueux, 1995; Van der Velden, 1991; Calta and Ural, 2001; Roy, et al., 2007). Most of previous studies shown that Ca was a more protective element than Mg, Na, SO_4 or CO_3 in SW against Cd toxicity to brook trout *Salvelinus fontinalis* (Carroll et al., 1979). Calcium had the ability to protect eggs of the teleost *Oryzias latipes* (Michibata et al., 1986) and *H. azteca,* more so than Mg in HW (Jackson et al., 2000). Calcium also inhibited Cu toxicity in rainbow trout and fathead minnow more than Mg in HW in less than 24 h (Naddy et al., 2002). Mirror carp required more than a 0.05 mg L^{-1} ambient Mg concentration to survive and develop successfully during the early life stage. Moreover, increase ambient Mg led also to increase Ca uptake in the carp eggs (Van der Velden, 1991; Calta and Ural, 2001).

The results obviously demonstrated that SW induced Cd accumulation with increasing time exposure after 8 weeks of exposure in, the haemolymph, hepatopancreas and the remaining tissue. There are different pathways to take up the toxicants to the body of aquatic crustacean. For example, gill, gut and the body surface (Kang et al., 2012). High Cd uptake in the gills led to increase haemolymph Cd (Wright, 1980b). In the current study, Cd in haemolymph, hepatopancreas and remaining tissues was 3 times in exposed to Cd. In hard water, Cd inhibited to less than 0.003 mg/mg wet weight in remaining tissues

and remaining similar to ambient Cd in hepatopancreas at 1 µg Cd L^{-1} after 8 weeks. Competition between Cd and hardness cations (Ca^{2+} and Mg^{2+}) decrease bioavailability and toxicity of Cd in ambient water. Abundance of Carbonate species in aquatic systems leads to form CdHCO3 or $CdCO_3$ as complexes, in addition to that metal speciation may lead to self-purification in many aquatic bodies for scavenging toxic metals (Korfali and Davies., 2004., Newman. 2010). Abundance of organic and inorganic ligands reduces toxic effect of free metal ion in water. Using different models to estimate and predicate metals speciation and complexes has been utilised successively in water in many aquatic systems (Korfali and Davies., 2004).

Cadmium accumulated in the body parts of juveniles in SW delay calcification may accelerate Cd uptake via thin cuticle. Accumulation of Cd in the remaining tissues is also a main process to reduce the toxicity of elements via deposit it in the exoskeleton during the moulting. It was found the midge larvae *Stichtochironomus histrio* lost both Zn and Cd during metamorphosis (de Nicola et al., 1993; Timmermans, 1993; Sornom et al., 2010).

The requirement of crustaceans for Ca especially during the moulting stage has led to increased Cd entrance to the body via the Ca pump (Rainbow and Dallinger, 1993). Furthermore, the interaction between Cd and Ca attributed to the similarity between the two elements in the outer shell of electron and ionic radii, Ca^{2+} (0.094 Å) and Cd^{2+} (0.92 Å) that facilitates entry of Cd through Ca channels (Rainbow, 1997; Hollis et al., 2000; Pellet et al., 2009; Newman, 2010). Wright and Frain (1981) found that the toxicity of Cd is related to Ca: Cd ratios and the feature of freshwater rather than the amphipods. Wright (1980a)

suggests that Cd accumulation in the freshwater *G. pulex* is attributed to 'accidental' active cadmium uptake.

Several previous studies have shown effect of Ca concentration on many aquatic organisms. Wright and Frain (1981) found that 200 mg Ca L^{-1} had an antagonistic effect on the Cd toxicity (0.5 mg Cd L^{-1}) of the adult freshwater *G. pulex* with increasing the mortality to lower than 50% after 120 h. The results of McCahon and Pascoe (1988b) illustrated that the toxicity of Cd reduced at 180 mg Ca L^{-1} in immediate post-moult of *G. pulex*. While the concentration 40 and 115 mg Ca L^{-1} did not exhibit influence on the adults of *G. pulex*. While, Increase Ca concentrations gradually to 3.5 mg Ca L^{-1}, 88 mg Ca L^{-1} and 174 mg Ca L^{-1} decreased Cd influxes in *G. pulex* (Pellet et al., 2009). An increase Ca concentration (1.0 mM) protected the teleost *Oryzias latipas* eggs exposed to 10 mg Cd L^{-1} (Michibata et al., 1986). Cadmium toxicity was inhibited in freshwater *H. azteca* at 6.5 mg/L of Ca (Stephenson and Mackie, 1989).

5.4.3 The growth of juveniles

There is no difference between the length of juveniles in SW and HW after 4 weeks of exposure. While, the weights of juveniles declined with increasing Cd concentrations in SW after 8 weeks. The juveniles reared in HW did not observe any differences in weights between exposed to Cd and the control at temperature 12 ± 1^{o}C. The juveniles reached to sexual maturity at the length 6 mm after 120 days (8 weeks at the beginning + 8 weeks exposure). Although, an increase temperature of water between 15 and 20^{o}C was contributed factor to accelerate the growth rate of the freshwater *G. pulex* to reach maturity in 120 days (Hynes, 1954; Bloor, 2010). For example, Welton and Clarke (1980) found

that the juveniles *G. pulex* reached sexual maturity at length 6 mm, required 133 days at 15^{o}C and 87.5 days at 20^{o}C.

Generally, newly hatched of juveniles have 5 segments in the primary antenna and the number increases gradually with growth. The maximum number of antennal segments in the males is 32 and 26 in females (Pinkster, 1970). Increase in segments is associated with increased length in males (1.6 mm) and females (1.4 mm) at each moult (Bloor, 2010; Sutcliff and Carrick, 1981). Cultured juveniles of *G. pulex* reached sexual maturity after 10 moults, with 5 mg wet weight, the body length 6 mm and 14–16 antennal segments (Nilsson, 1977; Sutcliff and Carrick, 1981; Bloor, 2010), that coincided with the present results, the juvenile *G. pulex* gained more than 5 mg w wt and 17 antennal in HW. Whereas, the weight of juveniles reduced to less than 5 mg w wt in SW at 1 µg Cd L^{-1}. In crustacean, shedding of the old exoskeleton coincides with increases in the size of the animal, this process is regulated by reabsorbing amount of Ca^{2+} from the old exoskeleton before and after moulting rapidly (Greenaway, 1985). The growth and survival of the juveniles *H. curvispina* reduced at Cd concentration 11.25 µg Cd L^{-1} (Giusto et al., 2012). Increased Ca and Mg concentrations in the haemolymph were associated with increasing growth of juveniles from HW and SW. It was also found that Ca concentrations in the haemolymph of the crayfish from SW and HW increased with increasing body size (Wheatly and Gannon, 1995).

5.4.4 Food consumption

The rate of food consumption reduced in the group of animals in SW after 8 weeks that coincided with the decrease the percentage of survival in SW. Food

and water are the main pathways to take up toxic metals in gammarids via the gills and the gut or through the cuticle especially after moulting (Wang, 2002; Kang et al., 2012; Vellinger et al., 2012b & c). In the present study, oak leaves as a source of food accumulated 16.98% of Cd more than *G. pulex* 15.91% at 0.001 mg Cd L^{-1} (Chapter 2). In addition to that, take up of Cd via food through the alimentary canal could have damaged tissue and cell organelles which are responsible for the synthesis and secretion of enzymes to digest and absorb food. In this current study, the hepatopancreas accumulated more than 2 fold of Cd in juveniles reared in SW at 0.5 and 1 µg Cd L^{-1}. Sublethal concentration (0.005 and 0.01 mg Cd L^{-1}) damaged microvilli and cell organelles in the hepatopancreas *G. pulex* after 10 days of exposure (Chapter 4).

The animals reared in low Ca failed to calcify their exoskeleton after moulting and were unable to eat due to the mouth parts not being hardened such as the mandibles, regardless of the abundance of oak leaves as food which are sources of Ca, but this may not have been enough in SW to compensate or regulate with increasing the growth rapidly (Wright and Frain, 1981; Greenaway, 1985; Rukke et al., 2002). The freshwater *G. pulex* depends upon oak leaves as a source of food rich with Ca (17.39 mg/g dry wet) to compensate losing Ca after moulting (Chamier and Sutcliffe, 1989).

Several researchers reported that the amphipod *G. pulex* prefers to eat conditioned leaf discs rich with microorganisms such as fungi and bacteria than unconditioned leaf discs (Marchant and Hynes, 1981; Graça et al., 1993a & b; Chamier and Willoughby, 2006; Bloor, 2010). In the current study, the juveniles *G. pulex* were reared on unconditioned oak leaves as a source of food for 4 and 8 weeks, using conditioned leaves could decrease microorganism growth or

increase Cd accumulation in case of exposure to Cd. Duddridge and Winwright, (1980) found the growth of aquatic fungi which grow on the food of *G. pulex* reduced due to accumulation of a large amount of Cd from the growth medium. Microbial activity in water with increasing discharge of pollutants may reduce the feeding rate of detritivores (Schaller et al., 2011b). Daily food consumption of juveniles reared in HW was better than SW after 8 weeks in the control group and exposure to Cd. Graca et al. (1994) found that conditioned leaves did not influence the growth of *G. pulex.* Hardness, alkalinity and dissolved oxygen in the water contribute to raising the rate of feeding in the freshwater *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda) (Pestana et al., 2007). Reduction of food consumption, in the juveniles reared in SW is an indicator for reducing the feeding rate. Abel and Bărlocher. (1988) reported that contaminated food of *G. fossarum* with Cd 1 mg/L in SW reduced survivorship from 95% to 66% in 12 days and 100% to 76% in HW.

In conclusion, hard water with 150 ppm as $CaCO_3$ inhibited Cd toxicity during long-term exposure to low Cd concentrations (0.5 and 1 µg Cd L^{-1}) and protected the juveniles at this stage. Soft water accelerated Cd accumulation in the body parts with time exposure. Low calcium in soft water led to delayed calcification of the exoskeleton rapidly after shedding the old exoskeleton. This adversely influenced food consumption and the weight of juveniles after 8 weeks. Soft water is an inappropriate medium to maintain the juveniles *G. pulex* at 15 ppm. The role of Ca and Mg in the function of the body parts throughout growth and exposure to toxicants is still not clear. This is the first report detailing these factors in juvenile *G. pulex* under chronic conditions of exposure

Chapter 6 Effect of sublethal concentrations of cadmium on Na^+/K^+-ATPase activity, ion concentrations in haemolymph, and cadmium- binding protein (metallothionein) in *Gammarus pulex* in hard and soft water.

