Palladium uptake and its effect on behavioural and molecular markers in the freshwater shrimp *Gammarus pulex*

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A thesis submitted in partial fulfilment of the requirement of Nottingham Trent University for the degree of Doctor of Philosophy

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Declaration
I hereby declare that this Ph.D. thesis has been compiled by me under the supervision of Dr Chris Lloyd Mills and Dr Alan Hargreaves, School of Science and Technology in Nottingham Trent University. This thesis has not been previously submitted for the award of any degree to any other University or Institution.
ABSTRACT
The expanded use of platinum group elements (PGEs), platinum (Pt), palladium (Pd), and rhodium (Rh), in automobile catalysts has led to increased levels of these metals in aquatic environments. However, data regarding acute toxicity and the sublethal effects of these metals on aquatic biota are limited. This study aimed to explore the response of the freshwater amphipod *Gammarus pulex* to PGEs. Investigations included the 96 LC$_{50}$ test and several behavioural (vertical movement and feeding activity) and biochemical (acetylcholinesterase (AChE); osmoregulation; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) reduction; reduced glutathione (GSH); glutathione-S-transferase (GST); thiobarbituric acid reactive substances (TBARS); heat shock proteins (HSPs) and protein phosphorylation) endpoints.

Pd caused a significant reduction in the survival of *G. pulex* with a 96 h LC$_{50}$ of 0.52 mg/L (4.89 µM). Pt or Rh increased the survival of *G. pulex* exposed to Pd. However, this was not associated with reduced Pd uptake.

Exposure for 24 h to 0.5 mg/L Pd (4.69 µM) significantly decreased the vertical movement (64%) and feeding activity (95%) of *G. pulex*. However, lower Pd concentrations ($\geq$0.25 mg/L (2.3 µM)) took 72 h to induce significant inhibition in feeding activity (50%). AChE activity was significantly increased (40%) following 72 h exposure to 0.5 mg/L Pd.

GST activity was significantly inhibited ($\geq$32%) following 72 h exposure to $\leq$0.25 mg/L Pd. The concentration of GSH was significantly increased (22 and 35%) following 72 h exposure 0.1 (0.94 µM) and 0.5 mg/L Pd$^{2+}$, respectively.

Exposure to $\leq$0.1 mg/L Pd caused a significant decrease ($\leq$27%) in MTT reduction and significant increase ($\leq$70%) in HSP60 content in mitochondrial extract (pellet) after 72 and 24 h exposure, respectively. The reactivity of anti-phosphoserine bodies with protein bands corresponding to 245 kDa in the pellet was significantly increased (38%) by exposure of gammarids to 0.5 mg/L Pd. Exposure to 0.1 mg/L Pd significantly increased (32%) the intensity of a 30 kDa anti-phosphothreonine reactive protein band in the post-mitochondrial extract, but higher Pd doses had no effect.

In conclusion, this study provides original data suggesting the mitochondria as a main target of Pd toxicity. Further studies of Pd toxicity, under both laboratory and field conditions, are needed including the effect of long-term exposure to low Pd concentrations.
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I am very grateful to Almighty Allah, most gracious, who in his infinite mercy has guided me to complete this PhD work. May peace and blessings of Allah be upon his prophet Muhammad (peace be upon him).

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Many thanks go to all my friends in Libya and the UK for being great friends. My completion of this thesis would not have been possible without their support.
Dedication

This thesis is lovingly dedicated to

My parents

My wife and children

My aunt

My brothers and sisters

Their support, encouragement, and constant love have sustained me throughout my life.
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<tr>
<td>2D-PAGE</td>
<td>two-dimensional Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AIDA</td>
<td>Advanced Image Data Analyser</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATCh</td>
<td>Acetylthiocholine iodide</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAF</td>
<td>bioaccumulation factor</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>COSHH</td>
<td>Control of Substances Hazardous to Health</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTNB</td>
<td>5, 5 dithiobis 2-nitrobenzoic acid</td>
</tr>
<tr>
<td>DW</td>
<td>Deionised water</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy dispersive X-ray</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSC</td>
<td>γ-glutamyl cysteine synthetase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S transferase</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HCs</td>
<td>Unburnt hydrocarbons</td>
</tr>
<tr>
<td>HPR</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSP60</td>
<td>Heat shock protein60</td>
</tr>
<tr>
<td>HSP70</td>
<td>Heat shock protein70</td>
</tr>
<tr>
<td>HSP90</td>
<td>Heat shock protein90</td>
</tr>
<tr>
<td>HSPs</td>
<td>Heat shock proteins</td>
</tr>
<tr>
<td>ICP-M)</td>
<td>inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively coupled plasma optical emission spectrometry</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDaltons</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>Median lethal concentration</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>mCB</td>
<td>Monochlorobimane</td>
</tr>
<tr>
<td>MDA</td>
<td>Malon dialdehyde</td>
</tr>
<tr>
<td>MFB</td>
<td>Multispecies Freshwater Biomonitor</td>
</tr>
<tr>
<td>MRGs</td>
<td>Metal rich granules</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>MTLP</td>
<td>Metallothionein like proteins</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NO&lt;sub&gt;x&lt;/sub&gt;</td>
<td>Nitrogen oxides</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PBS-EDTA</td>
<td>Phosphate buffer (PBS) plus 1 mM EDTA</td>
</tr>
<tr>
<td>PGEs</td>
<td>Platinum group elements</td>
</tr>
<tr>
<td>PM</td>
<td>Particulate matter</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase type 1</td>
</tr>
<tr>
<td>PP2</td>
<td>protein phosphatase type 2</td>
</tr>
<tr>
<td>ppm</td>
<td>Part per million</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SAM</td>
<td>Scheduled Ancient Monument</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBA</td>
<td>2-Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TCh</td>
<td>thiocholine</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'- Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TEP</td>
<td>1, 1, 3- tetraethoxypropane</td>
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<td>VECs</td>
<td>Vehicle exhaust catalysts</td>
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1 General Introduction

1.1 Platinum group elements and their emission from catalytic converters

The platinum group elements (PGEs) include the rare metals platinum (Pt), rhodium (Rh), palladium (Pd), osmium (Os), ruthenium (Ru) and iridium (Ir). These metals occur in nature at very low concentrations in the lithosphere, approximately 0.005 ppm for Pt; 0.0001 ppm for Rh; 0.015 ppm for Pd; 0.005 ppm for Os; 0.0001 ppm for Ru; and 0.001 ppm for Ir (Hartley, 1991; Greenwood and Earnshaw, 1997). Currently, PGEs are of considerable interest due to their high value and importance in industry. For example, Pd, Pt, and Rh have an important application as catalysts, increasing the efficiency of the production of petroleum and other fuels, and some chemicals from crude oil. Pt is also used in jewellery. Over the last few decades, the price of PGEs has significantly increased due to the expanding use of these metals (Kendall, 2004; Chen et al., 2009; Cowley, 2013). European consumption of Pd for automobile catalytic converters in 1993 was approximately only 8.4% of that in 2002 (Zereini et al., 2004). The worldwide demand for PGEs is expected to continue to increase as a consequence of expansion in the use of these metals in catalytic converters (Hilliard, 2003; Ravindra et al., 2004; Jollie, 2007). In 2013, 87.5 tons of Pt, 195.4 tons of Pd and 22.4 tons of Rh (37%, 72% and 78% of the worldwide demand, respectively) were used in the manufacture of catalytic converters (Cowley, 2013).

The first generation of catalytic converters (two-way), which contained Pt and Pd, was introduced in 1976. This aimed to control the emission of unburnt hydrocarbons (HCs) and carbon monoxide (CO). In 1979, a new generation of the catalytic converter was developed (three-way) by adding Rh to reduce the emission of nitrogen oxides (NOx) as well as HCs and CO. Since 1992, Pd has tended to replace Pt in catalytic
converters, due to Pd being less costly and less sensitive to catalytic poisoning than Pt (Ravindra et al., 2004; Matthey, 2012).

The emission of CO, HCs and NO\textsubscript{x} into the environment has significantly decreased as a result of the increasing use of PGEs in automobile catalysts (Ek et al., 2004; Kendall, 2004). More than 90% of HCs, CO and NO\textsubscript{x} are converted into carbon dioxide (CO\textsubscript{2}), water and nitrogen in modern vehicle exhaust catalysts (VECs) (Barefoot, 1997; Ravindra et al., 2004). Catalytic converters were first used in the USA and Japan, followed by Europe, Australia and parts of Asia in the 1980s, and developing countries, such as Brazil, Mexico, and India, in the 1990s. Since 1993, to comply with European Commission (EC) Stage I limits on emissions of Co, NO\textsubscript{x} and HCs, the use of VECs became mandatory in the UK (Hutchinson and Pearson, 2004). Most modern cars worldwide are now fitted with catalytic converters. It is expected that the use of catalytic converters will expand as a result of the stringent legislation that aims to control vehicle emissions, in most industrial and developing countries (e.g. 'Euro V' standards; European Directive 98/70/EEC of 13 October 1998) (Kendall, 2004; Jollie, 2007).

Whilst PGEs were introduced due to environmental concerns regarding vehicle emissions, the market may be negatively impacted by the growing awareness of the potential adverse effects of the emissions of these metals into the environment. There is accumulating evidence that the concentrations of PGEs have increased in environmental components, such as roadside dust (Lee et al., 2011), airborne particulate matter (Zereini et al., 2012), water bodies (Zereini et al., 1997), soil (Hutchinson et al., 2000) and vegetation (Leśniewska et al., 2004). This increase has led to a growing global concern that catalytic converters themselves may become a major source of pollutants (Ek et al., 2004; Ravindra et al., 2004). Although there are several emission sources of PGEs, catalytic converters are thought to be the major source (Palacios et al., 2000). PGEs that are released from refineries and industry have
limited and well-defined areas of impact, and these sources make only a relatively minor contribution to the contamination found in road dust and sewage sludge (Ek et al., 2004). In addition, a substantial effort is directed towards reducing PGE waste at all stages of mining processing, and reusing it in industry, due to the high cost of PGEs (Ravindra et al., 2004). The production of metals, such as chromium and nickel, has caused their local increases in environmental samples (Niskavaara et al., 2004; Rauch and Morrison, 2008). However, there is a lack in information regarding PGE emission rates from metal production activities (Rauch and Morrison, 2008). Hospitals represent another source of PGE emissions (e.g. via PGE-based pharmaceuticals such as the Pt containing anti-cancer drugs cisplatin and carboplatin). However, this is a minor source of emission and often passes immediately to communal sewage (Kümmerer et al., 1997). Consequently, catalytic converters are considered to be the most important emission source of PGEs (Pt, Pd and Rh) into the environment.

The abrasion and deterioration of the surface of catalytic converters leads to the release of Pt, Pd and Rh into the environment in particulate form, mainly in the (0) oxidation state or as oxides (\(\text{Pt}^{2+}\), \(\text{Pt}^{4+}\), \(\text{Pd}^{2+}\) and \(\text{Rh}^{3+}\)) (Moldovan et al., 2002). Thermal sintering (compacting and forming a solid mass of material by heat at a temperature below the melting point of the main constituent), evaporation and mechanical erosion may cause the emission of PGEs from catalytic converters (Palacios et al., 2000). Part of this material in road dust is bioavailable and can accumulate in organisms (Rauch and Morrison, 1999; Sures et al., 2001; Zimmermann et al., 2004, Zimmermann and Sures, 2004). These metals may enter the food chain through deposition on the environmental components adjacent to roads, such as soil, plants, and water bodies. It is estimated that the global emission of Pt from automobile catalysts is around 1.4 tonnes/year (Kümmerer and Helmers, 1999; Barbante et al., 2001; Ravindra et al., 2004). The PGE emission rate varies widely, depending on the speed of the automobile, type and
age of the catalyst and engine conditions (Artelt et al., 1999a; Ravindra et al., 2004). For instance, laboratory studies that directly collected exhaust emissions reported an emission rate of Pt ranging from 9–124 ng/km (Artelt et al., 1999a). These studies are often performed under optimal conditions with well-maintained engines operating over only a limited range of speeds (Artelt et al., 1999a). In contrast, the mean emission rate of PGEs estimated from analysis of the roadside environment is generally higher (Palacios et al., 2000). These authors reported an emission rate of Pt at 270 ng/km. Zimmermann and Sures (2004) reported that, depending on the age and type of the catalytic converter, an individual car can emit up to 200, 50 and 80 ng/km of Pd, Pt and Rh, respectively.

1.2 PGEs in environmental matrices

Numerous investigations have shown that PGEs released from catalytic converters accumulate in various environmental matrices (König et al., 1992; Zereini et al., 2001a, b; Dubiella-Jackowska et al., 2007). Most available data are for Pt. However, a number of studies have examined Pd and Rh concentrations in environmental compartments (WHO, 2002; Zimmermann et al., 2004).

Depending on the size and the morphology of the emitted PGE-containing particles, they may be deposited on roads, or transferred by the wind and then deposited on other environmental compartments (Zimmermann and Sures, 2004). Related traffic-emitted PGE deposited in road dust can be transported to river ecosystems via runoff. As a result, these pollutants may reach coastal, estuarine and freshwater ecosystems and enter the food chain by accumulation in organisms and sediments (Zimmermann and Sures, 2004; Haus et al., 2007). Therefore, further studies are needed in order to improve knowledge regarding the behaviour and mobility of PGEs and their potential risks to the environment.
1.2.1 PGEs in air and airborne particulate matter

Earlier studies in the USA and Europe reported that, prior to the introduction of catalytic converters, atmospheric PGE concentrations were below the detection limit of the atomic absorption spectroscopy (6 x 10^{-8} and 5 x 10^{-6} µg/m³ for Pd and Pt, respectively) (Johnson et al., 1975). However, later studies have shown elevated PGE concentrations in the air in urban areas (Table 1.1). Alt et al. (1993) studied Pt concentrations in airborne dust in Dortmund (Germany) and reported a Pt concentration range from 0.02 to 5.1 pg /m³. The same study found that the greatest proportion of observed Pt had particle sizes ranging between 0.5 and 8 µm. The presence of PGE-containing particles of this size range may increase the uptake of PGEs via inhalation. This represents an important route of PGE exposure in humans, thus increasing the potential threat to human health (Alsenz et al., 2009). The increase in ambient concentrations of PGE over time was shown by Zereini et al. (2001b), who found that Pt and Rh content in airborne particulate matter in Frankfurt (Germany) had significantly increased (46 and 27-fold, respectively) between 1988 and 1999 (Table 1.1). Alsenz et al. (2009) reported that the median Pd concentration in airborne particulate matter collected from urban areas in Germany was significantly higher than that found in samples collected from areas considered to represent background levels (0.6 and 14.8 pg/m³, respectively, Table 1.1). Similarly, Zereini et al. (2012) found that PGE levels in airborne particulate matter (PM_{2.5}) in rural areas were significantly lower than that in urban settings with Pd concentrations generally higher than that of Pt and Rh. They reported that the average concentrations of Pd, Pt and Rh in rural airborne particulate matter were 2.6, 2 and 0.3 pg/m³, respectively (Zereini et al., 2012). In urban airborne particulate matter, the mean PGE concentrations were 16.1, 9.4 and 1.8 pg/m³ for Pd, Pt and Rh, respectively. The same study also
Table 1.1 Platinum group element content in airborne samples from different locations. Studies are ordered according to the start time of samples collection. (PM10 are particles with aerodynamic diameter less than 10 µM).

<table>
<thead>
<tr>
<th>Place/Country</th>
<th>Year</th>
<th>Site description</th>
<th>Airborne PGE concentration (pg/m³)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>California (USA)</td>
<td>1975</td>
<td></td>
<td>Pt 0.05 &lt; 0.6 Pd 0.06</td>
<td>Johnson et al. (1975)</td>
</tr>
<tr>
<td>Frankfurt (Germany)</td>
<td>1988-1998</td>
<td>Urban areas</td>
<td>3 Pt 1.2 Pd 147 Rh 31.5</td>
<td>Zereini et al. (2001b)</td>
</tr>
<tr>
<td>Dortmund (Germany)</td>
<td>1991 -</td>
<td>Highway Urban areas</td>
<td>0.02-5.1 Pt 7.3 Pd 21.5</td>
<td>Alt et al. (1993)</td>
</tr>
<tr>
<td>Munich (Germany)</td>
<td>1993 - 1994</td>
<td>Urban areas</td>
<td>7.3 Pt 3.3 Pd 21.5</td>
<td>Schierl and Fruhmann, (1996)</td>
</tr>
<tr>
<td>Munich (Germany)</td>
<td>1993 - 1994</td>
<td>Buses and tramway (PM10)</td>
<td>7.3 Pt 3.3 Pd 21.5</td>
<td>Schierl, (2000)</td>
</tr>
<tr>
<td>Berlin (Germany)</td>
<td>1997</td>
<td>Urban areas</td>
<td>0.2-14.6 Pt 5.6 Pd 38.8 Rh 7.8</td>
<td>Tilch et al. (2000)</td>
</tr>
<tr>
<td>Madrid (Spain)</td>
<td>1998 - 1999</td>
<td>75000–100000 vehicles /day</td>
<td>9.18 Pt 3.3 Pd 2.2</td>
<td>Gomez et al. (2001)</td>
</tr>
<tr>
<td>Goteborg (Sweden)</td>
<td>1999</td>
<td>(&lt; 10000 vehicles /day)</td>
<td>2.1 Pt 1.5 Pd 0.6</td>
<td>Rauch et al. (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(PM10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(&gt; 30000-70000 vehicles /day) (PM10)</td>
<td>14.1 Pt 4.9 Pd 2.9</td>
<td></td>
</tr>
<tr>
<td>London (UK)</td>
<td></td>
<td>Downtown and ring-road (100000-100000 vehicles/day)</td>
<td>5.6 Pt 10.5 Pd 2.6</td>
<td>Gomez et al. (2002)</td>
</tr>
<tr>
<td>Munich (Germany)</td>
<td>1998 - 2000</td>
<td>Downtown and ring-road</td>
<td>4.1 Pt 51.4 Pd 3.0</td>
<td></td>
</tr>
<tr>
<td>Götéborg (Sweden)</td>
<td></td>
<td>(10000-100000 vehicles /day)</td>
<td>12.3 Pt 4.1 Pd 3.0</td>
<td></td>
</tr>
<tr>
<td>Madrid (Spain)</td>
<td></td>
<td></td>
<td>15.6 Pt 4.2</td>
<td></td>
</tr>
<tr>
<td>Sheffield (UK)</td>
<td></td>
<td></td>
<td>10.5 Pt 2.2</td>
<td></td>
</tr>
<tr>
<td>Rome (Italy)</td>
<td>2000</td>
<td>Urban areas</td>
<td>21.2-85.7 Pt 7.8-38.8 Rh 2.2-5.8</td>
<td>Petrucci et al. (2000)</td>
</tr>
<tr>
<td>Neuglobsow (Germany)</td>
<td>2008</td>
<td>Background areas</td>
<td>0.5 Pt 0.6</td>
<td>Alsenz et al. (2009)</td>
</tr>
<tr>
<td>Deuselbach (Germany)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frankfurt (Germany)</td>
<td>2008 - 2009</td>
<td>Urban area (32500 vehicles/day)</td>
<td>1.2-80.9 Pt 1.2-683 Rh 1.1-1.3</td>
<td>Zereini et al. (2012)</td>
</tr>
<tr>
<td>Frankfurt (Germany)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
observed that the PGE content in the samples collected during the winter was greater than that of summer samples, as a consequence of the frequency of thermal inversions which dominate during winter (Zereini et al., 2012).

Studies have indicated that the PGE content in the air of several cities has greatly increased over the last few decades (Table 1.1), due to increasing travel-related Pt, Pd and Rh emissions from catalytic converters (Alsenz et al., 2009; Zereini et al., 2012). Table 1.1 shows that PGE concentration depends on the sampling location and vehicular density. The PGE concentrations in samples collected from locations adjacent to heavy traffic routes are higher than those of low traffic locations. It is also evident that PGE concentrations in urban air have increased by more than two orders in a relatively short period of time (Schierl, 2000). This emphasises the importance of careful monitoring of the environmental concentrations of these metals in in the future, which can provide a more adequate basis for the assessment of exposure and related risks.

### 1.2.2 PGEs in soil and road dust

The natural levels of PGEs in environmental samples, including soil and road dust, are low. However, studies have observed increasing PGE concentrations in these environmental compartments (Table 1.2). PGE accumulation in environmental samples is affected by traffic conditions, wind direction, and traffic density (Ravindra et al., 2004). For example, Zereini et al. (2001) reported that the Pd content of soil adjacent to roads was significantly higher (up to 72-times) than the natural background level (Table 1.2). Similarly, Cubelic et al. (1997) found that the Pt concentration in roadside soils was ≥ 300 fold of background values. Lee et al. (2011) examined the Pt content of road dust and soil of heavy and light traffic roads in and around Seoul. High Pt concentrations in samples collected from heavy traffic roads reached 444 and 221 µg/kg for road dust and roadside soil, respectively, whilst
in the suburbs Pt levels were in the range of 2.3-5.2 µg/kg in road dust and 4-5.1 µg/kg in roadside soil (Table 1.2). PGE concentrations in the road dust of European cities reached concentrations up to 200, 500 and 2250 µg/kg for Rh, Pd and Pt, respectively (Zimmermann and Sures, 2004).

Table 1.2 Platinum group elements content in soil and road dust in different locations. Studies are ordered according to the start time of samples collection.

<table>
<thead>
<tr>
<th>Place/Country</th>
<th>Year</th>
<th>Sample</th>
<th>PGE concentration (µg/Kg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pt</td>
<td>Pd</td>
</tr>
<tr>
<td>San Diego (USA)</td>
<td>1986</td>
<td>Road dust/ heavy traffic</td>
<td>680</td>
<td>280</td>
</tr>
<tr>
<td>Nottingham (UK)</td>
<td>1982, 1996</td>
<td>Soil</td>
<td>0.27-1.37</td>
<td>0.64-0.99</td>
</tr>
<tr>
<td></td>
<td>1982</td>
<td>Road dust</td>
<td>0.46-1.58</td>
<td>0.69-4.92</td>
</tr>
<tr>
<td></td>
<td>1996</td>
<td>Road dust/ low traffic</td>
<td>0.82-6.59</td>
<td>0.19-1.43</td>
</tr>
<tr>
<td></td>
<td>1998</td>
<td>Road dust/ high traffic</td>
<td>7.3-298</td>
<td>5.6-556</td>
</tr>
<tr>
<td>Roma (Italy)</td>
<td>1992, 2001</td>
<td>Urban soils</td>
<td>0.8-6.3</td>
<td>7.0-23.7</td>
</tr>
<tr>
<td>Frankfurt (Germany)</td>
<td>1994-1995</td>
<td>Geogenic background</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1994-1995</td>
<td>Soil/ along highways</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>1999</td>
<td>Road dust/ along highways and a road</td>
<td>303±40</td>
<td>95±2</td>
</tr>
<tr>
<td>Bialystok (Poland)</td>
<td>2000-2001</td>
<td>Road dust</td>
<td>34.2-110</td>
<td>32.8</td>
</tr>
<tr>
<td>Perth (Australia)</td>
<td>2002</td>
<td>Road dust</td>
<td>54-419</td>
<td>58.2-293.5</td>
</tr>
<tr>
<td></td>
<td>2001</td>
<td>Soil</td>
<td>31-153.2</td>
<td>13.8-108.5</td>
</tr>
<tr>
<td>São Paulo (Brazil)</td>
<td>2002</td>
<td>Soil/adjacent to a major road</td>
<td>0.3-17</td>
<td>1.1-58</td>
</tr>
<tr>
<td>Seoul (Korea)</td>
<td>2005</td>
<td>Road dusts/ suburbs</td>
<td>2.3-5.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2005</td>
<td>Road dusts/ light traffic</td>
<td>22.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2005</td>
<td>Road dusts/ heavy traffic</td>
<td>132.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2005</td>
<td>Roadside soils/ suburbs</td>
<td>0.4-5.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2005</td>
<td>Roadside soils/ urban area</td>
<td>0.7-221</td>
<td></td>
</tr>
</tbody>
</table>
The PGE content in soil has increased over time. Cinti et al. (2002) reported that Pt concentrations in urban soils from Rome (Italy) in 2001 were up to 6-times higher than those measured in 1992 (Table 1.2). Hutchinson et al. (2000) found that Pt and Pd concentrations in the top layer (0-5 cm) of garden soils in Nottingham (UK) in 1996 were 1.3 and 3.6 fold higher than those reported in 1982 (0.61 and 0.8 µg/kg for Pt and 0.05 and 0.15 µg/kg for Pd in 1982 and 1996, respectively). The same study indicated that the Pt and Pd content in road dust in Nottingham in 1998 were approximately 108 and 75-times greater than concentrations observed in 1982 (0.9 and 96.8 µg/kg for Pt and 1.24 and 92.9 µg/kg for Pd in 1982 and 1998, respectively) (Hutchinson et al., 2000).

1.2.3 PGEs in water bodies
Studies have shown that water environments receiving road runoff are becoming polluted with traffic-related PGEs (Table 1.3). The PGEs observed in these ecosystems often have a Pt/Rh ratio similar to that of catalytic converters (5/1 – 8/1) (Zereini et al., 1997; Helmers et al., 1998). Early investigations on Pacific seawaters observed a low level of PGE (150 and 40 pg/L for Pt and Pd, respectively) (Lee, 1983; Goldberg et al., 1986). Hodge et al. (1986) reported that the Pt concentration in San Diego (USA) coastal seawater was 100 and 250 pg/L in surface water and at a depth of 4500 m, respectively (Table 1.3). Enhanced Pd concentrations were detected in freshly deposited coastal sediments in the moat that surrounds the Emperor’s Palace in Tokyo (Lee, 1983). This author suggested that enhancement in Pd concentration was a result of the Pd used in catalytic converters.

PGE concentrations in water runoff have increased since the introduction of catalytic converters. Rauch et al. (2004) reported that the annual
deposition rate of PGE in sediment samples collected from an urban lake near Boston (USA) were 0.8 and 11.2 μg/kg for Pt, 12.2 and 24.2 μg/kg for Pd and 1.6 and 2.1 μg/kg for Rh in 1987 and 2001, respectively.

Table 1.3 Platinum group elements content locations (PPb, if not indicated otherwise) in different water bodies and sediment in different

<table>
<thead>
<tr>
<th>Place/country</th>
<th>Sample</th>
<th>Year</th>
<th>PGE concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frankfurt</td>
<td>Surface water</td>
<td>9-78 ng/L</td>
<td></td>
<td>Zereini et al. (1997)</td>
</tr>
<tr>
<td>(Germany)</td>
<td>Urban water</td>
<td>0.4 ng/L</td>
<td></td>
<td>Eller et al. (1989)</td>
</tr>
<tr>
<td>Frankfurt</td>
<td>River sediment</td>
<td>9.8-80</td>
<td></td>
<td>Zereini et al. (1997)</td>
</tr>
<tr>
<td>(Germany)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweden</td>
<td>River sediment</td>
<td>1.0</td>
<td>13.9</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1984</td>
<td></td>
<td>Wei and Morrison, (1994a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1991</td>
<td>0.5-2.2</td>
<td>Rauch et al. (2000)</td>
</tr>
<tr>
<td>Sweden</td>
<td>Lake sediment</td>
<td>0.29-42</td>
<td>0.08-5.71</td>
<td>0.11-0.26</td>
</tr>
<tr>
<td>England</td>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Lake sediment</td>
<td>0.8</td>
<td>12.2</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1987</td>
<td></td>
<td>Rauch et al. (2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2001</td>
<td>11.2</td>
<td>24.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>California</td>
<td>Surface seawater</td>
<td>100 pg/L</td>
<td></td>
<td>Hodge et al. (1986)</td>
</tr>
<tr>
<td>(USA)</td>
<td>4500 m depth</td>
<td>250 pg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>seawater</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The above cited studies show increasing concentrations of PGEs in various parts of the water ecosystem.

1.3 Transport and transformation of PGEs in the environment

Following the emission of particles, PGEs accumulate on the road surface or in road dust. The concentration of accumulated PGEs in roadside soil is negatively correlated with distance from traffic and soil depth (Schafer et al., 1998; Hooda et al., 2007). These accumulated metals may then be washed from the soil and transferred to aquatic ecosystems (Rauch
and Morrison, 2008). The soluble portion of Pt in airborne particulate matter was 30-43% of the total amount of platinum (Alt et al., 1993). In a laboratory study, it was demonstrated that the water soluble portion of Pt and Pd emitted from catalytic converters was approximately 10% (Hill and Mayer, 1977). Moldovan et al. (2002) found that < 10% of PGEs emitted from fresh catalytic converters was soluble, though this portion was significantly higher in the case of old catalysts, especially for Pd and Rh. Therefore, the environmental Pd and Rh may be more mobile and bioavailable than Pt.