6.1 Introduction

Aquatic organisms face a lot of challenges for survival in ambient water influenced by alternation in daily and seasonally affected environmental factors. Osmoregulation is a main mechanism of adaptation for many aquatic organisms in freshwater, brackish water and sea water. The majority of freshwater organisms maintain their body fluids hyperosmotic to ambient water by increasing uptake of salts from dilute medium or producing urine hypoosmotic to haemolymph (Sutcliffe, 1967; Mantel and Farmer., 1983; Henry et al., 2012).

In crustaceans, the epithelial cell layers in gills, guts, integument and antennal glands play a vital role for osmoregulation (Ahearn et al., 1999; Lucu and Towle, 2003; Felten et al., 2008a). Gills developed in aquatic crustacean for regulating ions between the external and internal cell membrane and also as a site for ion transportation, gas exchanges, acid-base regulation, and nitrogen waste extraction (Péqueux, 1995; Lucu and Towle, 2003; Felten et al., 2008a; Soegianto et al., 1999; Henry et al., 2012; Li et al., 2012). Anthropogenic activities such as mining activities, agriculture, and industry led to increase the percent of pollutants in the aquatic environments, which represents a source of risk on the diversity of organisms in the aquatic bodies. Most heavy metals interfere with biological enzymes in several organs such as gills, hepatopancreas and haemolymph by disturbing ion regulation (Bury et al., 1999; Lignot et al., 2000; Li et al., 2012; Saglam et al., 2013).

ATPase enzymes such as Na^+/K^+-ATPase, and carbonic anhydrase act on regulating osmotic pressure, cellular volume and membrane permeability through membranes (Li et al., 2012; Saglam et al., 2013). It was found that the freshwater crustacean *G. pulex* expended approximately 11% of the total energy budget on osmoregulation (Sutcliffe, 1984). The sodium pump or Na^+/K^+-ATPase is a transport protein in plasma membranes of animal's cells, to transport the ions and maintain a symmetrical distribution of Na^+ and K^+ inside or outside lipid membrane of the cell (Kinne-Saffran et al., 1993; Lucu and Towel, 2003).

Previous studies have established that heavy metals disturb ion regulation in many aquatic organisms. For example, exposure to Cd changed the gill structure and Na^+/K^+-ATPase activity, in the brown shrimp *Crangon crangon* (Papathanassiou, 1985), it also inhibited Na^+/K^+-ATPase activity gills and hepatopancreas of the euryhaline crab *Scylla serrata* (Dhavale et al., 1988), and the gills and intestine of European eel *Anguilla anguilla* (Lionetto et al., 1998, 2000). Copper (Cu) inhibited Mg-ATPase and Na^+/K^+-ATPase activity of adult *G. pulex* (Brooks and Mills, 2003). Exposure to lead (Pb) disturbed Na^+/K^+-ATPase activity in the gill of the freshwater mussel *Elliptio complanata* after 28 days of exposure (Mosher et al., 2010). Therefore, there is considerable evidence for the susceptibility of aquatic crustaceans in both freshwater and marine environments to the adverse effects of these metals on osmoregulatory activity. Most of these studies, however, have been at concentrations of metals exceeding regulatory limits. There remains little information on the effects of very low, chronic doses under different conditions of water hardness and across different levels of biological organisation.

Metallothionein (MT) is highly studied as a biomarker for metal contamination in toxicological studies since it was extracted for the first time from equine (horse) kidney cortex by Margoshes and Vallee (1957), it is widely distributed among prokaryotes and eukaryotes, and highly concentrated in organs such as liver, hepatopancreas (crustaceans), kidney, lung and intestines (Klaassen et al., 1999; Newman, 2010). It is induced by essential metals such as Cu and Zn and non-essential metals such as Cd, Hg, and Ag (Roesijadi, 1992; 1994). It has a high content of cysteine (20–30%), which has a sulfhydryl group (–SH) important for binding of metals, it is of low molecular weight, e.g. Its sequence includes 58–60 amino acids (non aromatic) in crustaceans and 61 amino acids in mammals. It has a high affinity for essential elements (Zn and Cu) and non-essential elements (Cd and Hg). Metallothionein plays an important role for regulation Zn and Cu homeostasis, detoxification of non-essential metals such as Ag, Cd and Hg in the cell, and protection against free radicals (Bremner and Beattie., 1990; Roesijadi, 1992; Klaassen et al. 1999; Amiard et al., 2006; Newman, 2010). The thiol group (–SH) of cysteine has the ability to bind with the group IIB of heavy metals such as Cd, Hg, and Zn (Amiard, et al., 2006; Carpene et al., 2007; Shariati and Shariati, 2011), to detoxify heavy metal toxicity and protect cells and tissues (Klaassen et al., 1999; Amiard et al., 2006; Fraysse et al., 2006; Thirumoorthy et al., 2007; Nordberg and Nordberg, 2009).

Metallothionein (MT) plays an essential role in detoxifying heavy metals in a wide range of aquatic invertebrate species. For example, the American lobster *Homarus americanas* fed Cd rich oyster (88.5 µg/g w wt) for 28 days led to Cd and Cu binding metallothionein (Engel and Brouwer., 1986). There was an increase in Cd binding protein in the hepatopancreas, kidney and digestive

gland of the crab *Cyprinus carpio* after exposure to 5 and 30 mg Cd L^{-1} (Kito et al., 1986). Long-term exposure to Cd (0.1 and 0.2 mg Cd L^{-1}) and Zn (0.05 and 0.2 mg Zn L^{-1}) induced MT and MT- like proteins in the white shrimp *Litopenaeus vannamei* for 84 days (Wu and Chen., 2005). Metallothionein increased with an increasing Cd body burden in the freshwater crustacean *Daphnia magna* exposed to different Cd concentrations (3.13, 6.26, 12.5, 25 µg Cd L^{-1}) for 21 days (Fraysse et al., 2006). There was a positive relationship between Cd accumulation and MT induction in the gills and hepatopancreas of the freshwater crab *Sinopotamon henanense* for 5 days to 5.8; 11.6; 23.2 mg Cd L^{-1} (Ma et al., 2008).

This study aims to explore the influence of water quality (hard and soft water) and time of exposure on the ATPase responses in osmoregulation tissues (gills and hepatopancreas), haemolymph ions, and antioxidant level (metallothionein) during exposure to different Cd concentrations on the freshwater amphipod *G. pulex*.

6.2 Materials and methods

6.2.1 Experimental design

The animals were collected from the field in October 2011, transported to the lab to avoid low temperature during the winters which affect survival of these animals. They were kept in beakers of 2 L to acclimate to aquarium conditions, with a photoperiod of 12 light: 12 dark at 12^{o}C. They were aerated via glass pipettes, each beaker was covered with paraflim, and they were fed on unconditioned oak leaves, with dechlorinated tap water delivered directly from

the Aquatic Resource centre laboratories (Geoffrey Pop laboratories) which was changed every two weeks.

In this experiment 48 crystalline dishes were used (24) in hard water (HW) (150 ppm as $CaCO_3$) and 24 for soft water (SW) (15 ppm as $CaCO_3$) prepared as mentioned (Chapter 5), 5 animals per dish, the average length of animals was 10.08 ± 0.5 mm, wet weight 0.03 ± 0.004 g exposed to sublethal concentrations (0.005 mg and 0.01 Cd L^{-1}) of $CdCl_2$ (Fluka). The first group of animals was divided into two groups one for 7 days and another for 14 days. During the experiment, a pre weight of 0.29 g of dry oak leaves was dried in an oven for 48 h and then placed in Petri dishes with water to hydrate for 24 h to become suitable for the animals, as well as to prevent floating on the surface of water, the water was changed every 24 h, water parameters (temperature, dissolved oxygen, total hardness, pH) remain stable throughout the experiment.

6.2.2 Metallothionein assay

Metallothionein (MT) concentrations were measured by a spectrophotometer method according to Viarengo and his colleagues (1997) by measuring thiol concentration in the samples indirectly. The animals were exposed to Cd and non exposed a 0.5 g of the whole body of animals from each treatment was snap frozen in liquid nitrogen until analysis. The frozen tissue was ground with liquid nitrogen via cold mortar, and 5.98 ml of ice cold 1 mM L-Dithiothreitol (DTT) (Fluka) added and 110 µl of phenylmethylsulphonyl fluoride (PMSF) (Sigma) added, the mixture transferred to centrifuge tubes to sonicate for 15 mins, and then centrifuged at 4^{o}C for 70 mins at 55,000 rpm. The supernatant was transferred to eppendrorfs of 2 ml to freeze at –80 degree.

The frozen samples were thawed on ice, 500 µl of the sample transferred to 25 ml centrifuge tubes and 500 µl of –20^{o}C of absolute ethanol and 40 µl of chloroform was added. The mixture was vortexed, centrifuged at 7000 rpm for 12 mins, and the supernatant transferred to new centrifuge tubes, 3 ml of –20 absolute ethanol vortexed and placed for an hour to freeze at –20^{o}C, then recentrifuged at 7000 rpm for 12 mins at 4^{o}C. The pellet was washed with 2 ml of washing buffer (17.4 ml absolute ethanol, 200 µl chloroform and 2.4 ml Tris-Sucrose solution) the mixture vortexed and recentrifuged at 7000 rpm for 12 mins at 4^{o}C. The supernatant was poured away and the pellet dissolved in 300 µl of Tris- EDTA buffer (0.302 g Trisma-base, 0.146 g EDTA, 500 ml distilled water adjusted to pH 7), 4.2 ml of Ellman`s reagent or DTNB [5,5$'$ -Dithiobis-(2-nitrobenzoic acid)] was added to the standard (GSH) and the samples of the mixture was incubated at room temperature for 15 mins. The concentrations were calculated from the standard curve at 412 nm via spectrophotometer (Shimadzu). The amount of MT was calculated according to the equation below (1).

$$MT = \frac{\frac{\text{Concentration in µg}}{\text{vol. sample used(ml)}} \text{ X Vol. of DDT used in prep(ml)}}{\text{Weight of sample weighed out in first stage(g)}}$$

(MT= µg/g wet weight tissue).

Table 6.1 Concentrations for preparing glutathione reduced (GSH) standards

Standard	GSH(µl)	Tris - EDTA (µl)	Concentration (µg)
1	0	300	0
2	5	295	2.5
3	10	290	5
4	20	280	10
5	40	260	20
6	80	220	40
7	160	140	80

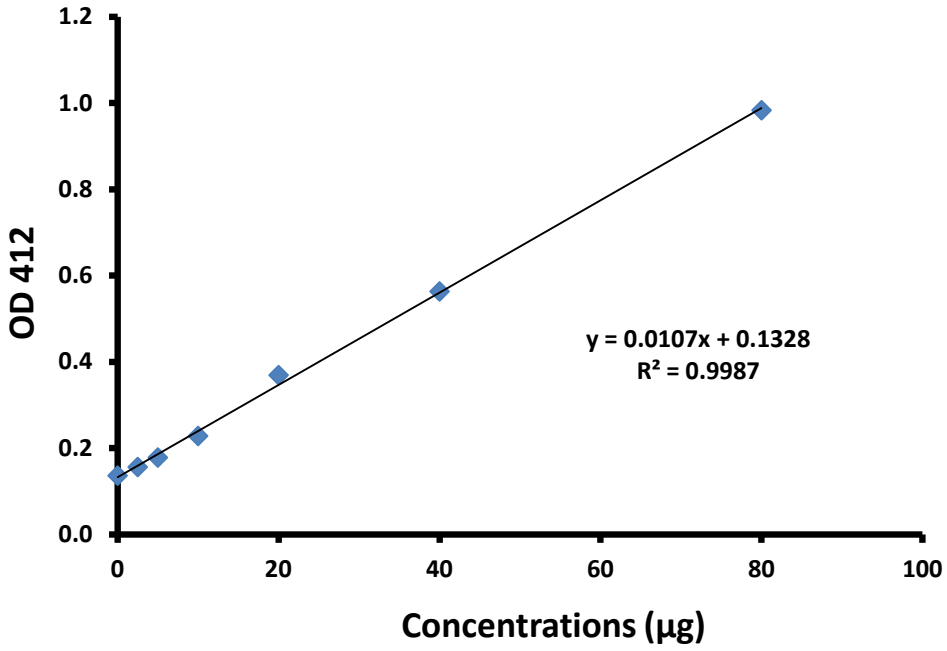

Figure 6.1 The standard curve of concentrations against the absorbance at 412 nm represents the R^2 and the equation to estimate the concentrations.

6.2.3 Na^+/K^+-ATPase assay

The gammarids were exposed to Cd (0.005 and 0.01 mg Cd L^{-1}) for two weeks. Na^+/K^+-ATPase was measured according to Brooks and Lloyd Mills (2003, 2006) method. In short, this assay divided into three parts as following: 1) homogenate the tissue. 2) Na^+/K^+-ATPase assay. 3) Protein assay. The pairs of gills were removed from the body and rinsed in ice-cold buffer (100 mM NaCl,

100 mM Hepes, 0.1% sodium deoxycholate, adjusted to pH 7.2), the gills were pooled from 5 animals in 500 µl in ice-cold buffer to homogenise on ice, centrifuged at 2000 g for 15 min at 4^{o}C, the extract enzyme was transferred to a new centrifuge tube and frozen at –80^{o}C. The frozen samples were thawed on ice, 30 µl added to a 1.5 ml centrifuge tube with 500 µl of incubation solution A (100 mM NaCl, 100 mM Hepes, 15 mM KCl, 10 mM $MgCl_2$, and adjusted to pH 7.2) and 30 µl of incubation solution B (incubation solution A + 10 mM ouabain), the mixture incubated in a water bath at 4^{o}C for 15 min, vortexed. Na^+/K^+-ATPase activity was estimated in the presence or absence of the inhibitor (ouabain). 5 mM Disodium ATP (Na_2ATP) was added to the incubation solution to block Na^+/K^+-ATPase activity. The mixture was vortexed and incubated for 20 min at 37^{o}C. Phosphate was released after adding 1 ml of freshly prepared Bonting solution (176 mM $FeSO_4$, 560 mM H_2SO_4, 8.1 mM ammonium molybdate) to stop the reaction at room temperature to raise the colour for 20 min. The absorbance was measured at 700 nm via spectrophotometer. The activity was measured by the difference between the incubation solution A and incubation solution B as Na^+/K^+-ATPase. Bradford assay (1976), Bio-Rad with bovine serum albumin (BSA) was used to measure protein as reference.

6.2.4 Haemolymph sampling

The haemolymph was taken from each individual animal which had been gently dried between tissues. Samples were taken from the haemolymph via syringe 0.3 ml from the end two segments of the telson. The samples were pooled from 5 animals in 100 µl ultrapure water in an eppendorf, frozen at –80^{o}C (according to Felten and Guerold, 2001). To measure ion

concentrations, haemolymph was defrosted, diluted (1:10 µl) and measured by Dionex ion chromatography (in individual poly vials). Haemolymph osmolality was measured with a vapour, pressure, osmometer (Wescor), calibrated using verified standard (100 mOsm kg^{-1}); each sample has taken 10 µl of the haemolymph.

6.2.5 Statistical analysis

Two Statistical programs were used, Excel, 2007 and SPSS (19.0) software to analysis the data as mean values ± standard error. One-way ANOVA was used to determine effects on metallothionein, sodium pump, haemolymph ion and haemolymph osmolality after exposure to Cd. Sheffes test used for significant differences between groups, Post Hoc multiple comparisons were assumed to be significant at a level of 0.05.

6.3 Results

6.3.1 Survival

Water quality was stable during the experiment, the total hardness of HW was 150 ± 0.01 ppm as $CaCO_3$ and SW 15 ± 0.05 ppm as $CaCO_3$, pH > 7.35 ± 0.08, temperature was 12 ± 1^{o}C during the experiment. The results indicate the percent of survival in HW after 7 days of exposure to 0.005 and 0.01 mg Cd L^{-1} was 100%. Increased time of exposure did not show an effect on the percent survival in HW after 14 days exposure (95 ± 0.25%) at 0.005 mg Cd L^{-1} and 95 ± 0.25% at 0.01 mg Cd L^{-1} (Fig. 6.1A). In the group of animals in SW, the percent of survival was less than in the group of animals in

HW, it was 95 ± 0.25% at 0.005 mg Cd L^{-1} and 90 ± 0.29% at 0.01 mg Cd L^{-1}, $p > 0.05$ after a week. Figure (6.1B) shows a high concentration (0.01 mg Cd L^{-1}) in SW led to decline the percent survival to 75 ± 0.25% at concentration 0.005 mg Cd L^{-1} as well as at 0.01 mg Cd L^{-1} (75 ± 0.95%) after 2 weeks.

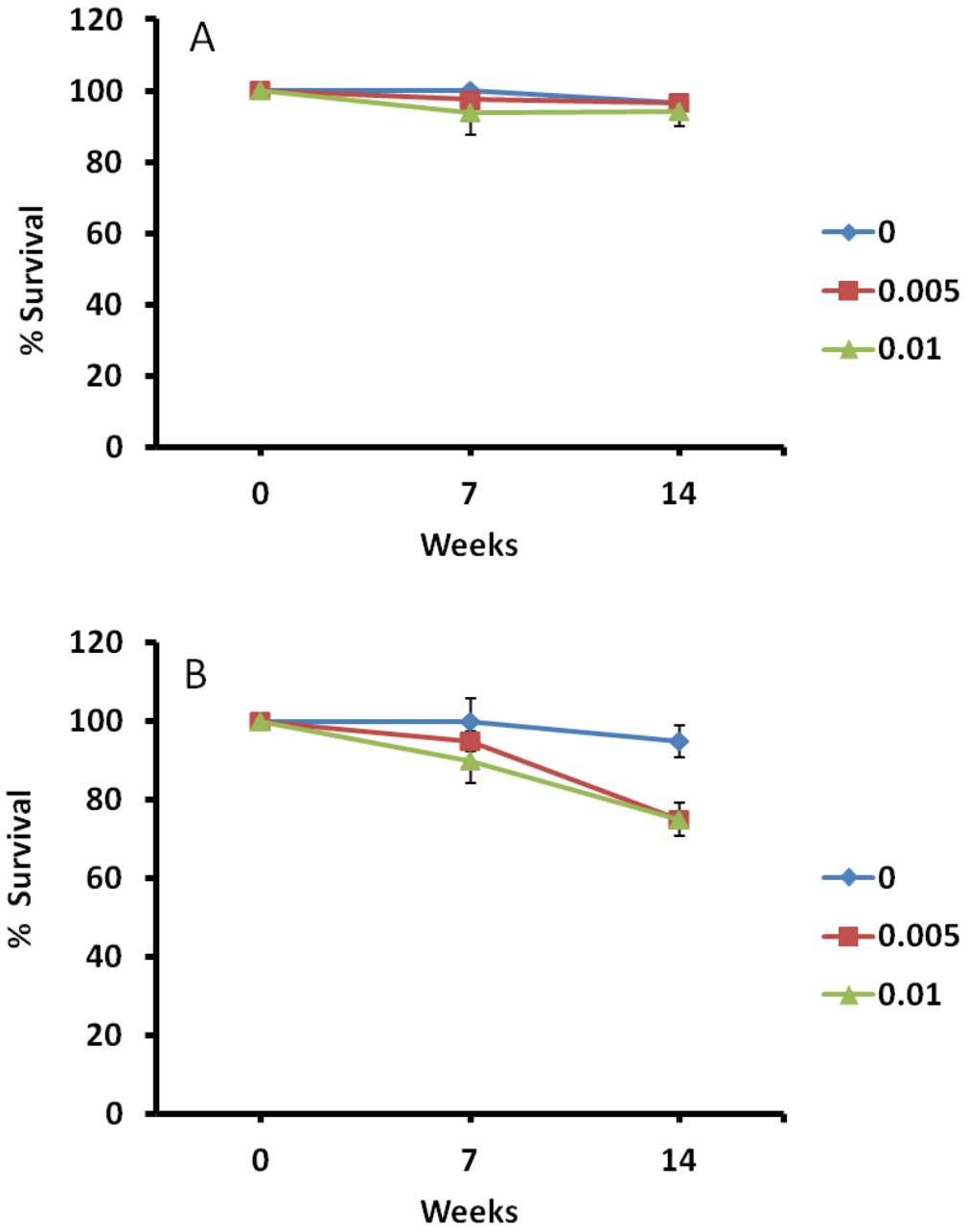

Figure 6.2 The percent of survival in, hard water (A) and soft water (B) after 7 and 14 days exposure to Cd, compared to time zero (Mean ± SEM; n = 45).

6.3.2 Metallothionein

The results show that the mean values of MT increased with the time of exposure in the group of amphipods nonexposed and exposed for 2 weeks. It elevated gradually (322.06 ± 47.28 µg MT/g w wt) at 0.005 mg Cd L^{-1} after 7 days to 373.54 ± 10.9 µg MT/g w wt ($p < 0.01$) after 14 days. While, the level of MT reached the highest concentration of 408.72 ± 13.72 µg MT/g w wt ($p < 0.001$) after 14 days of exposure to 0.01 mg Cd L^{-1} (Fig 6.3A).

As shown in Fig 6.3B, in soft water, MT increased (315.92 ± 30.0 µg MT/g w wt) in the whole body of amphipods exposed to 0.01 mg Cd L^{-1} after 7 days, and raised significantly 322.71 ± 6.3 µg MT/g w wt ($p < 0.05$) after 14 days. At the same time, the level of MT in nonexposed and exposed 0.005 mg Cd L^{-1} was higher than in the amphipods exposed to 0.01 mg Cd L^{-1}, after 14 days.