Although a small fraction of PGEs emitted from autocatalytic converters is soluble, it has been demonstrated that several factors can affect the solubility of these metals. For instance, Zereini et al. (1997) examined PGE solubility in soil under a range of different parameters such as pH, chloride and sulphur concentration, which can form complexes with PGE and thus enhance the solubility of these metals. The latter authors then compared the results with those obtained from several environmental materials (surface water, run-off sediment, tunnel dust and soil). No significant relationship was shown between the solubility of the examined metals and the chloride concentrations, whilst a positive correlation was observed between sulphur concentration and the solubility of Pt, but not Rh. The solubility of Pt and Rh in roadside soil under natural conditions (pH 5-8) was nearly constant with higher solubility for Rh than that of Pt (0.01-0.025% and 0.05%, respectively) (Zereini et al., 1997). The presence of siderophores, which are microbially produced iron-chelating ligands, increased the solubility of metallic and oxide forms of Pt and Pd in the environment (Dahlheimer et al., 2007; Rauch and Morrison, 2008). This increase in PGE solubility may be due to the presence of siderophores reducing the pH of the media. The solubility of PGEs was also found to be enhanced in the presence of complexing agents such as EDTA (Zimmermann et al., 2003) and humic substances (Lustig et al., 1998; Zimmermann et al., 2003).
This increase in PGE solubility may be due to the formation of water-soluble complexes with organic compounds (Zimmermann et al., 2003).

Soluble forms of PGEs may enter ecosystems and accumulate in organisms through food webs. Thus, these metals can be taken up and accumulated in plants, animals and humans, leading to ecotoxicological and health impacts. This increases the necessity of studies to examine the uptake of PGEs and their subsequent effect on biota. Watras et al., (1998) highlighted the strong interaction between the concepts of metal speciation, which involve both metal forms and different aqueous species, and bio-concentration in aquatic environments. These authors suggested that metal bioaccumulation includes two different processes, which are uptake from the surrounding environment into organisms at lower trophic levels (via the diet or permeable body surface) and transfer of these metals to organisms at higher trophic levels (Watras et al. 1998). They also reported that the latter process may also be affected by metal speciation. The uptake of metal by predators is influenced by metal distribution in prey organisms (Khan et al., 2010). Therefore, studying the sub-cellular distribution of metals can improve knowledge regarding their potential accumulation via food webs. Khan et al. (2010) showed that the bioavailability of metal stored within the cytosol (e.g. (metallothionein and metallothionein-like proteins) and soft tissue was higher than that bound to the exoskeleton or metal rich granules (MRGs). Metallothioneins (MTs) comprise a non-enzymatic and heat-stable family of proteins with low molecular mass, cysteine-rich content and the absence of aromatic amino acids (Amiard et al., 2006; Frank et al., 2008). The thiol groups (SH) of cysteine residues in these proteins increase their affinity to bind both essential and non-essential toxic metals (Amiard et al., 2006). Therefore, MTs are important players in the regulation of physiological metal (e.g. Zn and Cu) homeostasis as well as the detoxification of several non-essential xenobiotic metals (Frank et al., 2008).
1.4 PGEs in living organisms

It has been reported that PGEs are bioavailable to biota and can be accumulated in various organisms such as plants (Schafer et al., 1998), terrestrial animals (Zimmermann and Sures, 2004) and aquatic organisms (Sures, et al., 2002a, b). However, there is a lack of knowledge regarding the potential toxicological and ecotoxicological effects of these metals (Zimmermann and Sures, 2004).

1.4.1 PGEs in plants

Biological availability and accumulation of PGEs by plants has been reported in both laboratory and field studies (Messerschmidt et al., 1994, 1995; Veltz et al., 1996; Klueppel et al. 1998; Schafer et al., 1998; Verstraete et al. 1998; 2004; Ravindra et al., 2004; Zimmermann et al., 2005; Table 1.4). However, only a few studies have examined the consequences of PGE uptake on plants. For instance, Battke et al., (2008) observed that the leaf length of barley (Hordeum vulgare L) was significantly reduced as a response to 7 days of exposure to 5.32 mg/L Pd. The leaf length of H. vulgare was 120-125 and 105-115 mm at 0 and 5.32 mg/L Pd, respectively. Four weeks exposure to 0.7 mg/L PGEs (Pt, Pd and Rh combined) caused a significant decrease in plant dry weight, protein content, nitrate reductase activity and photosynthesis efficiency in lettuce (Lactuca sativa) (Odjegba et al., 2007).

The data presented in Table 1.4 show a wide range of PGE concentrations in plants. These concentrations may be affected by several factors such as plant species and the sampling location. Generally, PGE content in plants located in unexposed sites is lower than that in plants located near to road sites (Dan-Badjo et al., 2008).
**Table 1.4 Platinum group elements content in some plant samples collected from different locations**

<table>
<thead>
<tr>
<th>Place/Country</th>
<th>Sample</th>
<th>Site description</th>
<th>Year</th>
<th>PGE concentration (µg/kg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Norway spruce new needles</td>
<td></td>
<td></td>
<td>Pt: 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sitka spruce twigs</td>
<td></td>
<td></td>
<td>Pt: 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scots pine twigs</td>
<td></td>
<td></td>
<td>Pt: 14</td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>Dandelion (<em>Taraxacum officinale</em>)</td>
<td>Along highways</td>
<td>1999</td>
<td>Pt: 5.4 Pd: 0.83 Rd: 2.2</td>
<td>Djingova et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>Plantain (<em>Plantago lanceolata</em>)</td>
<td></td>
<td></td>
<td>Pt: 3.6 Pd: 0.45 Rh: 1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moss (<em>Rhytidiadelphus squarrosus</em>)</td>
<td></td>
<td></td>
<td>Pt: 30 Pd: 2.4 Rh: 5.4</td>
<td></td>
</tr>
<tr>
<td>UK</td>
<td>Tree bark</td>
<td>London city centre</td>
<td></td>
<td>Pt: 5.4</td>
<td>Becker et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sheffield city centre</td>
<td></td>
<td>Pt: 2</td>
<td></td>
</tr>
<tr>
<td>Bialystok (Poland)</td>
<td>Rough bluegrass (<em>Poa trivialis</em>)</td>
<td>along a road/20600 vehicles/day</td>
<td>2000</td>
<td>Pt: 8.63 Pd: 3.2 Rd: 0.65</td>
<td>Leśniewska et al. (2004)</td>
</tr>
<tr>
<td>Oulu (Finland)</td>
<td>Scots pine needles</td>
<td>along a road/43000 vehicles/day</td>
<td>2003</td>
<td>Pt: &lt; 0.5 Rh: &lt; 0.3</td>
<td>Niemelä et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>Moss (<em>Pleurozium schreberi</em>)</td>
<td></td>
<td></td>
<td>Pt: 27.4 Rh: 4.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dandelion (<em>Taraxacum vulgäre</em>) leaves</td>
<td></td>
<td></td>
<td>Pt: 1.2 Rh: &lt; 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grass (<em>Calamagrostis sp.</em>)</td>
<td></td>
<td></td>
<td>Pt: 1.5 Rh: 0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pine (Scots pine) (<em>Pinus sylvestris L</em>) needles</td>
<td></td>
<td></td>
<td>Pt: &lt; 0.5 Rh: &lt; 0.3</td>
<td></td>
</tr>
</tbody>
</table>
1.4.2 PGEs in terrestrial animals

Numerous studies have investigated the uptake of PGEs by terrestrial organisms. For instance, Moore et al. (1975) demonstrated that Pd and Pt were taken up by rabbits following oral and intravenous exposure. In another study, by Moore et al. (1974), Pd uptake by adult rats was observed after oral, intravenous, and intratracheal exposure. It was also found that Pd content in the tissue of exposed organisms was dependent on the route of exposure, with highest Pd concentration after an intravenous dose, followed by intratracheal and oral exposure respectively (Moore et al., 1975). Similarly, Artelt et al. (1999b) studied Pt uptake by female Lewis rats after two different routes of administration, intratracheal instillation and inhalation. These authors reported that after 24 h intratracheal instillation to 10 mg Al₂O₃/Pt, the total Pt concentrations in the liver, and kidneys of the exposed organisms were 2.48, and 4.68 μg/g, respectively. After 90 days of inhalation exposure to 12 mg/m³ Al₂O₃/Pt, Pt content in the examined tissue of the exposed organisms was 0.13 and 0.042 μg/g, respectively (Artelt et al., 1999b). The same study observed that during the inhalation exposure, 30% of the fine dispersed Pt deposited was bioavailable.

Birds have also been used as bioindicators of PGE concentrations in the environment. Jensen et al. (2002) measured PGE concentrations in the feathers of three raptor species in Sweden: peregrine falcon *Falco peregrinus*, the gyrfalcon *Falco rusticolus*, and the sparrowhawk *Accipiter nisus*. These raptors and their main prey were investigated to determine alterations in Pt, Pd and Rh tissue concentrations over the period 1917-1999. The main finding of the study was that PGE concentrations in the tested tissues had significantly increased since the mid-1980s, reaching 0.6, 1.8 and 2.1 μg/kg for Rh, Pt and Pd, respectively in 1999. They suggested that the increase in PGE concentrations in the examined tissues reflected the increase in the environmental PGE concentrations over time. Likewise, measurable
traffic-related PGE concentrations were reported in the kidney, liver and blood of the *F. peregrines* (Ek et al., 2004). The same study reported that PGE content in the eggs of *A. nisus* were 0.45, 0.51 0.48 µg/kg for Pt, Rh and Pd, respectively.

Only a few studies have investigated the effect of PGEs on terrestrial invertebrates. For example, it was found that traffic-related PGE reduce the reproduction of Folsomia candida (Hexapoda: Collembola) (Nemcova *et al.*, 2012; 2013). They reported that the relative order of toxicities is Pd$^{2+} >$Pt$^{4+} >$Rh$^{3+}$ with 28-day EC50 values of 21.1, 200.4 and 262 µM, respectively.

### 1.4.3 PGEs in aquatic organisms

The uptake of traffic related PGEs (Pt, Pd and Rh) by aquatic organisms has been reported in several studies. For example, it has been shown that PGEs are biologically available to a number of fish species (Sures *et al.*, 2002; Zimmermann *et al.*, 2002; Zimmermann *et al.*, 2005). PGE uptake by *Asellus aquaticus* (Isopoda: Asellidae) exposed to environmental samples (road dust, river sediments and tunnel dust) was positively correlated with the exposure period and PGE content in the exposure material (Moldovan *et al.*, 2001). They reported that the PGE content in unexposed *A. aquaticus* was 1.9 and 2.4 µg/kg dry weight for Pt and Rh, respectively. Following 24 h of exposure to road dust, the PGE content in the tested organisms was approximately 25 µg/kg dry weight for Pt and Rh (Moldovan *et al.*, 2001). After 96 h of exposure to the same material, PGE concentrations in the exposed animals increased to approximately 150 and 50 µg/kg dry weight for Pt and Rh, respectively (Moldovan *et al.*, 2001). Likewise, Osterauer *et al.* (2009) observed that bioaccumulation of Pt by *D. rerio* is dose-dependent. Following 96 h exposure to 0.73, 7.3 and 73 µg/L Pt$^{2+}$, Pt$^{2+}$ content in the treated organisms was 0.02, 0.04 and 1.06 µg/g, respectively (Osterauer *et al.*, 2009).
The available literature indicates that the biological availability of Pd to biota is higher than that of Pt and Rh. For instance, Moldovan et al. (2001) found that, in *A. aquaticus*, Pd bioaccumulation was much higher than for the other two PGE metals. PGE bioaccumulation factors (the ratio of the chemical concentration in the organism to the chemical concentration in the surrounding environment) in *A. aquaticus* exposed to soluble standard solutions of PGE for 24 h were 7, 85 and 150 for Rh, Pt and Pd, respectively (Moldovan et al., 2001). Similarly, bioaccumulation of Pd by *D. polymorpha* was higher than that of Pt, which in turn was higher than the bioaccumulation of Rh (Sures et al., 2001b; Sures and Zimmermann, 2007). A similar trend was observed when *D. polymorpha* was exposed to road dust as the source of PGE (Sures et al., 2002a). They found that, Pd concentration in the tissue of exposed animals was 2-times higher than Pt and 40-times as high as Rh, although Pt concentrations in exposed material was approximately 8-times higher than Pd. In a field study, Haus et al. (2007) found that the bioaccumulation of Pt by *Gammarus pulex* (Amphipoda: Gammaridae) was comparatively higher than that of a number of other traffic-related metals (Cd, Mo, Sb, Pb and Cr) and similar to the uptake rates of Zn, which is an essential metal. The tested organisms had bioaccumulation factors of 0.16, 0.15, 0.03, 0.008, 0.002, 0.002 and 0.001 for Pt, Zn, Cd, Cr, Pb, Sb and V, respectively (Haus et al., 2007).

The uptake of PGEs by aquatic biota may be affected by several factors, such as metal form and source of exposure. For example, Rauch and Morrison (1999) reported that Pt accumulation was valency dependent with accumulation of Pt (IV) (platinum in the 4+ oxidation state) in *A. aquaticus* higher than that of Pt (II) (platinum in the 2+ oxidation state). Following 96 h exposure to 100 μg/L Pt in the form Pt(II) or Pt(IV), total Pt content in the exposed organisms was approximately 200 and 500 μg/g, respectively (Rauch and Morrison, 1999). The uptake rates for the traffic-related PGEs (Pt, Pd and Rh) when *A. aquaticus* was exposed to catalyst materials (car catalyst powder with particle size < 63 μm) were 3-times higher than that observed in the environmental materials (river
sediments, road dust and tunnel dust) (Moldovan et al., 2001). The same study also found that the PGE ratio in A. aquaticus was similar to that in the catalytic converter samples. This finding agrees with that of previous studies reported that water bodies often have a PGE ratio similar to that of catalytic converters (Zereini et al., 1997; Helmers et al., 1998). However, the uptake rates varied when organisms were exposed to environmental materials, with the bioaccumulation rate of Pt higher than that for Rh (Moldovan et al., 2001). This finding suggests that the differences in the processes of PGE transformation under natural conditions produce different forms of PGE compounds leading to different uptake rates of PGEs by biota (Moldovan et al., 2001).

Interaction of PGE during their uptake by biota has been examined in some laboratory studies. For example, Yong et al. (2002) demonstrated that Pd uptake by resting cell suspensions of Desulfovibrio desulfuricans (a species of Gram negative sulphate-reducing bacteria) was inhibited by the presence of Pt but not Rh. It was also observed that Pt and Rh were bioavailable to D. desulfuricans when it was exposed to a single metal solution, but no uptake of these metals was detected in the presence of Pd (Yong et al., 2002). In contrast, Moldovan et al. (2001) found that no interaction occurred between the uptake of Pt, Pd and Rh by A. aquaticus, as the accumulation ratio of these metals was the same following exposure of the organism to either single or mixed PGE solutions.