6.3.3 Na^+/K^+-ATPase activity

6.3.3.1 Gills and hepatopancreas of *G. pulex* in hard water

In the gills, Na^+/K^+-ATPase activity was inhibited significantly 0.51 ± 0.01 µmol Pi mg $protein^{-1}$ h^{-1}, ($p < 0.001$); at 0.005 mg Cd L^{-1}, reached 0.57 ± 0.01 µmol Pi mg $protein^{-1}$ h^{-1} after 14 days. While, the concentration 0.01 mg Cd L^{-1} led to reduce the enzyme activity significantly 0.41 ± 0.003 µmol Pi mg $protein^{-1}$ h^{-1} ($p < 0.001$), after 7 days, then elevated 0.56 ± 0.01 µmol Pi mg $protein^{-1}$ h^{-1} after 14 days (Fig 6.4A).

In hepatopancreas, the enzyme activity was less than 0.40 µmol Pi mg $protein^{-1}$ h^{-1} in unexposed and exposed to Cd after a week of exposure then increased twice ($p < 0.001$) after 14 days (Fig 6.4C).

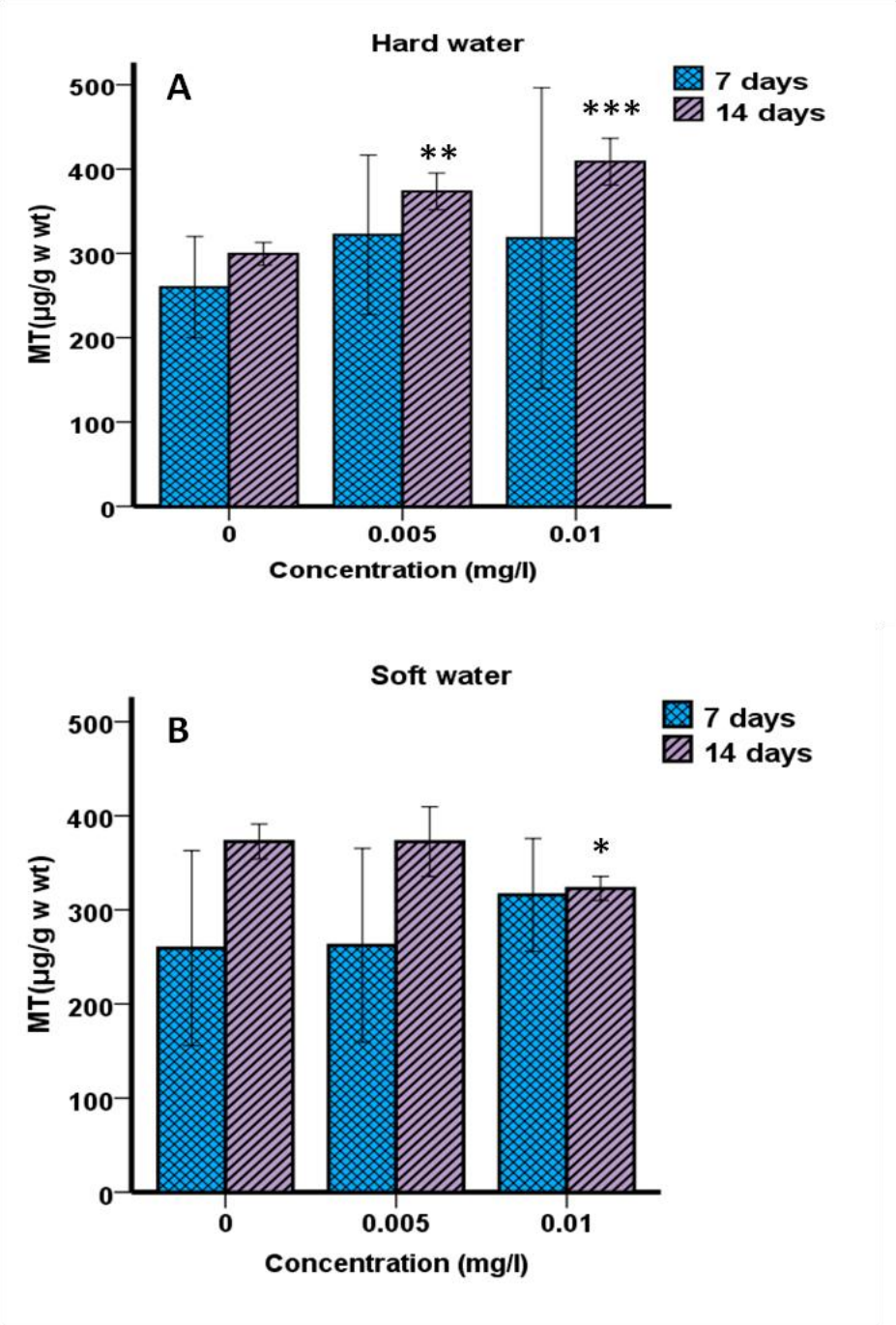

Figure 6.3 Metallothionein in the exposed animals in hard and soft water days 7 days (A) and 14 days (B) exposure to Cd (each point represents the mean ± SEM, n=6, ANOVA and Scheffe post hoc, *p< 0.05; **p < 0.01; ***p < 0.001).

6.3.3.2 Gills and hepatopancreas of *G. pulex* in soft water.

The activity of the enzyme in the gills and hepatopancreas was less than 0.30 µmol Pi mg protein^{-1} h^{-1} in unexposed and exposed to Cd. As shown (Fig 6.4B), the rate of enzyme activity reached 0.28 ± 0.01 µmol Pi mg protein^{-1} h^{-1}; ($p < 0.001$) at 0.01 mg Cd L^{-1} after 7 days to raise slightly 0.29 ± 0.003 µ mol Pi mg protein^{-1} h^{-1}; ($p < 0.001$), after 14 days.

The Na^+/K^+-ATPase activity of hepatopancreas was similar to the gills. It was 0.27 ± 0.002 µmol Pi mg protein^{-1} h^{-1} ($p < 0.001$) at 0.01 mg Cd L^{-1}; after 7 days. Following 14 days, the activity of enzyme increased 0.29 ± 0.002 µmol Pi mg protein^{-1} h^{-1} ($p < 0.05$) (Fig 6.4D).

6.3.4 Haemolymph ion concentrations

6.3.4.1 Haemolymph Cl^- and Na^+

The results demonstrate that haemolymph Cl^- and Na^+ increased with rising Cd concentrations, in the group of amphipod acclimated in hard water for 7 and 14 days. The concentration 0.01 mg Cd L^{-1} (hard water) led to increase the haemolymph Cl^- and Na^+ to 115.92 ± 0.02 mmol/l ($p < 0.001$) and 172.54 ± 0.49 mmol/l ($p < 0.001$), respectively after 14 days (Fig 6.5A and Fig 6.5C).

The low level of ions in soft water resulted in decreased haemolymph Cl^- and Na^+ in unexposed and exposed to Cd after 7 and 14 days. The highest concentration (0.01 mg Cd L^{-1}) reduced haemolymph Cl^- 25.22 ± 0.53 mmol/l ($p < 0.001$) and Na^+ to less than a 60 mmol/l, after 14 days (Fig. 6.5B and Fig. 6.5D).

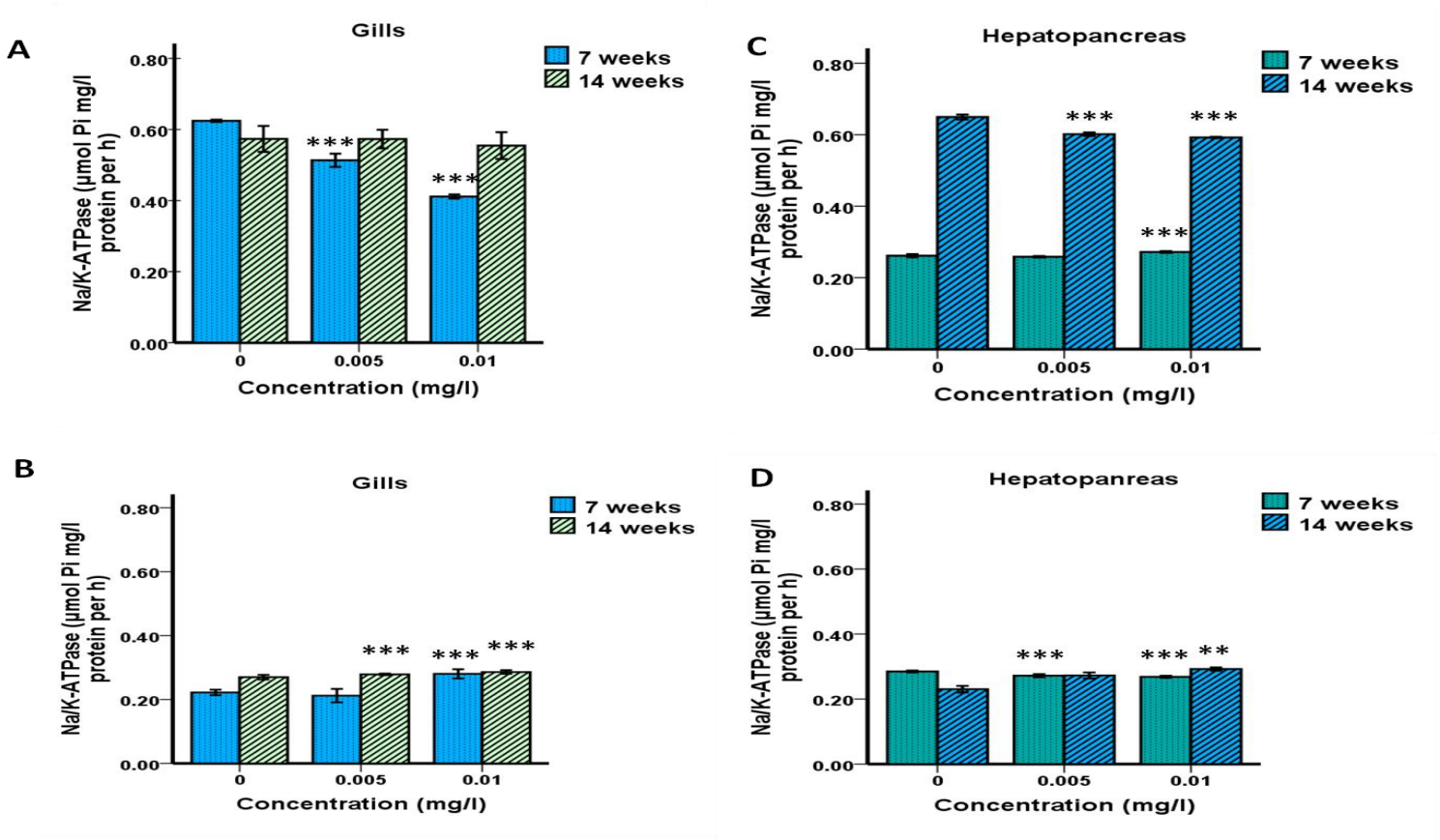

Figure 6.4 Na^+/K^+-ATPase, in gills of adults *G. pulex* exposed to Cd for 7 and 14 days in hard water (A) and soft water (B). Na^+/K^+-ATPase, in hepatopancreas of adults *G. pulex* exposed to Cd for 7 and 14 days in hard (C) and soft water (D) (each point represents the mean ± SEM, n= 8, ANOVA and Scheffe post hoc, ***p < 0.001, **p < 0.01).

6.3.4.2 Haemolymph Ca^{2+} and Mg^{2+}

In hard water; the level of haemolymph Ca^{2+} was higher in exposed to Cd than in control. The concentration 0.01 mg Cd L^{-1} resulted in increased haemolymph Ca^{2+} 22.45 ± 1.6 mmol/l ($p < 0.01$) after 7 days Cd exposure. Subsequently, it decreased slightly 18.45 ± 0.45 mmol/l ($p < 0.01$), after 2 weeks (Fig 6.6A). In addition, haemolymph Mg^{2+} in the control group and exposed to Cd was lower than 7 mmol/l at day 7. Increase time exposure after 14 days led to increase haemolymph Mg^{2+} 9.9 ± 0.28 mmol/l ($p < 0.001$) at 0.005 mg Cd L^{-1} and 9.0 ± 0.22 mmol/l ($p < 0.001$) at 0.01 mg Cd L^{-1}, after 2 weeks (Fig 6.6C).

In soft water; haemolymph Ca^{2+} reduced sharply after a week of exposure to 0.005 and 0.01 mg Cd L^{-1} ($p < 0.001$) then, it returned to increase significantly $p < 0.01$ (Fig 6.6B). Haemolymph Mg^{2+} was observed similar to Ca^{2+}, it reduced with increasing Cd concentrations, after 7 days of exposure, to rise twice after 2 weeks (Fig 6.6D).

6.3.4.3 Haemolymph K^{+}

In hard water, the highest haemolymph K^{+} was 44.06 ± 0.24 mmol/l ($p < 0.001$), after 14 days exposure to 0.005 mg Cd L^{-1} (Fig 6.7A). While, the group exposed to 0.01 mg Cd L^{-1} reduced 34.3 ± 0.19 mmol/l ($p < 0.001$) after 14 days of exposure. In soft water; haemolymph K^{+} reduced sharply with rising Cd concentration and then returned to increase significantly ($p < 0.001$) in the amphipod exposed to Cd (Fig 6.7B). Similarity, haemolymph Ca^{2+} and Mg^{2+}.

6.3.5 Haemolymph osmolality

Figure (6.8A) show that haemolymph osmolality increased significantly 65.33 ± 1.76 mOsm/kg ($p < 0.001$) at 0.005 mg Cd L^{-1}, and 65.67 ± 1.2 mOsm/kg at 0.01 mg Cd L^{-1}, in the group of amphipods acclimated in hard water for 14 days. In soft water; the haemolymph osmolality of the amphipods *G. pulex* tended to decline with increasing Cd concentrations. Subsequently, it recovered after 2 weeks (Fig 6.8B), the results show that the haemolymph osmolality was similar to trend haemolymph ions (Ca^{2+}, Mg^{2+} and K^{+}).

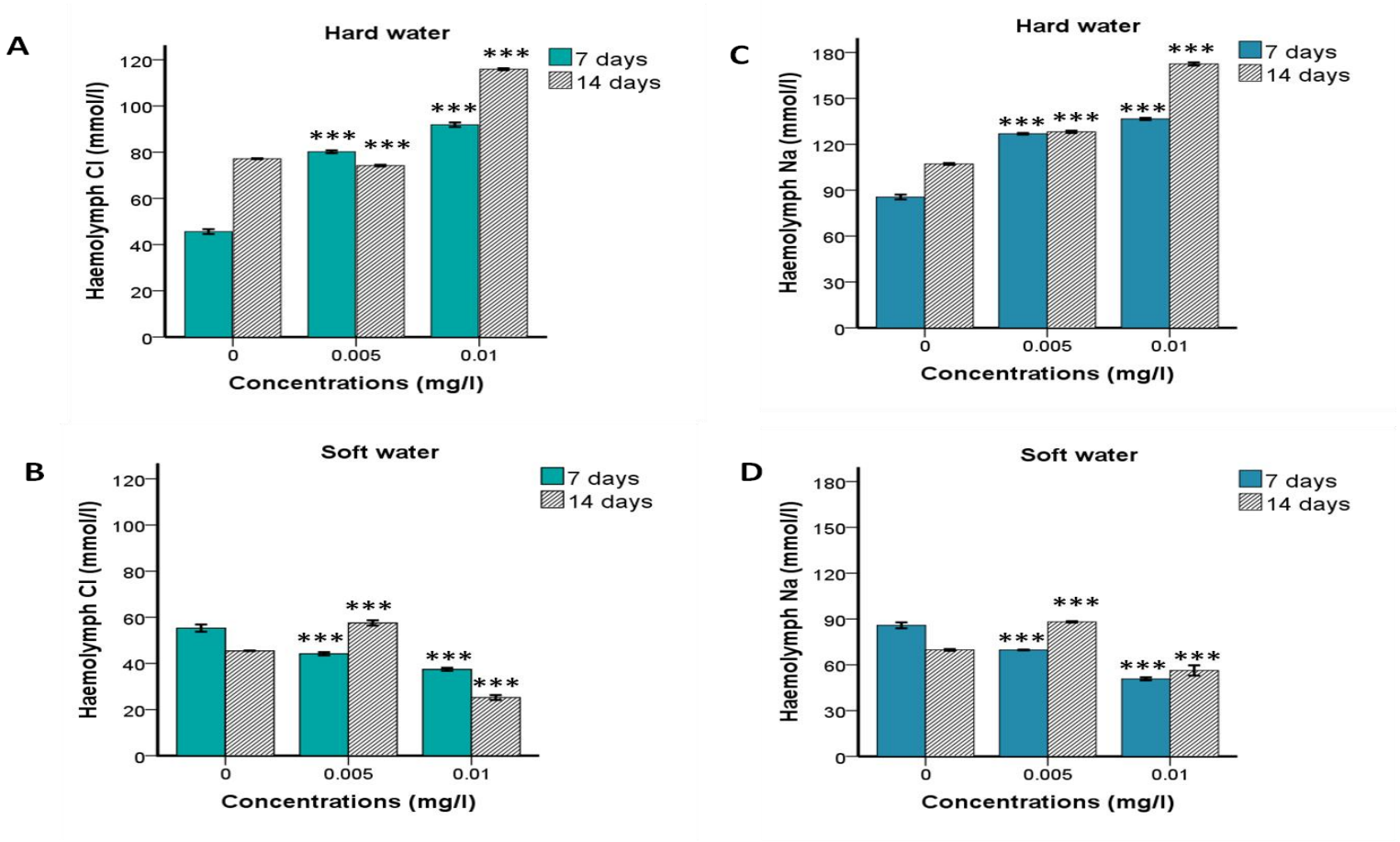

Figure 6.5 Haemolymph Cl^- ion concentrations of *G. pulex* exposed to Cd in hard (A) and soft water (B). Haemolymph Na^+ ion concentrations in hard (C) and soft water (D), after 7 and 14 days (each point represents the mean ± SEM, n=8, ANOVA and Scheffe post hoc, ***p < 0.001).

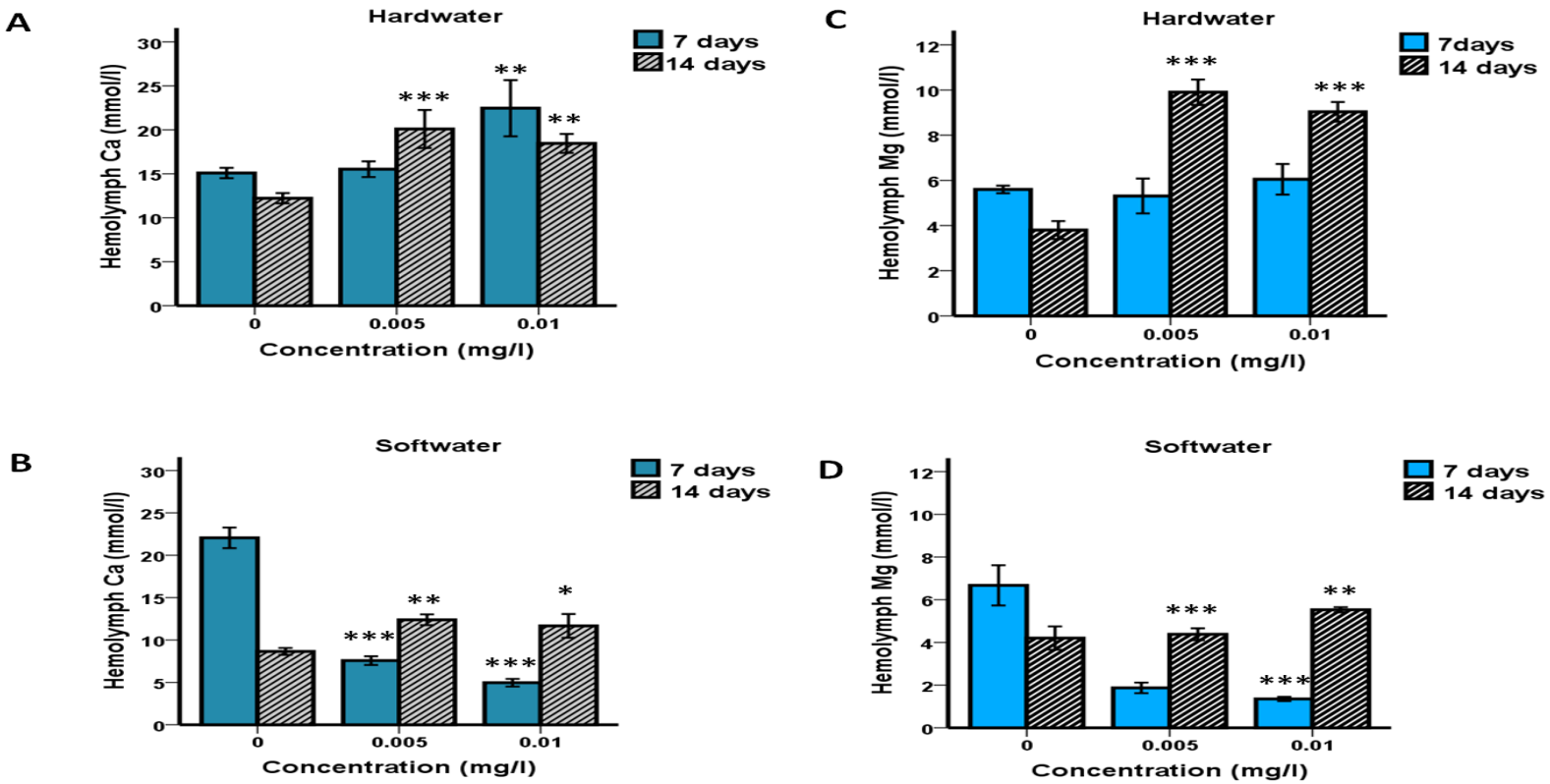

Figure 6.6 Haemolymph Ca^{2+} ion concentrations of *G. pulex* exposed to Cd in hard (A) and soft water (B). Haemolymph Mg^{2+} ion concentrations of *G. pulex* exposed to Cd in hard water (C) and soft water (D) after 7 and 14 days (each point represents the mean ± SEM, n=8, ANOVA and Scheffe post hoc, *p < 0.05; **p < 0.01; ***p < 0.001).