The uptake of PGEs by aquatic biota may also be affected by water quality. Studies have shown that PGE bioavailability can be enhanced or reduced by the presence of humic substances. Humic substances can interact with PGEs to form soluble and/or insoluble complexes and thus affect PGE bioavailability to aquatic biota (Sures and Zimmermann, 2007). They reported that the solubility of Pt was higher in dechlorinated water than in the humic water of a bog lake, whilst contrasting results were observed for Pd and Rh. The same study observed that the
presence of humic substances increased the bioaccumulation of Pt by *D. polymorpha*, whereas the opposite effect was shown for Pd and no obvious trend was observed for Rh. This contrasts with Zimmermann *et al.* (2005) who found that the uptake and bioaccumulation of Pt, Pd and Rh by the *D. polymorpha* in humic water was higher than that in tap water. Zimmermann *et al.* (2004) reported that the uptake of Pt and Rh by European eels *Anguilla anguilla* (Anguilliformes: Anguillidae) in humic water was lower than that in tap water. They reported that the presence of fulvic acid increases the water solubility of Pd but does not affect Pt solubility. The latter metal can interact with humic acid forming insoluble Pt-humic complexes (Sures and Zimmermann, 2007).

Studies have also investigated the PGE concentrations in the tissues of exposed organisms. For example, Sures *et al.* (2001) investigated the uptake of PGEs by *A. anguilla* treated with water containing 10% w/v road dust for 28 days. It was found that the Pd concentration was higher in the liver than in the other tested organs, at approximately 0.18 µg/kg (wet weight), whilst in the kidney the level of Pd could not be detected. Similarly, Essumang (2008) studied PGE levels in the tissues of *Stenella* sp. dolphins (Cetacea: Delphinidae) caught along the Ghanaian coastline. The liver content was 0.040-0.481, 0.239-0.946 and 0.011-0.037 µg/g fresh weight for Pd, Pt and Rh, respectively. The lower Pd content in dolphins, when compare to that in *A. anguilla*, may be due to different exposure conditions (e.g. Pd concentration), and/or differences in the ratio of body volume to metal permeable surface area of examined organisms.

### 1.4.4 PGEs in humans

Several studies have investigated PGE concentrations in human fluids and tissue. The Pt concentration in the whole blood, hair and urine of 21 adults from Sydney (Australia) was 0.56 µg/L, 3.84 µg/kg and 0.18 µg/L, respectively (Vaughan and Florence, 1992). They found that diet was the main pathway of Pt uptake, with an average daily Pt intake of 1.73
and 1.15 µg by adult males and females, respectively. The Pt and Pd content of human urine (10 volunteers from DiisseldorJ, Germany) was examined by Begerow et al. (1997), who reported that the Pd concentration in test samples ranged from 0.02 to 0.08 µg/L (mean 0.039 µg/L). They observed that Pt concentrations were below the detection limit of their method (0.07 µg/L).

A number of researchers have examined the relation between PGE content in human fluids and traffic density. For instance, Caroli et al. (2001) showed that the average urinary concentrations of PGE in samples collected from 310 schoolchildren from urban and suburban areas of Rome (Italy) were 7.5, 0.9 and 8.5 µg/kg for Pd, Pt and Rh, respectively. A major finding of that study was a strong positive relationship between Pd and Rh content in test samples and traffic density. In contrast, Iavicoli et al. (2004) examined the Pt concentration in the urine of two groups of the Rome city (Italy) police force, one involved in patrolling streets and the other engaged only in office work. These researchers examined the Pt concentration in urine samples for the tested groups at the beginning and at the end of the 7 h outdoor and indoor work shifts. No significant difference was found between the Pt in the urine of the examined groups with mean concentrations of 4.45 and 4.56 ng/L, respectively (Iavicoli et al., 2004). It was therefore suggested that the duration of the work shift was much shorter than that needed to eliminate and excrete Pt with urine.

Investigations with human cell cultures have shown that PGEs have significant toxic impacts that are similar to other toxic metals. Schmid et al. (2007) found that the viability of the human bronchial epithelial cell line BEAS-2B was considerably lower after 24 h exposure to 0.05 mM Pt⁴⁺ (9.8 mg/L). They also observed that 2 h exposure to 0.012 mM Pt²⁺ (2.34 mg/L) caused a significant reduction in the induction of reactive oxygen species (ROS), suggesting that Pt affects basic cell metabolism. The same study showed that these effects following Pt²⁺, Pt⁴⁺ and Pd²⁺ exposure are comparable to those resulting from exposure
to a number of other toxic metals such as Cd\(^{2+}\) and Cr\(^{2+}\). The 24 h LC\(_{50}\) concentration for bronchial epithelial cells was 0.4 mM for Pt\(^{2+}\) and Pd\(^{2+}\), 0.05 mM for Pt\(^{4+}\), 0.02 mM for Cr\(^{2+}\) and 0.005 mM for Cd\(^{2+}\) (Schmid et al., 2007).

### 1.4.5 PG E toxicity to living organisms

Pt is toxic to a wide range of aquatic organisms, such as the water flea *Daphnia magna* (Cladocera: Daphniidae) (Biesinge and Christen, 1972), coho salmon *Oncorhynchus kisutch* (Salmoniformes: Salmonidae) (Ferreira Jr and Wolke, 1979), the invertebrate *Hyalella Azteca* (Amphipoda: Dogielinotidae) (Borgmann et al., 2005), zebrafish *Danio rerio* (Cypriniformes: Cyprinidae) (Osterauer et al., 2009) as well as humans (Rauch and Morrison, 1999). Organisms may therefore be affected by increasing Pt concentrations in the environment and subsequent bioaccumulation via food chains (Vaughan and Florence, 1992).

Data regarding Pd toxicity is somewhat limited. Only a few studies have examined the toxic impact of Pd on aquatic organisms, for example, *Eichhornia crassipes* (Commelinales: Pontederiaceae), *Lumbriculus variegatus* Müller (Lumbriculida: Lumbriculidae), *H. azteca*, the zebra mussel *Dreissena polymorpha* (Veneroida: Dreissenidae) and the green alga *Pseudokirchneriella subcapitata* (Sphaeropleales: Selenastraceae) (Farago and Parsons, 1994; Veltz et al., 1996; Borgmann et al., 2005; Singer et al., 2005; Bednarova et al., 2012) or terrestrial organisms (e.g. outbred albino rats) (Moore et al., 1975). If the data regarding Pd toxicity on aquatic organisms are limited then the studies regarding Pd toxicity in humans are even more so. Kielhorn et al. (2002), report that human health may be negatively impacted by Pd exposure. These authors reported that concentrations as low as 1 mM (0.1 mg/L) can cause allergic reactions in exposed individuals. Based on the information highlighted above, further studies are needed to improve knowledge of the potential toxic impact of Pd.
1.5 Biomonitoring of environmental pollution

Biomonitoring, which refers to the use of biological responses to evaluate environmental quality, is recognised as a useful approach to examining water quality (Depledge et al., 1995). Studying the physical and chemical components of water bodies, whilst informative, should not be considered as the only approach to evaluating the health of aquatic ecosystems. Chemical surveys can only offer information on the concentrations of pollutants in aquatic ecosystems but do not provide information on the toxicity and the possible negative effects of contaminants on the ecosystems. In this context, sentinel species of aquatic animals can be used to obtain valuable information regarding contaminants in aquatic environments, particularly at low concentrations (Chapman, 1996). This assumes that the condition and health of biota can reflect the health of aquatic systems (Wepener, 2008). Biomonitoring comprises two approaches: bioassays, which are based on laboratory monitoring, and bioassessments, which are based on field monitoring (Roux et al., 1993). The latter approach can involve passive and/or active biomonitoring (Herricks et al., 1989; Chapman, 1996). Passive biomonitoring refers to laboratory analyses of those biota naturally occurring in the test area, collected from the wild. Alternatively, organisms of known response, collected from one location, can be transferred to an area of interest for the purpose of measuring their physiological and/or biochemical responses, in order to monitor water quality. This is known as active monitoring (Smolders et al., 2003).

Biomonitoring takes into account the variation in absorption, bioavailability, excretion and detoxification, which may affect chemical toxicity (Gil and Pla, 2001). Metal accumulation varies between metals and between organisms (Rainbow, 2005, 2007). Since increasing total metal concentrations in the environment may not be associated with increase in metal bioavailability (John and Leventhal, 1995), investigation of metal concentration in tissues of exposed organisms would provide a better estimation of metal bioavailability. However, total
concentrations of accumulated metals in an organism may not reflect metal toxicity, as it is influenced by several biological processes such as metal detoxification (Rainbow, 2007; Rosabal et al., 2014). Rainbow (2007) demonstrated that metals were toxic when they accumulated at a rate higher than that of detoxification and excretion.

Metals taken up by aquatic crustaceans (a group of invertebrates), mainly via the diet or permeable body surface, enter in a form that is metabolically available. They can bind to molecules in the receiving cell, and are also transferred via the haemolymph to elsewhere in the body (Rainbow, 2007). Some of these metals may play an essential role in metabolism (e.g. as enzyme co-factors), or bind to sites where they will disturb normal metabolic functioning, and thus play a toxic role (Marsden and Rainbow, 2004). Non-essential metals and over-accumulated essential metal, in metabolically available forms, have the potential to play a toxic role unless they are detoxified or excreted (Rainbow, 2002). Metal could be detoxified via binding either in an insoluble form, such as granules or deposits in the organism’s tissues (Rainbow, 2002) or in the soluble phase, such as metallothioneins (Amiard et al., 2006).

Environmental contaminant concentrations are generally lower than the LC$_{50}$ (median lethal concentration) values obtained in the laboratory (Gaudy et al., 1991). Therefore, only limited information regarding the effects of pollutants in natural settings can be obtained using acute toxicity studies. Thus, investigating the influence of sublethal doses of pollutants on behavioural, physiological and/or biochemical responses at the suborganismal or organismal level can improve the understanding of possible environmental effects of toxic pollutants (Roast et al., 1999; Jensen et al., 2001).

Investigation of the effects of environmental stresses on different levels of biological organisation (e.g. molecule, cell, tissue, organism, population, community etc.) is a commonly used approach in bio-
monitoring (Wallace and Estephan, 2004; Singer et al., 2005; Lloyd Mills et al., 2006; Schmid et al., 2007). Valuable information can be obtained by studying the alteration in an ecosystem at community and population levels, but this may not reflect the direct causes of the impact at the ecosystem level. On the other hand, assessment of alterations in biomarkers from the molecular to the organism level can provide a sensitive response to various environmental stresses. Additionally, alteration of biomarkers occurs earlier than change at the population and community levels (Tsangaris et al., 2007). This may increase the utility of biomarkers in toxicity studies as tools to detect stress and early signs of damage at the ecosystem level.

Biomarkers examine the stress on organisms and thus are more biologically relevant than chemical monitoring. The organisms’ responses integrate the temporal and spatial environmental effects, and reflect the impact of contaminants (Chapman, 1996). Organismal responses also combine the conflicting and potential effects of the full range of pollutants and stresses encountered in an ecosystem (Bealey et al., 2008). Consequently, the cost and time of environmental monitoring can be reduced compared to chemical monitoring of individual elements (Adriaanse et al., 1995).

Many biotic groups (e.g. benthic macro-invertebrates, fish and plants) have been used as bioindicators in the aquatic environment (Lenat, 1984; Berkman et al., 1986; Doust et al., 1994; Michels et al., 1999; Stephens and Farris, 2004). Biological monitoring include a range of biochemical and/or behavioural responses. Different behavioural endpoints, such as feeding inhibition and alterations in swimming or ventilation have been frequently employed to produce dose response curve data that are statistically analysable (Maltby et al., 2002; Wallace and Estephan, 2004; Lloyd Mills et al., 2006; Alonso et al., 2009; Kunz et al., 2010; Gerhardt, 2011).
Biochemical biomarkers, such as alteration in acetylcholinesterase (AChE) activity and induction of heat shock proteins (HSPs), have also been examined and thus developed to provide an early sign of environmental toxicity and the biological impact of pollutants (Lam and Gray, 2003; Xuereb et al., 2009a,b). These biomarkers are easy to examine using inexpensive assays and can act as useful indicators of the fitness of the tested organisms.

1.5.1 *Gammarus pulex* as a freshwater biomonitor

Gammaridean amphipods, which are among the most abundant crustaceans of the order Amphipoda, are known metal accumulators (Scott, 1996). These amphipods are commonly employed as biomonitor due to their widespread occurrence in marine, brackish and freshwater environments (Rainbow, 1995; Rinderhagen et al., 2000). They perform a key function in aquatic food webs as a trophic link from primary producers to higher level consumers such as fish and birds (Chiaravalle et al., 1997; Lloyd Mills et al., 2006). Furthermore, these organisms can be readily employed in laboratory studies.

The freshwater amphipod *G. pulex* has been commonly used as a biomonitor to assess the quality of freshwater ecosystems (McLoughlin et al., 2000; Mills et al., 2006; Felten et al., 2012). *G. pulex* is extensively dispersed and numerically abundant in European freshwater habitats, and is sensitive to numerous toxins (Guven et al., 1999; Ek et al., 2004; Lloyd Mills et al., 2006). The lifespan of *G. pulex* is up to 23 months for females and up to 30 months for males (Welton and Clarke, 1980). The total length of adult *G. pulex* may reach 21 mm for males and 14 mm for females (Pinkster, 1970). McCahon and Pascoe (1988) found that approximately 70% of laboratory cultured *G. pulex* survived to reach sexual maturity within 130 days at 13°C. During the life cycle of *G. pulex*, the average production of females is between 2 and 5 clutches, and may reach 7, each one consisting of 10 to 26 eggs (Welton and Clarke, 1980; McCahon and Pascoe, 1988). A negative relationship
was observed between the development time of eggs of *Gammarus lacustris* (Crustacea: Amphipoda) and temperature over a range of 4-20°C (Wilhelm and Schindler, 2000). These authors also reported that at a given temperature the development time of the eggs has a positive relationship with the size of the eggs. In the current study, adult field-collected *G. pulex* were used in order to reduce the time needed for a breeding programme.

### 1.5.2 Median lethal concentration (LC$_{50}$) toxicity studies

A number of methods have been employed to assess the toxicity of chemicals on aquatic biota (Boateng *et al.*, 2009). The purpose of toxicity tests is to obtain data regarding the harmful effect of chemicals on test organisms (Stephan, 1977). Acute toxicity tests, such as LC$_{50}$ (a test measures the concentration of a chemical that is lethal to 50% of test organisms within the test period), have been widely used as an initial tool to examine the harmful effects of environmental contaminants on aquatic biota. This may be due to the ease of performing such experiments under laboratory conditions (Mishra *et al.*, 2011). In the LC$_{50}$, each group of test organisms is exposed to a single concentration of a chemical for a particular period of time. This test is generally designed to obtain initial information on the toxic nature of a chemical. Such information is needed to select chemical concentrations used in short term exposure, sublethal and sub-chronic toxicity tests.

Many studies have examined the acute toxicity of various different chemicals (e.g. pesticides, medications and metals) to *G. pulex* (Abel, 1980; Green *et al.*, 1986; McCahon and Pascoe, 1988; Girling *et al.*, 2000; Watts *et al.* 2001; Cold and Forbes, 2004; Felten *et al.* 2008). Ashauer *et al.* (2011) reported that the sensitivity of *G. pulex* to the neonicotinoid insecticides acetamiprid, imidacloprid and thiacloprid was 100 to 1000-times higher than that of *D. magna*, which is known to be highly sensitive to toxic substances (Tyagi *et al.*, 2007). Studies also indicate that *G. pulex* may be more sensitive than *D. magna* to the
synthetic pyrethroid, esfenvalerate. The 48 h LC$_{50}$ value of esfenvalerate was 0.14 µg/L for _G. pulex_ (Cold and Forbes, 2004) and 0.27 µg/L for _D. magna_ (Fairchild _et al._, 1992). However, differences in response of exposed organisms in the previously mentioned studies may also be due to differences in exposure conditions (e.g. water quality, temperature etc.). The LC$_{50}$ values for a range of metals, for _G. pulex_ and some other freshwater invertebrates, are shown in Table 1.5. The data presented in the table indicates that gammarids may be highly sensitive to metals.

A number of factors may affect the toxicity of metals toward gammarids. For example, Felten _et al._ (2008) reported a 48 h LC$_{50}$ value of Cd$^{2+}$ to adult field-collected _G. pulex_ was 0.49 mg/L, while adult cultured _G. pulex_ showed approximately a 10-fold greater survival response with a 48 h LC$_{50}$ of 4.7 mg/L Cd$^{2+}$ (McCahon and Pascoe, 1988; Table 1.5). The latter authors reported that mortality of _G. pulex_ exposed to Cd$^{2+}$ depended on the age group of the exposed organisms. In a similar study, Felten _et al._ (2008) found that the effect of Cd$^{2+}$ on the survival of _G. pulex_ was both time and concentration dependent (Table 1.5). Muskó _et al._ (1990) showed that toxicity of Cd$^{2+}$ to _G. fossarum_ was enhanced by increasing the pH of the surrounding medium. They observed that, at pH 6, the LC$_{50}$ values were 0.62, 0.08 and 0.007 mg/L Cd$^{2+}$ at 24, 48 and 96 h, respectively. At pH 8, the LC$_{50}$ values were reduced to 0.082, 0.015, 0.0062 mg/L Cd$^{2+}$ at 24, 48, and 96 h, respectively (Table 1.5). Bat _et al._ (2000) showed that the toxicity of Zn$^{2+}$, Cu$^{2+}$ and Pb$^{2+}$ to _G. pulex_ was positively correlated with temperature within a range of 15-25 ºC (Table 1.5). Metal toxicity is also influenced by water quality. Borgmann _et al._ (2005) demonstrated that the toxicity of Pt and Rh to _H. azteca_ in soft water was lower than in hard water. The same study showed the opposite result for Pd, which was more toxic in hard water.
### Table 1.5 A summary of some studies that reported median lethal concentration (LC₅₀) of some metals for *G. pulex* and other freshwater invertebrates

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>Organism</th>
<th>Life stage</th>
<th>Time (hr)</th>
<th>LC₅₀ (mg/L)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium (Cd²⁺)</td>
<td><em>Gammarus pulex</em></td>
<td>Juvenile</td>
<td>48</td>
<td>0.019</td>
<td>McCahon and Pascoe (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult</td>
<td>48</td>
<td>4.7</td>
<td>Felten et al. (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>1.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48</td>
<td>0.494</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96</td>
<td>0.0821</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>120</td>
<td>0.0371</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>168</td>
<td>0.0216</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>264</td>
<td>0.0105</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Echinogammarus tibaldi</em></td>
<td>7 - 9 mm long</td>
<td>96</td>
<td>1.1</td>
<td>Pantani et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>(Amphipoda: Gammaridae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Gammarus italicus</em></td>
<td>Adult</td>
<td>96</td>
<td>9.1</td>
<td>Muskó et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>(Amphipoda: Gammaridae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Gammarus fossarum</em></td>
<td>Adult</td>
<td>24 at pH 6</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Amphipoda: Gammaridae)</td>
<td></td>
<td>96 at pH 6</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 at pH 8</td>
<td>0.082</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96 at pH 8</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Copper (Cu²⁺)</td>
<td><em>Gammarus pulex</em></td>
<td>Juvenile</td>
<td>24</td>
<td>0.2</td>
<td>Guven et al. (1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72</td>
<td>0.12</td>
<td>Bat et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96 at 15ºC</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96 at 20ºC</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96 at 25ºC</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Daphnia magna</em></td>
<td>&lt; 24 h old</td>
<td>72</td>
<td>0.09</td>
<td>Winner and Farrell (1976)</td>
</tr>
<tr>
<td>Lead (Pb²⁺)</td>
<td><em>Chironomus decorus</em></td>
<td>4th instar</td>
<td>48</td>
<td>0.74</td>
<td>Kosalwat and Knight (1987)</td>
</tr>
<tr>
<td></td>
<td>(Diptera: Chironomidae)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td><em>Gammarus pulex</em></td>
<td>5 - 8 mm long</td>
<td>96</td>
<td>0.39</td>
<td>Kutlu and Sümér (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96 at 15ºC</td>
<td>23.2</td>
<td>Bat et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96 at 20ºC</td>
<td>16.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96 at 25ºC</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Daphnia pulex</em></td>
<td>Adult</td>
<td>48</td>
<td>4</td>
<td>Theegala et al. (2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Daphnia magna</em></td>
<td>Adult</td>
<td>48</td>
<td>0.45</td>
<td>Biesinger et al. (1972)</td>
</tr>
<tr>
<td>Zinc (Zn²⁺)</td>
<td><em>Gammarus pulex</em></td>
<td>96 at 15ºC</td>
<td>12.1</td>
<td>9.3</td>
<td>Bat et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96 at 20ºC</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Data regarding the acute toxicity of PGEs, especially Pd, to aquatic organisms is limited. Sawasdee and Koehler (2010) found that the 96 h LC$_{50}$ of Pd$^{2+}$ in the ramshorn snail *Marisa cornuarietis* (Architaenioglossa: Ampullariidae), was approximately 0.5 mg/L. The 96 h LC$_{50}$ values of Pd$^{2+}$ in the freshwater tubificid worm *Tubifex tubifex* (Annelida: Oligochaeta) and the rainbow trout *Oncorhynchus mykiss* (Salmoniformes: Salmonidae) were 0.09 and 0.19 mg/L, respectively (WHO, 2002). WHO (2002) also reported that in the freshwater fish medaka *Oryzias latipes* (Beloniformes: Adrianichthyidae) the 24 h LC$_{50}$ for Pd$^{2+}$ was 4.2 mg/L. The 168 h LC$_{50}$ of PGEs in *H. azteca* were 0.13 and 0.22 mg/L for Pt and 0.98 and 3.15 mg/L for Rh and 1 and 0.57 mg/L for Pd, in soft water and hard water, respectively (Borgmann et al., 2005). More investigations are needed to improve the understanding regarding toxic concentrations of PGEs and determine the risk potential of PGEs exposure. Therefore, the current study examined the survival response of *G. pulex* to PGEs. The doses that could be employed to examine the sublethal responses of *G. pulex* were also established.

### 1.5.3 Feeding behaviour

Feeding inhibition in aquatic biota has been widely employed as a general stress response (Semlitsch et al., 1995; Lotufo and Fleeger, 1996; Donkin et al., 1997; Jensen et al., 2001; McWilliam and Baird, 2002). This may be due to the ease and low cost of such tests in comparison to physiological tests. In addition, feeding reduction provides one of the first responses to environmental stresses (McLoughlin et al., 2000), being as sensitive as physiological responses (Maltby and Crane, 1994). Studies have shown that feeding assays can be employed under both field and laboratory conditions. These have highlighted that the feeding rate of some aquatic organisms can be adversely influenced by toxicants even at sublethal concentrations (Cowles, 1983; Farke et al., 1985). This can significantly reduce the rates of growth and reproduction when organisms are exposed to environmental stressors (Maltby and Crane, 1994). Therefore, alteration
in the feeding behaviour of individuals may affect populations and vital ecosystem functions (Forrow and Maltby, 2000). Maltby et al. (2002) observed that reduction in feeding activity of *G. pulex* was associated with a decrease in its abundance, detritus processing and macro-invertebrate diversity in polluted environments.

However, feeding behaviour is affected by numerous internal and external factors, such as parasite load (Pascoe *et al.*, 1995), source population (Maltby and Crane, 1994), body size (Nilsson, 1974), temperature (Maltby *et al.*, 1990), pH (Naylor *et al.*, 1989) and dissolved oxygen concentration (Maltby *et al.*, 1990). Agatz and Brown (2014) showed that the feeding activity of *G. pulex* was influenced by: parasite infection; body mass; food source; food quality and acclimation time. They indicated that the feeding activity of small organisms (< 5 mg dry weight) was approximately 3-times higher than that of larger organisms (> 5 mg dry weight). They also found that the acclimation of organisms, for at least 24 h prior to the feeding test, reduced the variation in their feeding responses. Andrew *et al.* (1989) found that the feeding rate of *G. pulex* infected with acanthocephalan parasites was lower than that of uninfected gammarids, suggesting that the use of infected organisms would reduce the chance of measuring the adverse effects of environmental stressors. Geffard *et al.* (2013) reported that the feeding activity of *G. fossarum* depends on temperature. They suggested that seasonal differences in feeding activity might reflect the influence of temperature on the production and activity of digestive enzymes (Geffard *et al.*, 2013). Similarly, Baillieul and Blust (1999) reported that swimming activity might be affected by body size. Therefore, the feeding activity of an organism reflects its status and the conditions of exposure media (Maltby *et al.*, 2002). In order to interpret feeding responses correctly all the appropriate confounding factors need to be taken into account.

Although *Gammarus* spp. are traditionally viewed as shredders under the functional feeding group classification (Cummins and Klug, 1979), it
has been reported that they can also serve as detritivores, predators, and even cannibals in aquatic ecosystems (MacNeil et al., 1997). Several studies have reported alterations in feeding activity of *G. pulex* as a response to environmental stressors. For example, under laboratory conditions the feeding activity of *G. pulex* was significantly reduced by Cd$^{2+}$ (Brown and Pascoe, 1989; Taylor et al., 1993; Felten et al., 2008; Alonso et al., 2009) and Cu$^{2+}$ (Blockwell et al., 1998). Taylor et al. (1993) reported that feeding reduction might be correlated with the dose and duration of exposure. In an *in situ* study, Maltby et al. (2002) reported significant inhibition (27 to 99.6%) in the feeding activity of *G. pulex* deployed at sites contaminated by a wide range of pollutants, including metals, ammonia, and organophosphorus, organochlorine and pyrethroid pesticides. Similarly, a considerable decrease (56%) in the feeding rate of *G. pulex* was observed when organisms were located downstream of highway runoff, thus polluted by metals such as Cd, Cr, Pb and Zn as well as aromatic hydrocarbons, as indicated by Forrow and Maltby (2000). This study suggested that water contaminators affect the feeding activity of the exposed animals either directly, via behaviour or physiology change, or indirectly, by change in food quality. In another study, Bloor and Banks (2006) examined the feeding response of *G. pulex* employing *in situ* and *ex situ* feeding tests. Test organisms were transplanted to several sampling sites along an undisclosed stream, which was polluted with leachate discharge from an unlined, disused British industrial dumping ground. They reported a significant decrease in the feeding activity of experimental organisms following both test conditions with greater reduction in the *in situ* tests. This may be due to the effect of complex mixture and other environmental factors, such as the water temperature, water flow and dissolved oxygen levels (Bloor and Banks, 2006).

The above data suggest that alteration in the feeding activity of *Gammarus* might present a useful toxicological endpoint. However, the effect of Pd exposure on the feeding activity of aquatic organisms has
not been investigated. Therefore, part of the current study was designed to examine the usefulness of the feeding behaviour of *G. pulex* as a response to exposure to sublethal Pd\textsuperscript{2+} concentrations.

### 1.5.4 Movement behaviour

Movement is an important ecologically relevant behavioural marker that is linked to energy metabolism and required for various important intra- and interspecific interactions (e.g. predator avoidance, find food and reproduction). Locomotion is energetically costly as it dependent mainly on muscular activity. At the same time, movement enables organisms to find food and to escape from predators. Hence, alteration in movement behaviour may offer an indicator of physiological damage and/or alteration in energy allocation patterns. Additionally, movement assays are generally inexpensive, quick, sensitive and easy to perform (Felten *et al.*, 2008). However, movement response may be influenced by several internal (e.g., physiological stress) or external (e.g., environmental instability) factors (Chon *et al.*, 2005).

Over the last few decades, several movement behaviours have been employed to examine the effects of contaminants on several aquatic test species (Little and Finger, 1990; Roast, 2000a, b; Roast *et al.*, 2001). For example, it was shown that monitoring of the swimming endurance of *Gammarus* could be considered a reliable technique for assessing the effect of water contamination on aquatic biota (Vellinger *et al.*, 2012). The sublethal effect of copper, pentachlorophenol and benzo[a]pyrene on pleopod beat frequency and the swimming stamina of *Gammarus duebeni* (Crustacea: Amphipoda) was investigated by Lawrence and Poulter (1998). Pleopods are specialised abdominal appendages in malacostraceans used both for swimming and for facilitating respiratory gas exchanges across the tegument (Vellinger *et al.*, 2012). It was found that the pleopods beat and the swimming stamina of the exposed organisms were significantly reduced as a consequence of 168 h exposure to 50 and 45 µg/L copper, respectively (Lawrence and Poulter,
These authors also observed a significant decrease in swimming stamina of *G. duebeni* following 136 h exposure to 20 µg/L pentachlorophenol or 8 µg/L benzo[a]pyrene. Similarly, Vellinger *et al.* (2012) reported a significant reduction in the swimming ability of *G. pulex* after 96 h exposure to sublethal doses of cadmium, arsenate or binary mixtures of these metals. In another study, the alteration in the horizontal and vertical movement activity of *Gammarus lawrencianus* (Crustacea: Amphipoda) as a response to cadmium exposure was investigated (Wallace and Estephan, 2004). They observed a significant reduction (50%) in vertical swimming following 72 h exposure to 62 µg/L, whilst horizontal movement was not significantly affected at up to 125 µg/L, but dramatically decreased (50%) following exposure to 250 µg/L. It was concluded that vertical movement was more sensitive to Cd\(^{2+}\) exposure than horizontal movement. This may be due to vertical movement requiring more energy than horizontal movement. Lloyd Mills *et al.* (2006) observed significant decrease in the horizontal movement activity of *G. pulex* treated with 10 µg/L copper. In another investigation, DeLange *et al.* (2006a) reported a reduction in time spent on the locomotor activity of *G. pulex* following exposure to low concentrations (10–100 ng/L) of three pharmaceuticals, fluoxetine, ibuprofen, and carbamazepine, and one cationic surfactant, cetyltrimethylammonium bromide (CTAB).