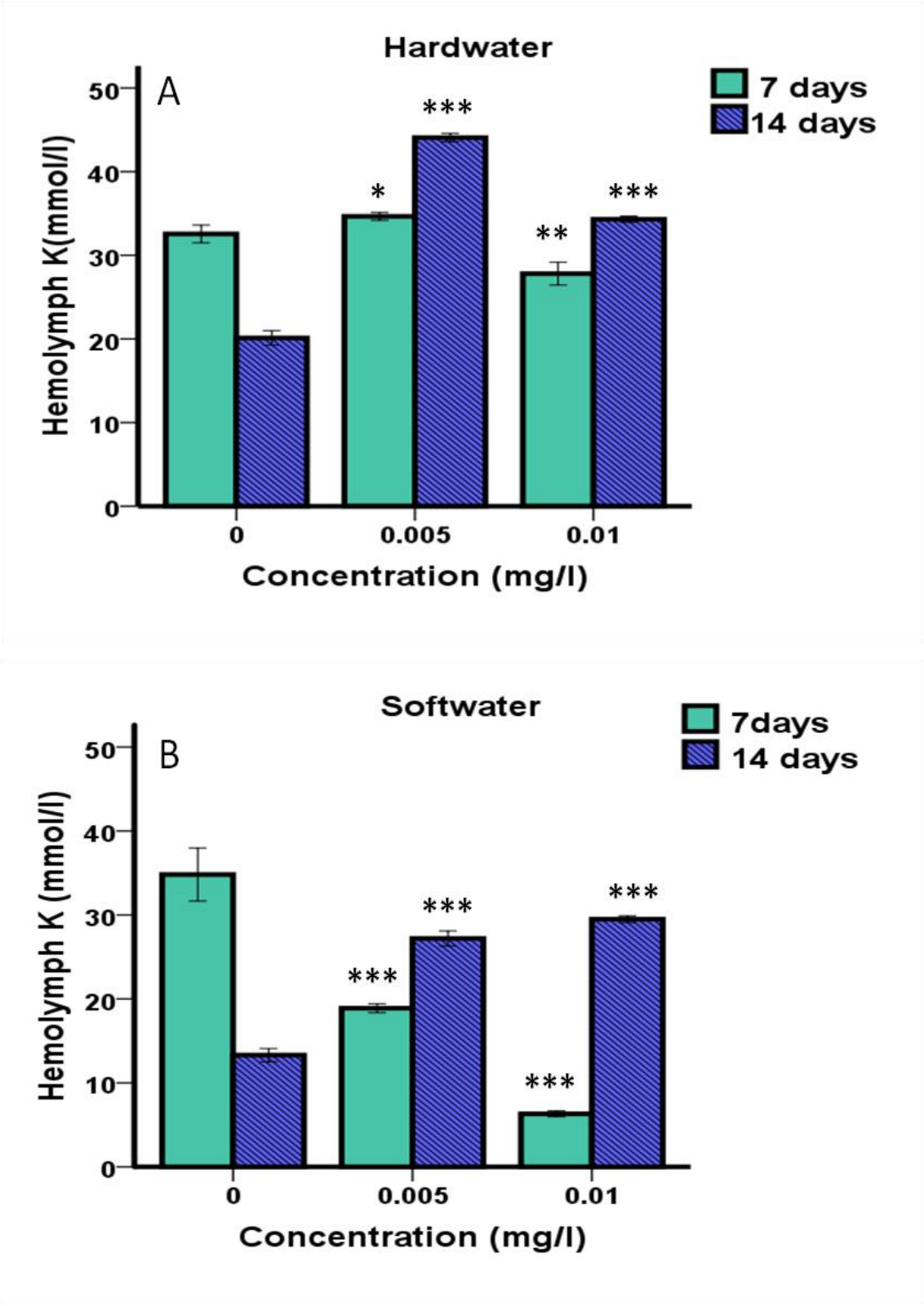

Figure 6.7 Haemolymph K^+ ion concentrations of *G. pulex* exposed to Cd in hard water (A) and soft water (B) after 7 and 14 days (each point represents the mean ± SEM, n=8, ANOVA and Scheffe post hoc, *p < 0.05; **p < 0.01; ***p < 0.001).

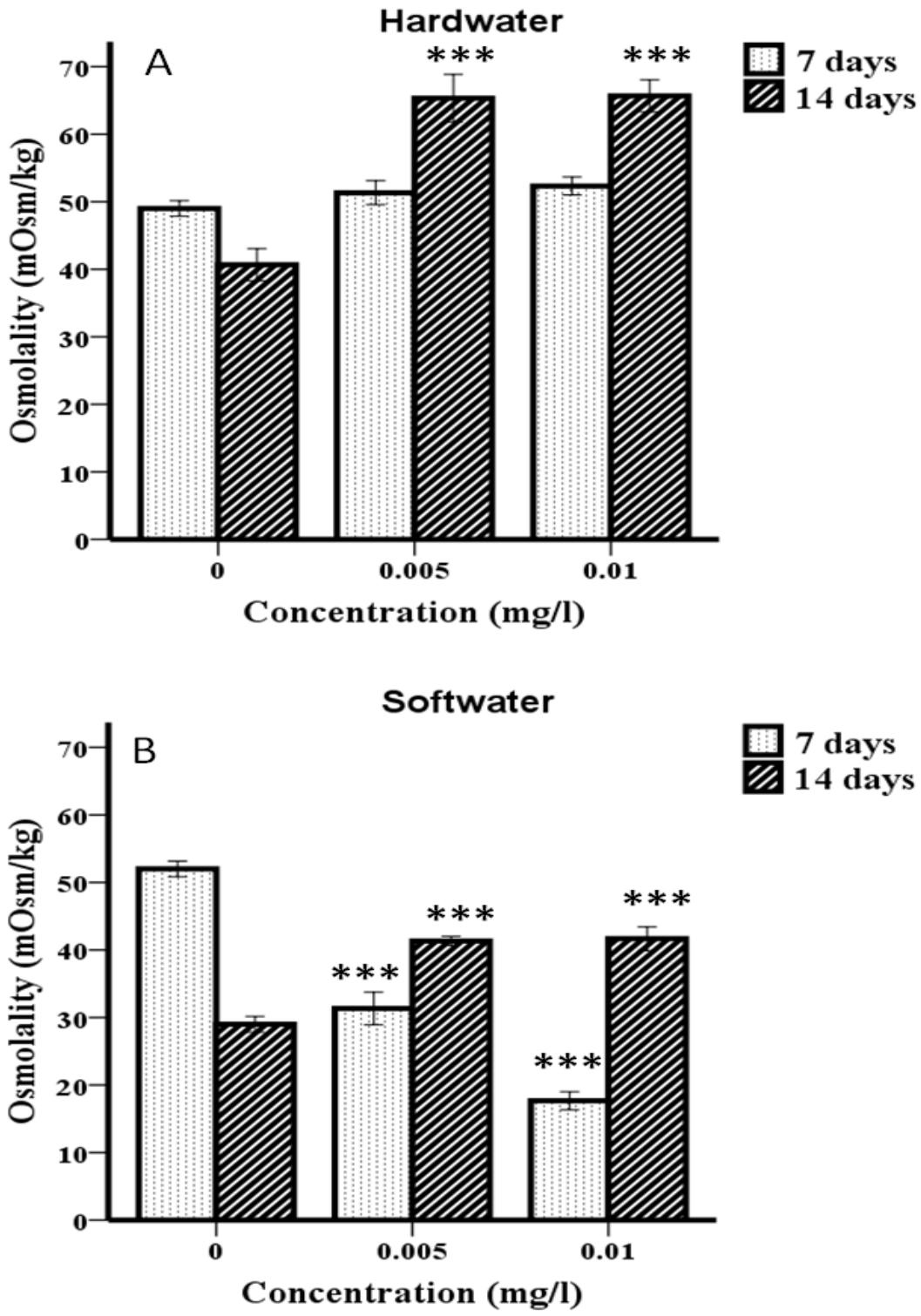

Figure 6.8 Haemolymph osmolality (mOsm/kg) of *G. pulex* exposed to Cd in hard water (A) and soft water (B) for 7 and 14 days (each point represents the mean ± SEM, ANOVA and Scheffe post hoc, ***p < 0.001).

6.4 Discussion

6.4.1 Survival

The results show that hard water is a contributing factor to inhibiting Cd toxicity and results in an increase in the percent of survival above 94% in the freshwater *G. pulex* exposed to Cd for 7 and 14 days. It was also associated with increasing MT in the group of animals in hard water. Many authors reported that the toxicity of metals is related to physico-chemical features of aquatic systems such as salinity, pH, temperature and hardness (Rainbow and Dallinger., 1993; Jackson et al., 2000; Saglam et al., 2013). For example, abundance of Ca and Mg in hard water are also contributing elements to inhibiting Cd toxicity (Carroll et al., 1979; Rathore and Khangarot., 2003). It was found that the percentage of survivorship of *Daphnia magna* increased with increasing water hardness from 57 ppm to 115 ppm as $CaCO_3$ when exposed to 15 μg Cd L^{-1} for 40 days (Winner and Gauss., 1986).

In the present study, the results suggest that the amphipods have acclimated to soft water, because the percent of survival was as high as 75% after 14 days equally at concentrations of 0.005 and 0.01 mg Cd L^{-1}. The soft water (SW) used in these experiments has low Ca. In SW, Cd accumulated in the amphipods and led to disordered physiological activities, potentially because, Cd competes with Ca on the same binding sites on the active Ca regulatory mechanism (Carroll et al., 1979; Wright, 1979). In addition to that the concentration of Ca in the amphipods depends on the status of the animal during the molting cycle. For example, post-molt *G. pulex* were more sensitive to Cd in the than intermolt (Wright, 1977; Wright and Frain., 1981). It was found

that Cd accumulation in the whole body in excess of the storage capacity led to reduced rates of survival (Rainbow and White, 1989; de Nicola et al., 1993).