The above information suggests changes occur in movement behaviour of the *Gammarus* species as a rapid response to alteration in water quality. To the best of the author’s knowledge, no published data are available regarding the effect of PGEs on the movement behaviour of aquatic biota. Therefore, this study investigated the utility of the vertical movement of *G. pulex* as a marker of Pd\(^{2+}\) toxicity. This can improve knowledge regarding the possible environmental effects of Pd\(^{2+}\).
1.5.5 Acetylcholinesterase

Acetylcholinesterase (AChE) is an essential enzyme in the nervous system of animals that breaks down the neurotransmitter acetylcholine (ACh). It is released by the nerve fibre into the synaptic cleft and plays an important role in controlling muscle contraction. ACh is hydrolysed into choline and acetic acid by AChE, at cholinergic synapses and neuromuscular junctions. The binding of this neurotransmitter to ACh receptors in the muscle fibre membrane opens ion channels in the muscle fibre (Clarke and Trim, 2013). This allows ions to enter muscle cells and alter the resting membrane potential. A wave of depolarisation, thus produced, spreads down the muscle fibre, mobilises Ca$^{2+}$ ions, which bind to troponin, removing inhibition from actin-myosin leading to muscle contraction (Clarke, and Trim, 2013). Calcium plays an important role in the release of neurotransmitters, as well as in non-neural secretory processes (Katz and Miledi, 1970). Ca$^{2+}$ entry into the presynaptic neuron is essential to the release of neurotransmitters by an action potential (Zucker et al., 1986). Action potentials arriving at a presynaptic terminal open voltage gated Ca$^{2+}$ channels lead to a rapid increase in intracellular Ca$^{2+}$ concentration at the presynaptic active zone. Transmitter release is then triggered by Ca$^{2+}$ activating proteins of the exocytotic machinery, including synaptotagmins (Sudhof, 2012). After ACh is secreted into the synaptic cleft it is rapidly degraded by AChE. Therefore, inhibition of AChE will lead to ACh accumulation in the synaptic cleft and can cause abnormal activity in both neuronal and muscular tissue (Roex et al., 2003). This can subsequently lead to dysfunction in the impacted organisms, such as alterations in behaviour, paralysis and mortality (Fulton and Key, 2001; Xuereb et al., 2007).

In *G. pulex*, AChE activity in the head of is higher than that in the body (Xuereb et al., 2007). This is because the supraoesophageal ganglion (brain) of *Gammarus* is located in the head (MacPherson and Steele, 1980a, b). These authors reported that the supraoesophageal ganglion of *Gammarus* is anterior to the alimentary canal, between the eyes and
slightly posterior and ventral to the anterior and dorsal limits of the eyes in the head somite. The brain has a high density of cholinergic neurons and thus a higher content of AChE.

Several studies have found that AChE is associated with behavioural alterations. For example, Xuereb et al. (2009a) reported that, following 96 h exposure to the organophosphorus pesticide chlorpyrifos and the carbamate pesticide methomyl, the alteration in feeding and locomotor activity of adult male G. fossarum was significantly correlated with AChE inhibition. Likewise, reduction in AChE activity of the mysid (Neomysis integer: Crustacea, Mysidacea) by chlorpyrifos disturbed its swimming behaviour (Roast et al., 2000b). They reported that the pesticide-treated organisms became hyperactive than the control group. In another study, Jensen et al. (1997) reported that a 27% inhibition in AChE of the carabid beetle Pterostichus cupreus (Coleoptera: Carabidae), subsequent to 24 h exposure to the organophosphorous insecticide dimethoate, was associated with alteration in the locomotor behaviour of these organisms.

AChE inhibition has been widely employed as a specific biomarker to monitor organophosphate and carbamate pesticides (Bocquene and Galgani, 1991; Hamza-Chaffai et al., 1998; Najimi et al., 1997; McLoughlin et al., 2000). However, studies have shown that inhibition in AChE activity can also be used as a marker of metal toxicity (Olson and Christensen, 1980; Gill et al., 1991; Hamza-Chaffai et al., 1998; Martinez-Tabche et al., 2001; Table 1.6).

In contrast, several studies have reported that acute exposure to specific metals causes an increase in AChE activity in aquatic organisms. This has been observed in the brains of Rosy barb Barbus conchonius (Chordata: Cyprinidae) exposed to 7.7 mg/L Cd$^{2+}$ for 48 h (Gill et al., 1991). They found that AChE activity in the tested tissue of the exposed organisms was approximately 121% of the control value (Table 1.6).
Similarly, Thaker and Haritos (1989) demonstrated that AChE activity in *Pestarella tyrrhena* (Crustacea: Decapoda) increased significantly (145%) after organisms were exposed to 0.4 mg/L mercury (Hg) for 6 days (Table 1.6). Jebali *et al.* (2006) found that the brain AChE of *Seriola dumerili* (Perciformes: Carangidae) increased by 150% as a consequence of 48 h exposure to 50 μg/L Cd\(^{2+}\) (Table 1.6). Similarly, Cunha *et al.* (2007) reported that *in vivo* exposure to 1.53 mg/L Cd\(^{2+}\) significantly enhanced AChE activity in the dogwhelk *Nucella lapillus* (Mollusca: Gastropoda) (Table 1.6).

### Table 1.6 A summary of some studies that reported alteration in AChE activity in aquatic organisms by metal exposure

<table>
<thead>
<tr>
<th>Metals</th>
<th>Tissue/organisms</th>
<th>Exposure conditions</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercury</td>
<td><em>Pestarella tyrrhena</em></td>
<td>0.4 mg/L for 6 days</td>
<td>Significant increase</td>
<td>Thaker and Haritos</td>
</tr>
<tr>
<td></td>
<td>The freshwater fish <em>Cyprinus carpio</em> (Chordata: Cypriniformes)</td>
<td>100 μg/L for 24 h</td>
<td>Significant reduction</td>
<td>Suresh <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>Zinc</td>
<td><em>Osilinus lineatus</em> (Mollusca: Gastropoda)</td>
<td>600 μg/L for 24 h</td>
<td>Significant reduction</td>
<td>Cunha <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>Copper</td>
<td><em>Nucella lapillus</em></td>
<td><em>in vitro</em> exposure</td>
<td>Significant reduction (IC(_{50}) = 5.87 mg/L)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Significant reduction (IC(_{50}) = 12.17 mg/L)</td>
<td></td>
</tr>
<tr>
<td>Cadmium</td>
<td>The fish <em>Seriola dumerilli</em> (Chordata: Perciformes)</td>
<td>100 μg/L for 48 h</td>
<td>Reduction by 37%</td>
<td>Jebali <em>et al.</em> (2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250 μg/L for 48 h</td>
<td>Reduction by 85%</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Oreochromis niloticus</em> (Perciformes: Cichlidae)</td>
<td>100 μg/L for 28 h</td>
<td>Reduction by 24-32%</td>
<td>Chandrasekara <em>et al.</em> (2008)</td>
</tr>
<tr>
<td></td>
<td><em>Nucella lapillus</em></td>
<td>5-30 μg/L for 28 day</td>
<td>Reduction by 21-34%</td>
<td>Silva and Pathiratne</td>
</tr>
<tr>
<td></td>
<td><em>Oreochromis niloticus</em></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td><em>Barbus conchonius</em></td>
<td>7.7 mg/L for 48 h</td>
<td>Increase by 121%</td>
<td>Gill <em>et al.</em> (1991)</td>
</tr>
<tr>
<td></td>
<td><em>Seriola dumerili</em></td>
<td>50 μg/L for 48</td>
<td>Significant increase (150%)</td>
<td>Jebali <em>et al.</em> (2006)</td>
</tr>
<tr>
<td></td>
<td><em>Nucella lapillus</em></td>
<td>1.53 mg/L for 96 h</td>
<td>Significant increase (145%)</td>
<td>Cunha <em>et al.</em> (2007)</td>
</tr>
</tbody>
</table>
Previous studies have indicated that AChE activity could be employed to examine metal toxicity and that change in enzyme activity might be related to behavioural changes. Inhibition of AChE in *Gammarus* due to pesticide exposure is well documented (Kuhn and Streit, 1994; McLoughlin *et al.*, 2000; Xuereb *et al.*, 2007; Xuereb *et al.*, 2009), but very few studies have examined the potential of AChE in *Gammarus* as a biomarker of metal toxicity (Shirvan *et al.*, 2013). Therefore, more studies are needed to improve knowledge regarding metal impact on AChE in this genus. The effect of PGEs on AChE activity is not reported in the literature. Thus, it was considered important to examine whether Pd$^{2+}$ could affect AChE activity in *G. pulex* and whether it might account for any behavioural disturbances induced by Pd$^{2+}$.

### 1.5.6 Oxidative stress

Oxidative stress refers to a disruption of the balance between production of reactive oxygen species (ROS) and antioxidant defences in cells in favour of prooxidants leading to potential damage (Kochhann *et al.*, 2009). ROS are normal products of aerobic cell metabolism, especially mitochondrial respiration which presents an important endogenous source of ROS. However, the induction of ROS by chemicals such as pesticides and transitional metals (metals that have an incomplete penultimate electron shell and tend to exhibit more than one valency) has been previously reported (Sevcikova *et al.*, 2011). ROS are reactive molecules and free radicals, which are unstable highly reactive molecules that possess one or more unpaired electrons, derived from molecular oxygen. The enhanced production of ROS, such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH-) and the superoxide radical (O$_2^-$), can lead to cell damage (e.g. DNA damage, oxidation of lipids and proteins, and changes in gene expression) (Kochhann *et al.*, 2009; Rajkumar and Milton, 2011; Sevcikova *et al.*, 2011).

Metals can stimulate the Fenton reaction (Figure 1.1), in which the superoxide anion and H$_2$O$_2$ are converted to hydroxyl radicals, thus
inducing oxidative degradation of lipids (also known as lipid peroxidation) (Valko et al., 2005). Lipid peroxidation refers to the degradation of membrane phospholipids that contain polyunsaturated fatty acids by strong oxidants. Redox active metals can generate ROS through redox cycling of redox active metals, while the induction of ROS by redox-inactive metals can be attributed to their impact on antioxidant defences, especially those involving enzymes and thiol-containing antioxidants (Sevcikova et al., 2011). Metals can also facilitate the formation of free radicals under physiological conditions, inducing oxidative stress (Valko et al., 2005). To combat oxidative damage, cells maintain a complex web of enzymatic antioxidants (e.g. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione-S-transferase (GST)) and non-enzymatic antioxidants (e.g. vitamin E and C, and glutathione (GSH)) antioxidant defence systems (Trenzado et al., 2006).

\[
\begin{align*}
\text{Me}^{n+1} + \text{O}_2^{*^-} & \rightarrow \text{Me}^n + \text{O}_2 \\
\text{Me}^n + \text{H}_2\text{O}_2 & \rightarrow \text{Me}^{n+1} + \text{OH}^- + \cdot \text{OH}
\end{align*}
\]

Figure 1.1 Fenton reaction. Adapted from Valko et al. (2005). (Me\(^n\) and Me\(^{n+1}\) are the reduced and oxidised form of metal, respectively).

Lipid peroxidation occurs when the negatively charged head groups of phospholipids in cell membranes bind to metal ions, which are positively charged (Repetto et al., 2012). As a result of this binding, biological membranes may be adversely affected, impacting the cell and organism (Jemec et al., 2012).

The short half-lives of both free radicals and several compounds that are formed by free radical attack on electron-rich compounds (e.g. polyunsaturated fatty acids) complicates the assessment of oxidative stress. Therefore, the measurement of oxidative stress is mainly based on the quantification of the breakdown products of the oxidative
degradation of cell membrane lipids, such as malondialdehyde (MDA) (Oakes et al., 2003). MDA is a low-molecular weight by-product that is produced during the decomposition of lipid peroxidation products (Piotrowsk et al., 2004). One of the commonly used methods to estimate lipid peroxidation is measuring the level of thiobarbituric acid reactive substances (TBARS). This arises from the reaction of MDA with 2-thiobarbituric acid (Oakes et al., 2003; Pretto et al., 2010; 2011). Alternatively, lipid peroxidation can be measured by the determination of diene conjugation from the polyunsaturated fatty acids as indicated by Valavanidis et al. (2006). These authors, however, reported that this method was relatively insensitive to small alterations in the lipid peroxide level.

Metal exposure can increases the TBARS level in aquatic organisms (Table 1.7). In contrast, several studies have reported decreases in TBARS activity in test organisms as a result of metal exposure. This was observed in the tissues of the catfish *Rhamdia quelen* (Chordata: Anostomidae), exposed to 236 µg/L Cd^{2+} for 7 days (Pretto et al., 2010; 2011), and in the brain, muscle and liver of the fish, *Leporinus obtusidens* (Chordata: Anostomidae) exposed to 40 µg/L Cu^{2+} for up to

<table>
<thead>
<tr>
<th>Toxic</th>
<th>Tissue/ organisms</th>
<th>Exposure conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>The brain/ <em>Rhamdia quelen</em></td>
<td>236 µg/L for 14 days</td>
<td>Pretto et al. (2010; 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>414 µg/L for 7 days</td>
<td></td>
</tr>
<tr>
<td>copper pyrithione</td>
<td>The gill tissue/ juvenile brook trout <em>Salvelinus fontinalis</em> (Chordata: Salmonidae)</td>
<td>16 µg/L for 2 h</td>
<td>Borg and Trombetta (2010)</td>
</tr>
<tr>
<td>zinc</td>
<td>The brain, muscle and liver/ <em>Leporinus obtusidens</em></td>
<td>20 µg/L for 45 days</td>
<td>Gioda et al. (2007)</td>
</tr>
<tr>
<td>titanium dioxide nanoparticles</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>1000 µg/L for 14 days</td>
<td>Federici et al. (2007)</td>
</tr>
<tr>
<td>copper</td>
<td><em>Asellus aquaticus and Dreissena polymorpha</em></td>
<td>100 µg/L for 7 days</td>
<td>Bouskill et al. (2006)</td>
</tr>
</tbody>
</table>
45 days (Gioda et al., 2007). No significant alteration in TBARS activity was observed in *A. aquaticus* and *D. polymorpha* following 7 days of exposure to 80 µg/L As²⁺ (Bouskill et al., 2006).

Another approach that can be employed to investigate the effect of metals on the oxidation state is to examine the alteration in the concentration of GSH. This is an intracellular tripeptide, composed of glutamic acid, cysteine, and glycine. It is involved in several central processes in the cell, including maintaining cellular homeostasis and the protection of cells against the impact of xenobiotics, reactive electrophiles and oxidative stress (Pompella et al., 2003; Niedźwiecka et al., 2011). GSH is an important non-enzymatic antioxidant that can scavenge oxygen radicals and thus minimise lipid peroxidation in cell membranes (Regoli and Principato, 1995). The thiol group in cysteine performs varied functions in GSH, allowing GSH to chelate metals and participate in redox cycling (Jozefczak et al., 2012). GSH may form a stable complex with metals reducing their ability to induce oxidative stress (Canesi et al., 1999; Wang et al., 2008). Singhal et al. (1987) reported that GSH may represent the first line of defence against metal toxicity (e.g. Cd²⁺). The induction of GSH due to metal exposure has been reported in mammalian (Woods and Ellis, 1995) and fish tissues (Thomas and Juedes, 1992). In contrast, Canesi et al. (1999) reported a reduction in GSH content in the gills and digestive gland of the mussel *Mytilus galloprovincialis* (Mytiloida: Mytilidae) following 24 h exposure to either 0.04 mg/L Cu²⁺ or 0.046 mg/L CH₃HgCl. The same study, however, observed that 0.4 mg/L inorganic Hg²⁺ did not cause a significant change in GSH concentrations in the examined tissues (Canesi et al., 1999). This difference in GSH responses may reflect the higher availability of Hg in the organic form than that of the chloride form. Reduction in GSH concentration may be a consequence of a stimulation of GST activity (Canesi et al., 1999). GST is a family of enzymes that catalyse the conjunction of reduced glutathione (GSH) to a variety of electrophilic substrates and are implicated in reducing
oxidative damage by conjugation of the products of lipid peroxides to GSH (Belabed and Soltani, 2013).

Induction of GST activity has also been proposed as an indicator of oxidative stress (Veal et al., 2002). Belabed and Soltani (2013) reported that GST activity in the marine bivalve Donax trunculus (Veneroida: Donacidae) was significantly increased following 24 h exposure to 2.59 mg/L Cd. In another study, Farombi et al. (2007) examined the effect of metal exposure on GST activity in the heart, liver and kidney of African catfish Clarias gariepinus (Siluriformes: Clariidae). Samples were collected from either a river polluted with metals (Zn, Cu, Pb, As and Cd) or from an unpolluted fish farm (Farombi et al. 2007). They found that the metal concentrations and GST activities in all tested tissues were significantly higher in organisms collected from the polluted river than that of the unpolluted samples. With regard to the use of GST as a biomarker in crustaceans, the majority of available data have examined the enzyme response to pesticides exposure (Blat et al., 1998; McLoughlin et al., 2000; Forcella et al., 2007). Therefore, more studies with different substances, such as metals, are needed to test the efficacy of GST as a general stress marker in crustaceans.

The previous studies mentioned above showed that a variety of oxidative parameters can be employed to investigate the effect of chemical exposure on aquatic organisms. However, Pd-induced oxidative stress in aquatic biota has not been reported in the literature. Thus, the usefulness of several oxidative markers, TBARS, GST, GSH and total thiol group, in G. pulex as markers of Pd\(^{2+}\) toxicity was examined in the current thesis. These assays are relatively sensitive, quick and inexpensive.

1.5.7 Osmoregulation

Osmoregulation is a core energetically expensive regulatory function that enables aquatic invertebrates to survive in freshwater (Vellinger et
al., 2012). The survival of freshwater organisms requires maintenance of their body’s osmotic concentration at a level higher than that of the external medium. All gammarid species, including *G. pulex*, are hyperosmotic regulators. They can keep their haemolymph concentration higher than that of the surrounding medium (Felten *et al*., 2008). In fresh water, hyperosmotic regulators are challenged by water ingress and ion losses. To maintain the body osmolality, organisms such as crustaceans may produce copious volumes of diluted urine to excrete excess water. In addition, active ion uptake from the external medium across the gills into the body fluids is required (Felten *et al*., 2008). The gills contain cells specialized in ionic trans-membrane transport, and play central roles in gaseous exchange, the acid–base balance and osmoregulation (Péqueux, 1995; Vellinger *et al*., 2012).

Crustaceans may also restrict the surface area of permeable surfaces to reduce ion loss and water gain (Brooks and Lloyd Mills, 2003). Osmotic gradients could induce stresses on the intercellular junctions that hold the gill epithelial cells together. These junctions play a key role in the maintaining osmotic gradients between the haemolymph and external environment and thus it is expected that their morphological characteristics are correlated to the level of the stress imposed (Shires *et al*., 1994). For example, *G. pulex*, which maintains a gradient of some 300 mOsmol across the gill epithelium, possessing a well-developed junctional microtubular system with microtubules on both sides of relatively long septate junctions (Shires *et al*., 1995). This reinforce the structure properties of the septate junctions makes it more resistant to osmoregulation stresses. In contrast, the less euryhaline species, such as *G. duebeni*, which is found in brackish water habitats, show a shorter length of septate junctions accompanied by one or sometimes by a double row of microtubules (Shires *et al*., 1995).

Whereas aquatic organisms can reduce the general permeability of their bodies, some permeable surfaces, such as gills, are still needed for the
exchange of dissolved gases (Brooks and Lloyd Mills, 2003). The gills of aquatic crustaceans are also an important site of metal diffusion and uptake. Ion losses can also be offset by the activity of some active transport enzymes, such as Na\(^+\), K\(^+\)-ATPase (Brooks and Lloyd Mills, 2006). This latter enzyme regulates the exchange of Na\(^+\) and K\(^+\) across the basolateral membrane and thus comprises a significant proportion of the energy required for the active uptake of Na\(^+\) and Cl\(^-\) (Morgan et al., 2005). In most crustaceans, Na\(^+\) and Cl\(^-\) provide more than 90% of the osmotic pressure of the haemolymph (Felten et al., 2008). Based on the model of crabs by Towle and Weihrauch (2001), a modified model of gill cell osmoregulation in *G. pulex* is shown in Figure 1.2.

The gills of freshwater crustaceans have an apical vacuolar-type H\(^+\)-ATPase (V-ATPase) pump (Figure 1.2), which makes the inside of the cell more negatively charged with respect to the extracellular fluid (Towle and Weihrauch, 2001). Therefore, Na\(^+\) can be taken up by the gill epithelial cells via apical sodium channels, against a Na\(^+\) concentration gradient (Onken and Riestenpatt, 1998; Brooks and Lloyd Mills, 2003). Na\(^+\), K\(^+\)-ATPase activity pumps 3 Na\(^+\) ions out of cells into the haemolymph and 2 K\(^+\) ions into cells, across the basolateral membranes. Consequently, Na\(^+\), within the gill epithelial cells, is maintained at a lower concentration than that of the surrounding extracellular fluid, enabling cells to maintain their osmotic balance (Towle and Weihrauch, 2001).

Potassium that enters the cells is recycled by basolateral K\(^+\) channels (Figure 1.2). These channels also contribute to the membrane potential of the cell, which plays a key role in the passive movement of ions across the plasma membrane (Cotton, 2000). In basolateral membranes, Cl\(^-\) channels may also play a crucial role in the maintenance of the intracellular Cl\(^-\) and the membrane potential of the cell (Figure 1.2) by recycling the cellular Cl\(^-\) (Halm, 2005).
Figure 1.2 Proposed cell model of osmoregulation in *G. pulex*. Adapted from Towle and Weihrauch (2001). CA1 and CA2 are the basolateral and cytosolic form of carbonic anhydrase. Pumps are indicated by ● and exchangers by ○.

The Na\(^+\)/H\(^+\) exchanger is also involved in ion regulation in freshwater crustaceans (Figure 1.2). This protein mediates the movement of Na\(^+\) and H\(^+\) across the plasma membrane according to their electrochemical gradient (Towle and Weihrauch, 2001). It is not currently known whether *G. pulex* have apical Na\(^+\) channels and/or N\(^+\),K\(^+\) exchangers. H\(^+\),K\(^+\) ATPase is another enzyme that may be involved in osmoregulation (Figure 1.2). This enzyme uses the energy of ATP to regulate the exchange of cytoplasmic hydrogen ions and external potassium ions.
against their concentration gradients (Dunbar and Caplan, 2001). The latter action can reduce the acidity inside cells. Other proteins, such as Na\(^+\)/K\(^+\)/2Cl\(^-\) co-transporter, which transports Na\(^+\), K\(^+\), and 2 Cl\(^-\) ions into the cytoplasm, depending on an electrochemical gradient for Na\(^+\), may play a role in ionoregulation (Towle and Weihrauch, 2001). In the freshwater gammarid G. pulex, the Na\(^+\)/K\(^+\)/2Cl\(^-\) co-transporter is probably not present, as the Na\(^+\) gradient may not be sufficient to drive ion uptake from fresh water (Towle and Weihrauch, 2001).

Carbonic anhydrase is involved in active ion transport across crustacean gills (Pavičić-Hamer et al., 2003). Basolateral membrane associated carbonic anhydrase converts HCO\(_3\) and H\(^+\) into CO\(_2\) and H\(_2\)O. The CO\(_2\) then diffuses across the basolateral membrane (Figure 1.2). The cytosolic form of carbonic anhydrase catalyses the hydration of CO\(_2\) within gill cells (Figure 1.2), providing the counter-ions for Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\)\(^-\)-exchangers (Towle and Weihrauch, 2001; Henry, 2006). They respectively aid Na\(^+\) and Cl\(^-\) uptake from the water (Figure 1.2).

A reduction in Na\(^+\) content in the haemolymph of G. pulex can activate Na\(^+\) uptake mechanisms in the gills and antennal gland (Brooks and Lloyd Mills, 2003). In crustaceans, the potential role of the antennal gland in ion regulation has been previously reported (Lin et al., 2000; Chung et al., 2006). The main function of this organ is urine production. By filtering the haemolymph, urine is produced. In G. pulex, the antennal gland reabsorbs ions from the urine, so that it is markedly hypotonic to the haemolymph and thus participates in osmoregulation by reducing ion loss from the haemolymph (Brooks and Lloyd Mills, 2003; Chung et al., 2006).

Osmoregulation disruption has been described as a physiological biomarker of various stresses, including metals, in crustaceans (Lignon et al., 2000). Osmoregulation of G. pulex was impacted by exposure to Cu\(^{2+}\) (0.1 mg/L for 4 h; Brooks and Lloyd Mills, 2003), zinc (2 mg/L for
4 days) (Spicer et al., 1998) and Cd^{2+} (0.0075 mg/L for 5 days) (Felten et al., 2008). Metals can impact osmoregulation by increasing the rates of sodium loss and/or reducing sodium uptake (Brooks and Lloyd Mills, 2003). In fresh water, whilst urine produced by unstressed *G. pulex* is more dilute than haemolymph, this urine is still highly concentrated compared to fresh water (Lockwood, 1961). As a result, organisms will lose ions, including sodium, to the environment via urine. To reduce the loss rate of Na^{+} to the external environment, Na^{+} may be reabsorbed from the urine in the antennal gland (Péqueux, 1995). Towle (1981) observed the high activity of Na^{+}/K^{+} ATPase in the gills and the antennal gland of three terrestrial crabs (Crustacea: Decapoda): *Cardisoma carnifex*, *Gecarcoidea lalandii*, and *Birgus latro*. Brooks and Lloyd Mills (2003) suggested that a higher sodium loss rate may, in part, occur when a metal inhibits Na^{+}/K^{+} ATPase activity. They also reported that reduction in Na^{+}/K^{+} ATPase activity would also reduce Na^{+} uptake from the surrounding medium. In addition, metal-induced damage to the structural integrity of epithelial cells may induce copious urine production due to excess water ingress. This may, in turn, heighten the loss of Na^{+} from the body, particularly if antennal gland Na^{+}/K^{+} ATPase is inhibited. The impact of metal exposure on Na^{+}/K^{+} ATPase activity has been observed in previous investigations. For example, a significant inhibition (50-60%) in the enzyme activity in the shore crab *Carcinus maenas* (Crustacea: Decapoda), was observed following 7-days exposure to 4.7 mg/L copper (Hansen et al., 1992). Similarly, Na^{+}/K^{+} ATPase in *G. pulex* was significantly inhibited by copper (*in vitro* exposure to 10 µg/L) (Brooks and Lloyd Mills, 2003). In contrast, Felten et al. (2008) reported a significant increase in the enzyme activity (19%) in *G. pulex* following 7 days exposure to 7.5 µg/L cadmium. This may have arisen via an increase in the amount of Na^{+}/K^{+} ATPase in an attempt to compensate for cadmium-induced osmotic stress.

Metals can also inhibit carbonic anhydrase, reducing the intracellular supply of H^{+} and HCO_{3}^{-}, which are needed for ion exchangers (N^{+}/H^{+} and
HCO$_3^−$/Cl$^−$ (Henry et al., 2012). Vitale et al. (1999) reported a significant inhibition of carbonic anhydrase (50%) and haemolymph Na$^+$ concentration (6%) in the estuarine crab Chasmagnathus granulate (Crustacea: Decapoda), following 96 h in vivo exposure to 1.25 mg/L Cd$^{2+}$. The same study reported that the enzyme activity was inhibited by 50% following in vitro exposure to 0.24, 0.1 and 0.238 µg/L Cd$^{2+}$, Zn$^{2+}$ and Cu$^{2+}$, respectively. Skaggs and Henry (2002) reported the cytoplasmic carbonic anhydrase form in the gills of crabs Callinectes sapidus (Crustacea: Decapoda), was inhibited following in vitro exposure to 0.056, 0.022, 0.005 and 0.126 mg/L of Cd$^{2+}$, Cu$^{2+}$, Ag$^{2+}$ and Zn$^+$, respectively. The same study reported that the cytoplasmic form of the enzyme in the gills of crabs of C. maenas was about 1000-times less sensitive to the examined metals than that of C. sapidus (Skaggs and Henry, 2002).

The effect of metals on enzyme activities, such as Na$^+/K^+$ ATPase and carbonic anhydrase, may result from oxidation of thiol groups in an enzyme by metal ions. As they have high affinity and attraction for sulphur, metals can break the disulphide bridges and bond to the sulphide functional groups, leading to protein denaturation and thus dysfunction (Brooks and Lloyd Mills, 2003; Reger et al., 2009). Alternatively, a toxic metal can competitively displace the resident metal of a metalloprotein, disturbing its structure and function (Brooks and Lloyd Mills, 2003). The formation of metal complexes and/or salts with proteins is another potential mechanism of how metal can affect enzyme activity (Brooks and Lloyd Mills, 2003).

The competition for uptake sites is another potential mechanism of metal impact on osmoregulation capability in aquatic biota (Spicer et al., 1998). Silver (Ag$^+$) exposure caused a significant inhibition in Na$^+$ uptake by D. magna, as indicated by Bianchini and Wood (2003), who observed that this inhibition was, at least partly, due to the competition between Ag$^+$ and Na$^+$ at the Na$^+$ uptake channel. The same study
reported that Ag\(^+\) did not affect Cl\(^-\) concentrations in the exposed organisms (Bianchini and Wood, 2003). McGeer et al. (2000) reported that several metals, such as Zn\(^{2+}\), Cu\(^{2+}\) and Cu\(^{2+}\), can inhibit Ca\(^{2+}\) influx, at least partly via competition at the apical calcium channels.