In the present study, Cd did not kill all tested animals in soft water after 7 and 14 days. Binding Cd protein (MT) may reduce Cd toxicity. Previous studies showed that soft water resulted in toxicity for many kinds of aquatic species, although many of these studies used very high concentrations of Cd. Cadmium killed all the amphipod *G. fossarum* in less than 12 days when exposed to 1 mg Cd L^{-1} (Abel and Bärlocher, 1988), the juveniles *Tilapia sparrmanii* exposed to 20 mg Cd L^{-1} for 96 h (Van Aardt and Booysen., 2004) and the freshwater fish *Oreochromis niloticus* died when exposed to Cu for 12 days (Saglam et al., 2013). Bioavailability of Cd is clearly higher in soft water than in hard water (Van Aardt and Booysen., 2004).

In addition to the possibility of competition with Ca pumps, many authors pointed out that Cd accumulation induced Cd-bound protein (MT) in the amphipods to detoxify Cd to a less toxic form (Stuhlbacher and Maltby., 1992; Fraysse et al., 2006).

6.4.2 Metallothionein

In the present study, MT increased in the whole body of *G. pulex* exposed to Cd for 14 days in hard water at both 0.005 and 0.01 mg Cd L^{-1}, but this was not seen in SW, where MT concentrations were much more variable. It should be noted that the method of measurement of MT used here included both MT and GSH and hence values will be high interpretation of the results modified accordingly. Pavicic et al (1994) described that the tolerance of embryo-larvae *Mytilus galloprovincialis* to 2.75 µg Cd ml^{-1} resulting to a competitive interaction

for metal – binding sites on MT. Stuhlbacher and Maltby (1992) established that pre exposure of the amphipod *G. pulex* to low concentrations of Cd (500 µg Cd L^{-1}) for 48 h induced MT to increase resistance and tolerance. Previous studies observed a strong relationship between Cd concentrations in water and MT content in a wide range of aquatic organisms. For example in the hepatopancreas of the freshwater crayfish *Procambarus clarkii* after 12 h exposure to 10 mg Cd L^{-1} (Martinez et al., 1993), the brine shrimp *Artemia parthenogenetica* and the freshwater crayfish *Procambarus clarkii* hepatopancreas following 48 h exposure to 5, 10 and 20 mg Cd L^{-1} (Del Ramo et al., 1995). It was also found a strong relationship between Cd accumulation and MT induction in the gills and hepatopancreas of the freshwater crab *Sinopotamon henanense* when exposed to different Cd concentration (5.8–23.2 mg Cd L^{-1}) for 5 days (Ma et al., 2008). It is likely therefore that the increased MT in the whole body that occurred here after 14 days occurred as a mechanism of defence against Cd toxicity. Although, *G. pulex* has the ability to accumulate Cd rapidly from ambient water with increasing Cd concentrations, it could reduce it to less toxic form by binding with the sulfhydryl group (–SH) of the polypeptides in MT (Lionetto et al., 1998; Klaassen et al., 2009). Binding Cd to protein (MT) represents a sequestration process to prevent interaction with other proteins as protection against Cd toxicity at the cellular level (Roesigadi, 1992; Fraysse et al., 2006).

The level of MT also elevates as a response to excess oxidative stress, in isolated digestive cells gland and entire body of *Mytilus galloprovincialis* exposed to 200 µg Cd L^{-1} for 7 days (Viarengo et al., 1999). As observed in the present study (Chapter 2) Cd induced increased lipid peroxidation after 120 h with increasing concentrations. Several studies have pointed out that the

concentration of MT is also linked to the physiological condition of the organism, reproductive status, sex, weight, growth rate, and season (Correia et al., 2003; Geffard et al., 2007; Henry et al., 2012). Correia et al. (2002a) interpreted the variability of MT levels after exposure the amphipods *G. locusta* to 4 µg Cu L^{-1} to the fact that he had utilised the whole body tissue to extract his samples and his organisms were all at different stages in the moulting cycle.

6.4.3 Na^+/K^+-ATPase activity

The results presented here showed variable effects of Cd depending on the hardness of the water and duration of exposure. In HW, initial decreases in gill and hepatopancreas Na^+/K^+-ATPase activity had increased again after 14 weeks, suggesting a degree of adaptation. In SW, the situation was less clear. Activity was lower in all cases than in HW, with both increases and decreases in activity.

Gills of aquatic crustaceans are frequently the first targets of pollutants, as they are in direct contact with ambient water (Marsden and Rainbow, 2004; Felten et al., 2008a). The epithelial tissue of gills play an important role for active transport and osmoregulation in most crustaceans through Na^+/K^+-ATPase (Dhavale et al., 1988; Ahearn et al., 1999; Lucu and Towle, 2003). The ATPase enzymes maintain Na^+ and K^+ gradients, regulate cell volume, osmotic pressure and membrane permeability between cells and the surrounding environment (Li et al., 2012; Saglam et al., 2013). Lionetto et al. (1998, and 2000) found two different isoenzymes, causing the enzyme activity of gills to be more sensitive to Cd than in the intestinal enzyme of European eel *A. anguilla* (in vitro) exposed to 0.5–50 µM $CdCl_2$ for an hour incubation.

Cadmium has a high affinity for sulfhydryl groups and hence can by interfere with enzyme-ATP complexes that contain these, leading to inhibition of Na^+/K^+-ATPase activity (Viarengo, 1985; Dhavale et al., 1988; Brooks and Mills., 2003). Reduction in Na^+/K^+-ATPase activity was observed in the gills and hepatopancreas of crab *Scylla serrata* after exposure to a range of Cd concentrations (0.3–1.5 mg Cd L^{-1}) for 10 days, related to changes in the ionic concentrations in the blood and tissue damage (Dhavale et al., 1988). Na^+/K^+-ATPase activity reduced in the gill tissues of the freshwater crab *Sinoptamon yangtsekiense* after exposure to 58 and 87 mg Cd L^{-1} for 96 h which coincided with increased malonyldialdehyde (Li et al., 2012). This is similar to the results seen here in Chapter 2. Many heavy metals act by inhibiting enzyme activities for osmoregulation in aquatic organisms (Saglam et al., 2013). For example, silver inhibited Na^+/K^+-ATPase activity of rainbow trout acclimated to SW (Bury et al., 1999), lead nitrate (245 µg/l) reduced Na^+/K^+-ATPase activity in the freshwater mussel *Elliptio complanata* after 28 days exposure, associated with increasing haemolymph Ca^{2+} concentration (Mosher, et al., 2010).

After 14 days, the enzyme activity started increasing in the gills and hepatopancreas to higher than 0.50 µmol Pi mg $protein^{-1}$ h^{-1} in the animals from HW, as a mechanism to compensate elements by increasing surface permeability (Lin et al., 1993; Lingot et al., 2000). Felten, et al. (2008a) and Saglam et al, (2013) suggested that increase Na^+/K^+-ATPase activity as a mechanism to compensate linked to disturbed ion regulations, in order to recover ions lost after exposure of the amphipod *G. pulex* and the freshwater fish *Oreochromis niloticus* to different Cd concentrations. Exposing the Chinese mitten crab *Eriocheir sinensis* to acute Cd concentrations decreased

Na^+/K^+-ATPase activity after 3 days, which is thought to be the mechanism of osmoregulation recovery after transporting the crabs to low Cd concentrations for a month, as acclimation to low metal contamination (Silvestre et al., 2005).

The activity of Na^+/K^+-ATPase was lower in the gills of the amphipods from SW than in the animals from HW. Low Na^+ and Cl^- in SW associated with impairing Na^+/K^+-ATPase activity in the gills and hepatopancreas after 7 and 14 days, in control and exposed to Cd. It might be a mechanism to reduce energy expenditure in osmoregulation to tolerant hypoosmotic environments (Brooks and Mills, 2006). Reducing ventilation rate in *G. pulex* was a method to redirect energy for ion regulation after exposure to Cd (Felten et al., 2008a; Vellinger et al., 2012c). Na^+/K^+-ATPase activity in the intestine of the European eel *A. anguilla* resulted in breakdown of the active transport mechanism for sodium dependent absorption of nutrients and metabolites when exposed to Cd range from 3.15×10^{-8} – 3.24×10^{-6} M (Lionetto et al., 2000). Decrease enzyme activity was related to increase Cd accumulation in the tissue of the gills and hepatopancreas which led to tissue damaged (Saglam et al., 2013).

In the present study (see, Chapter 4) it was also found that sublethal concentrations of Cd (0.005 and 0.01 mg Cd L^{-1}) damaged the tissue and cell organelles of the gills of *G. pulex*, especially mitochondria through lysis of the outer and inner membrane. The damage disturbs a site of energy needed for osmoregulation and respiration and coincided with a decrease in the ventilation rate. Issartel et al. (2010) found that Cd (15 µg Cd L^{-1}) for 3 and 7 days damaged gill structures of the amphipod *G. fossarum* associated with decreasing intensity of Na^+/K^+-ATPase fluorescence.

Cadmium leads to an increase in membrane permeability and ion efflux with increasing lipid peroxidation (Bertin and Averbeck, 2006). It was also noticed in the present study (Chapter 2) that lipid peroxidation increased with increasing Cd exposure to 0.005 and 0.01 mg Cd L^{-1}.

6.4.4 Haemolymph ions

The results indicate that HW increased haemolymph Cl^- and Na^+ ions significantly during exposure to Cd (0.005 and 0.01 mg Cd L^{-1}) after 7 and 14 days, while, SW led to reduce both ions in the haemolymph. Decrease blood plasma chlorides were also observed in the freshwater fish *Tilapia sparrmanii* exposed to 20 mg Cd L^{-1} for 96 h in SW (Van Aardt and Booysen., 2004). In the present, Cd reduced haemolymph Na^+, Cl^-, Ca^{2+}, Mg^{2+} and K^+ after a week of exposure, but, the animals recovered the ions lost after 14 days, as a kind of acclimation in low ions (soft water) to survive. That means the animals can survive in dilute media throughout rainy seasons in streams and lakes. Sutcliffe. (1967) reported that a decline in the blood Na concentration in the freshwater *G. pulex* leads to activating an uptake mechanism at the body surface and the antennary glands and producing more dilute urine. Vincent (1963 and 1969) reported that organisms able to live in ambient water in low Ca^{2+} concentration (1.5–1.8 mg Ca L^{-1}). By altering electrochemical gradient, permeability and active transport to facilitate uptake Ca from aquatic environment or acting on shifting Ca from exoskeleton to the blood or gastrointestinal tract before moulting (Neuffld and Cameron, 1993).

6.4.5 Haemolymph osmolality

Exposure to sublethal concentrations increased haemolymph osmolality in the group of the amphipods in HW associated with increasing haemolymph Na^+ and Cl^-. While, the amphipods acclimated to SW, osmolality reduced sharply in the amphipods exposed to Cd to recover after 2 weeks. Several studies reported that Cd reduced osmolality the freshwater amphipods *G. pulex* when exposed to 7.5 and 15 µg Cd L^{-1} for 120 and 168 h (Felten et al., 2008a), and the Chinese mitten crab, *Eriocheir sinensis* exposed to 500 µg Cd L^{-1} for 72 h (Silvestre et al., 2005). The low level of free amino acids led to reduce osmolality the amphipod *G. pulex* when exposed to 14 µg Cd L^{-1} for 2 days (Xu, 1995).

In conclusion, the present data demonstrated that the percent of survival, Na^+/K^+-ATPase activity and haemolymph ions concentration depend on water chemistry and time of exposure. The results of this study were highly variable, but one consistent fact was that the survival of the animals was close to 100% in all test conditions in hard water, but dropped to less than 75% in soft water, despite this concentration being within the water quality limits of Cd in the environment, These results illustrate the complex task in understanding the biological effects of Cd to freshwater organisms and how to use this information to set environmental quality standards in changing and variable water quality conditions.

Chapter 7 General discussion

Increase anthropogenic activities leads to the release of pollutants such as Cd to the aquatic environment. This work has investigated the biological effects of Cd on the amphipod *G. pulex* through a series of experiments under laboratory conditions. These studies have shown the following:

Hypothesis 1: Cadmium will induce biological effects, including effects on lipid peroxidation, DNA damage and behaviour at lethal and sublethal concentrations

Exposure to sublethal Cd concentrations induced oxidative damage in whole tissues of animals, resulting in increased lipid peroxidation and DNA damage to haemolymph cells, associated with increase Cd accumulation in the whole body of *G. pulex* with rising Cd concentrations. This means that the animal was unable to regulate Cd, or eliminate it rapidly (**Chapter 2**). Transport of the metal into the food chain in this way could raise the level of risk to other organisms; in particular, the amphipod *G. pulex* is a source of food for many species in the aquatic environment. In addition to that, unconditioned oak leaves used as a source of food also accumulated higher amount of Cd than in the amphipod *G. pulex.* Transport of contaminated food to the alimentary canal led to damage to tissue and cell structures associated with feeding. Since these animals play a major role in decomposing leaf litter in aquatic bodies, this may lead to a decline in the number of these species.

Behavioural studies showed that (**Chapter 3**), the males are more tolerant to the sublethal effects of Cd concentrations than females. This may be because

the difference in size between males and females and their age could create variation in feeding rate. There was a reduction of horizontal movement associated with hypoventilation in males and females. The uptake of Cd and its accumulation in the gills are contributing factors to disordered ventilation rate and locomotion activities. The inclusion of behavioural studies in this study has allowed a link to be made between cellular and chemical effects and toxic impacts of Cd on the organisms in aquatic bodies.

The results from behavioural toxicity data can be used as signal to detect the hazardous materials in aquatic environment and effects on survival, health and fitness of organisms. Changes in the behaviour of the amphipods may lead to diminished ability to survive, grow and reproduce. That affects on the natural population in aquatic environments.

Hypothesis 2: Cadmium at sublethal concentrations below the environmental quality standard will affect cell and tissue structures

Histological study illustrates (**Chapter 4**) that Cd damaged the apical infolding system (AIS) in the epithelial layer under the cuticle which is responsible for transporting ions from the external environment to haemolymph, the ultrastructure of *G. pulex* is maintained by numerous mitochondria which release energy for osmoregulation and respiration. Exposure to Cd caused swelling and lysis of the outer and inner membrane of mitochondria in the gill and hepatopancreas cells. Cadmium destroyed the epithelial layer with increasing vacuolation which disrupted osmoregulation mechanism and reduced the ventilation rate. Contaminated food with Cd is the main pathway to the digestive system of the animal. It caused degeneration of microvilli of the

hepatopancreas that influence the absorption and digestion of food and the feeding rate. The hepatopancreas of crustacea are a site to detoxify heavy metals to less toxic forms in the cell. Programmed cell death was clearly observed in hepatopancreas cells. Scanning electron microscope showed that exposure to Cd had an influence also on the myoepithelium of the hepatopancreas causing swelling and contraction after exposure to Cd. These muscles act on contraction and movement of food during digestion. Cadmium interacted with Ca metabolism which is responsible for extension and contraction of myoepithelial in the hepatopancreas.

The results of histological study showed that gills and hepatopancreas of the freshwater *G. pulex* high response to pollutants and can be used as monitoring organs to assess effect of pollutants on the cells and tissues of aquatic organisms in the field.

Hypothesis 3: Cadmium will induce greater toxicity in soft water than hard water during chronic exposures.

Long term exposure of juvenile *G. pulex* acclimated to hard water to low Cd concentrations, showed that the hard water protected animals completely from the risk of Cd toxicity (**Chapter 5**). While soft water led to a decrease in the percent of survival in exposed and unexposed organisms. The lack of elements in the ambient water consumed meant that the animals most likely expended a lot of energy for osmoregulation to survive in diluted media. For example, heavy rains lead to decrease the amount of dissolved salts in water such as Ca, Mg, Na and Cl by inducing Cd accumulation rapidly. Furthermore, the length and the number of segments (primary antenna) did not show any effect on the rate of growth in SW. Exposure to a high concentration Cd reduced the weight of

juveniles in SW associated with reducing food consumption. Soft water was also a contributing factor to increase Cd accumulation in the body parts of the juveniles in SW. While, Cd accumulation was less in the body parts of juveniles acclimated in HW. The remaining tissues of the juveniles represented a place to accumulate higher amount of Mg^{2+} and Ca^{2+} than haemolymph and hepatopancreas. It seems that Ca concentration in SW was not enough to complete the calcification process rapidly, in addition to vulnerability to cannibalism. Shedding and ingestion of an old exoskeleton after moulting and oak leaves are a source of Ca to animals reared in SW, to compensate for insufficient Ca available from ambient water. In contrast, Ca concentration in HW was sufficient for calcification of exoskeleton rapidly. The weights of juveniles reared in HW increased without observing any difference between unexposed and exposed to Cd. However, the weights of juveniles in SW declined at high concentration. Despite that the juveniles reached a mature stage after 8 weeks.

Long term exposure (chronic) provides us with a guideline for assessing water quality criteria for protecting the early life stage of aquatic organisms. Reduction in the hardness of water is a contributing factor to increase Cd accumulation in the body parts with increasing growth of animals. That means the secondary consumers in the top of the food chain accumulate high amount of water pollutants because, they fed on primary consumers in food chain which already accumulated amount of Cd in their body. Abundance of organic and inorganic ligands in water column and sediment can bind with free metal ion to form complexes which make them less hazardous for aquatic organisms.

Hypothesis 4: Cadmium will alter Na^+/K^+-ATPase activity and metallothionein concentrations and ion regulation differently in hard and soft waters

The Cd - binding protein (MT) is utilised in toxicological studies as a biomarker of metal exposure. Sublethal concentrations of Cd induced MT in the whole body tissue gradually in the amphipods acclimated in HW with increasing Cd concentrations (**Chapter 6**). Whilst in the amphipods acclimated to SW, Cd - binding protein varied in unexposed and exposed to Cd. It is not clear how the role of MT as an antioxidant against Cd toxicity and abundance or lack of Ca contributed to increase or decrease Cd toxicity. Furthermore, HW showed a positive effect on the adult *G. pulex* to tolerate Cd toxicity, in regard to the animal in SW the percent of survival was less.

Sodium potassium pump activity in the gills reduced with increasing Cd concentrations after 7 days of exposure and return to rise after 14 days in HW. In contrast, insufficient elements in SW such as Cl^- and Na^+ cause decreased Na^+/K^+-ATPase activity in the gills and hepatopancreas after 7 and 14 days associated with declining haemolymph Ca^{2+}, Mg^{2+} and K^+ after a week, to recover after 14 days the ions lost. This could represent an adaptation to allow the animals to survive when there are insufficient elements in the surrounding environment. Dilution of urine maintains the percentage of the elements in the body above that found in the ambient water. Gills of the freshwater *G. pulex* are in direct contact to ambient water and can be used as a tool to assess physiological condition during exposure to pollutants. These results provide a better understanding of how a priority pollutant behaves and is detoxified in aquatic environments.

Conclusion

This thesis has considered how a priority pollutant, Cd, affects an ecologically important aquatic organism across the lifecycle and under different environmental conditions. By studying these factors, it was intended that scientific knowledge would be improved about the conditions in natural waters that contribute towards toxicity. A particular feature of this work was to study concentrations of Cd well below the environmental quality standard (EQS) commonly applied to allow for environmental protection of aquatic water bodies.

Sublethal concentrations of Cd decreased the percent of survival with increasing Cd concentrations, in dechlorinated tap water (75 ppm as $CaCO_3$) that leads to increase lipid peroxidation in the whole body tissues, Cd accumulation in whole body and leaves as a source of food and causes DNA damage to haemolymph cells. The sublethal effects of Cd damaged gills and hepatopancreas tissue and cell organelles via ambient water or food. These studies confirmed and extended our understanding of how exposure to Cd through food can cause detrimental effects to the health of the animals.

Water chemistry is a contributing factor to reducing or increasing Cd toxicity in *G. pulex*, hard water (150 ppm as $CaCO_3$) inhibited Cd toxicity in chronic exposure to the juveniles at 0.5 and 1 µg Cd L^{-1} and adults at concentrations (0.005 and 0.01 mg Cd L^{-1}). Any reduction in hardness may accelerate Cd accumulation in the body parts of animals. These results are important in understanding the survival of this species in hard and soft water bodies in the wider environment and may be relevant to predicting the effects of climate change and other anthropogenic stressors. The remaining tissues represented a place to concentrate Ca^{2+} and Mg^{2+} more than in the haemolymph and

hepatopancreas. Distribution of Ca^{2+} and Mg^{2+} in the body parts of juveniles needs more study to understand the role of these elements during growth and effect of contaminants on the sensitive juvenile life stage in the aquatic environment.

Cadmium disordered osmoregulation in the gills and hepatopancreas in HW and inhibited it in SW in exposed and nonexposed to Cd. The role of antioxidant MT in body tissues of *G. pulex* in both media during exposure to Cd was not clear as a defence against Cd toxicity, but is certainly deserving of further study

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