Another hypothesis is that metals can disturb gill structure and thus function (e.g. they become leakier) (Brooks and Lloyd Mills, 2003; Issartel et al., 2010). This can lead to a consequent reduction in Na\(^+\) concentration in the haemolymph, disrupting osmoregulation (Brooks and Lloyd Mills, 2003).

There is a lack of knowledge regarding the effect of PGEs on the osmoregulation capability of aquatic biota. To the best of the author’s knowledge, the effect of Pd\(^{2+}\) exposure on the osmoregulatory function of crustaceans has not been investigated. In \textit{G. pulex}, osmoregulation consumes approximately 11\% of the total energy budget (Sutcliffe, 1984). Hence, a significant increase in the energetic cost of metabolism may occur when stresses cause a functional disturbance in the osmoregulation of this organism, even if mortality is not observed (Brooks and Lloyd Mills, 2003). To better assess and understand the impact and action mechanisms of Pd\(^{2+}\), part the current thesis examines the potential effect of Pd\(^{2+}\) exposure on concentration of the main haemolymph cations (Na\(^+\), K\(^+\), Ca\(^{2+}\) and Mg\(^{2+}\)) as an indicator of osmoregulatory damage in \textit{G. pulex}. These cations, especially Na\(^+\), play important roles in osmotic regulation in aquatic crustaceans; thus alteration in their concentrations in the haemolymph has been suggested as a marker of disruption of ionic/osmoregulation (Felten et al., 2008).

\textbf{1.5.8 Cell viability}

Cytotoxicity endpoints have been frequently examined with metabolic viability assays, such as the reduction of tetrazolium salt in the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. It
was initially thought that MTT reduction was based on mitochondrial succinate dehydrogenase (SDH) activity, thus reflecting the number of living cells with functional mitochondria (Slater et al., 1963). Therefore, this assay has been widely employed to examine cell proliferation and viability. Later studies, however, have shown that a significant proportion of MTT reduction can occur outside the mitochondrial inner membrane (Berridge and Tan, 1993). MTT reduction can be associated with the cytoplasm and with non-mitochondrial membranes including the endosome/lysosome compartment and the plasma membrane (Berridge et al., 2005), and involves the pyridine nucleotide cofactors NADH and NADPH (Berridge and Tan, 1993). Thus, MTT reduction can be employed as an indicator of cellular metabolic activity.

SDH is an enzyme that plays important roles in mitochondrial metabolism, as it is involved in both the Krebs cycle, which is an important pathway of ATP generation, and the respiratory electron transport chain. These processes involve conversion of succinate into fumarate in the mitochondrial matrix and the ubiquinone reduction in the mitochondrial inner membrane. SDH is inhibited by the binding of oxaloacetate, and is activated by ATP (Huang and Millar, 2013).

The MTT assay has been employed in several studies to examine the cytotoxic impacts of chemicals on cell viability. For example, the toxic impact of up to 0.3 mg/L Cd$^{2+}$ on the hepatoma cell line, HepG2, was examined employing four cytotoxicity assays: the neutral red assay, the lactate dehydrogenase leakage assay (LDH), a protein assay and the MTT assay (Fotakis and Timbrell, 2006). They found that, in comparison to other tested assays, MTT reduction showed the highest sensitivity to metal exposure. Wataha et al. (1992) used MTT reduction in cultured murine fibroblasts (Balb/c 3T3) to assess single exposures to several metal cations. They found that the toxic effect of Cd$^{2+}$, Cu$^{2+}$, Ga$^{3+}$, and Ni$^{2+}$ were positively correlated to the period of exposure, whereas the toxicity of Ag$^+$ and Zn$^{2+}$ were not time dependent. In an in vitro study, 2 h exposure to either 0.011 mg/L Cd$^{2+}$ or 0.63 mg/L Cu$^{2+}$ reduced the
conversion of MTT in the sea bass *Dicentrarchus labrax* (Chordata: Moronidae) blood cells by 75% and 50%, respectively (Vazzana *et al.*, 2010). Similarly, Kang *et al.* (2010) reported that 24 h exposure to 5-50 µg/L Cd^{2+} caused a significant dose-dependent decrease in MTT reduction in spermatogonic cells of the oriental river prawn, *Macrobrachium nipponense* (Crustacea, Decapoda). MTT reduction in the established RF fish cell line, developed from fin tissue of the freshwater fish, rohu, *Labeo rohita* (Chordata: Cyprinidae) was inhibited as a response to *in vitro* exposure to Zn^{2+} or Cd^{2+} salts (Goswami *et al.*, 2013). They reported that the half-maximal inhibitory concentration values were 53.38, 26.44, 20.26, 5.17, 4.97 mg/L for ZnSO_{4}, Zn(NO_{3})_{2}, ZnCl_{2}, CdSO_{4}, CdCl_{2}, respectively, indicating that Cd^{2+} was significantly more cytotoxic than Zn^{2+}.

Although previous studies indicated the utility of the MTT assay to investigate the cytotoxic impact of metals, these studies mainly employed *in vitro* systems using isolated or/and cultured cells. *In vitro* models ignore any potential alteration that the toxicant may undergo as it is taken up by the organisms (e.g. excretion and detoxification). It is of interest to employ *in vivo* systems to examine the cytotoxic effects of chemicals. This approach would provide more environmentally relevant data regarding chemical toxicity. Therefore, part of the present study aimed to investigate the impact of *in vivo* Pd^{2+} exposure on metabolic activity in *G. pulex*, employing the MTT assay.

### 1.5.9 Heat shock proteins

Heat shock proteins (HSPs), also known as stress proteins, are families of proteins expressed by organisms in response to a wide range of environmental stresses. These proteins were first found to be induced due to a sudden rise in temperature, hence their designation as HSPs (Harboe and Quayle, 1991). HSPs are classified into several groups according to their molecular weight in kiloDaltons (kDa) (e.g. HSP40, HSP60, HSP70, etc.) (Bornfeldt, 2000).
HSPs are expressed in cells under normal conditions and play key roles in several fundamental processes in the cell including the stabilisation, assembly and folding of denatured and newly synthesised proteins and their translocation across cell membranes (Hartl and Martin, 1995; Feder and Hofmann, 1999; De Maio, 1999; Buchanan, 2000). These proteins are also involved in the stimulation of the innate and acquired immune systems (Pockley, 2003; Javid et al., 2007). The heat shock response is also a mechanism of stress adaptation to protect cellular proteins from the potential damage caused by a range of stressors (e.g. extremes of temperature, acidity, toxin exposure, hypoxia and starvation (Choresh et al., 2001; Kunz et al., 2010 heat, and metals). Stressful environmental conditions may lead to protein misfolding, and consequently increase the synthesis of several HSPs in an effort to reduce adverse effects. In such situations, HSPs play a cytodefensive role through their functions in protein folding and degradation (Wu and Tanguay, 2006). Bensaude et al. (1990) reported that HSPs are soluble under unstressed conditions and may become insoluble under stress conditions. HSPs may bind to the hydrophobic domains of temporarily denatured proteins, leading to structural alteration in HSPs or the overall proteins in stressed cells. Studies have reported that the induction of HSPs can be linked to a reduction in individual fitness, such as growth (Feder et al., 1992), development rate (Krebs and Feder, 1997) and fertility (Silbermann and Tatar, 2000).

HSP70 is one of the most abundant families of HSP. Studies have shown the production of various isoforms of HSP70 by different organisms. This group of proteins performs key functions in several subcellular compartments (e.g. mitochondria, endoplasmic reticulum, cytosol, lysosomes) and extracellular compartments (Stetler et al., 2010). This family of HSPs is involved in fundamental processes in the cell, including regulation of the folding of nascent polypeptides and partially unfolded proteins and the repair or refolding of denatured proteins (Bačkor et al., 2006).
HSP60 is another group of HSPs. The inducible form of HSP60 is located mainly in the mitochondrial matrix (Werner and Nagel, 1997). Following the introduction of some mitochondrially targeted proteins into the mitochondrial matrix, their refolding may interact with and be catalysed by HSP60. Therefore, dysfunction of these proteins may lead to aggregation of imported proteins into insoluble complexes as a result of incorrect folding (Hallberg et al., 1993).

HSP90 is a family of proteins with a molecular weight range between 82 and 90 kDa (Lindquist, 1992) that are highly expressed in the cells of eukaryotes. This family of proteins is abundantly expressed and found in several subcellular compartments, such as mitochondria, cytosol and the endoplasmic reticulum (ER) (Stetler et al., 2010; Zuehlke and Johnson, 2010). HSP90 may interact with HSP70 due to the overlap in the location and expression of these two HSP families. HSP90 plays an important role in the regulation of protein activity, mainly via multichaperone complex binding and sequestration of substrate proteins in active or inactive forms (Stetler et al., 2010). Under normal conditions, these proteins are associated with numerous intracellular proteins including actin, protein kinases, calmodulin, tubulin, and several receptor proteins (Gupta et al., 2010).

The induction of HSPs has been investigated in a wide range of aquatic biota including protozoa, echinoderms, molluscs, crustaceans and fish (Sanders, 1993). In fish, for example, HSP70 induction has been observed in extracts from different types of fish cells including primary cell cultures, cell lines and the tissues of whole organisms (Iwama et al., 1999). The induction of HSPs in aquatic biota can be considered a potential biomarkers of exposure to environmental stressors including metals. For instance, Werner and Nagel (1997) observed an induction of HSP60 and HSP70 in three species of aquatic amphipods, *Ampelisca abdita* (Amphipoda: Ampeliscidae), *Rhepoxynius abronius* (Amphipoda: Phoxocephalidae) and *H. azteca*, following 24 h of Cd$^{2+}$ exposure.
Similarly, HSP70 content in *G. fossarum* significantly increased following 120 h exposure to 8 µg/L Cd²⁺ (Schill *et al.*, 2003). In the gill tissue of male *C. maenas*, 14 days of exposure of up to 0.1 mg/L Cu²⁺ only significantly increased HSP70 levels at 0.1 mg/L (Vedel and Depledget, 1995). They concluded that HSP70 was not a sensitive indicator for Cu²⁺ exposure in this particular species. Radlowska and Pempkowiak (2000) reported that 8 days exposure to 0.2 mg/L Cd²⁺, Pb²⁺ or Cu²⁺ increased HSP70 levels in the blue mussel *Mytilus edulis* (Mytiloida: Mytilidae). They also observed a higher HSP70 response when *M. edulis* was exposed to a combination of Cu²⁺ (0.1 mg/L) and Cd²⁺ (0.1 mg/L) in comparison to that resulting from exposure to comparable concentrations of single metals (0.2 mg/L Cu²⁺ or Pb²⁺). This finding suggests that Cu²⁺ and Pb²⁺ stimulated the uptake and/or reduce the elimination of each other. It might also be that Cu²⁺ and Pb²⁺ formed soluble complexes and thus increases the bioavailability of these metals to the exposed organisms.

The effect of 70 days exposure to PGEs, Cd or Pb on the expression of HSP70 in *D. polymorpha* was examined by Singer *et al.* (2005). They observed positive relationships between HSP70 levels and concentrations of metals in all tested groups. However, at day 39, all exposed groups had their highest levels of HSP70 but this reduced to initial concentrations over 70 days, suggesting some forms of adaptation had occurred. Rios-Arana *et al.* (2005) reported that induction of HSP60 in the rotifer *Plationus patulus* (Rotifera: Brachionidae) could be a useful indicator of both single and combination exposure to several metals (Cr, Cu, Pb, Ni, Zn, and As). HSP60 concentrations in *P. patulus* after 20 min of exposure to Cr, Cu, Pb, Ni, or Zn (50 µg/L except for Pb 100 µg/L) were approximately 2-fold higher than that of untreated groups, which was in turn 2-fold higher than that of the 50 µg/L As group, suggesting that some metals may induce HSPs while others depress them.

The induction of HSPs has been observed in numerous aquatic species including *Gammarus*, and as a response to a wide range of metals
including PGEs. However, information regarding the induction of HSPs in *Gammarus* as a response to PGE exposure is too limited. Sures and Radszuweit (2007) observed a 3-fold increase in HSP70 concentration in *Gammarus roeseli* following 24 days exposure to 10 µg/L Pd. To date, the effects of PGE exposure on HSPs in *G. pulex* has not been investigated. Hence, it was important to examine the usefulness of using HSP responses in *G. pulex* as biomarkers of Pd$^{2+}$ exposure in the present study. Such an investigation can offer a better understanding with regard to the toxicity of Pd$^{2+}$ which is needed to determine the risks associated with Pd$^{2+}$ exposure.

### 1.5.10 Protein phosphorylation

Phosphorylation plays a central role in the regulation of the majority of cellular processes, such as cellular division, growth, signal transduction, differentiation, cell motility, immunity, learning and memory, cytoskeletal regulation, membrane fusion and transport and metabolism (Groban et al., 2006; Ubersax and Ferrell, 2007; Szöör, 2010; Pearlman et al., 2011; Leach and Brown, 2012). Protein phosphorylation is a ubiquitous type of post-translational modification used in signal transduction. A protein is phosphorylated when a phosphate group is transferred, often from ATP, to one of its amino acid side chains. In eukaryotes, serine, threonine, and tyrosine are the most common sites of protein phosphorylation. These amino acids have a hydroxyl (−OH) group, which presents the main target for the transferred phosphate group (Groban et al., 2006; Mullis, 2008). In contrast, a phosphorylated protein may be switched back to its original conformation by removing the added phosphate group. This reaction is known as dephosphorylation and is catalysed by a group of enzymes called phosphatases, whilst protein kinases are the group of proteins that catalyse protein phosphorylation (Cohen and Cohen, 1989; Purves et al., 2001). Covalently bound phosphate is present in approximately 30% of human proteins, and the human genome encodes approximately 500 protein kinases and more than 150 protein phosphatases (Cohen, 2000).
Both phosphatases and kinases can be divided into two groups, tyrosine and serine/threonine specific. Although the overall structure of proteins in each group of kinases is very similar, the presence of certain short amino acid stretches in the enzyme’s structure can be used to predict whether it would affect the phosphorylation state of serine/threonine or tyrosine (Hanks et al., 1988). Generally, protein phosphatases are classified into two broad families, protein phosphatase type 1, (PP1), and protein phosphatase type 2, (PP2). One of the roles of protein phosphatases in the body is to reverse the effects of unregulated protein kinase C activation, and thus reduce the induction of tumours (Mullis, 2008). PP1 phosphatases are involved in the regulation of several cell processes including glycogen metabolism, protein synthesis and intracellular transport (Bollen and Stalmans, 1992). PP2 plays a role in cell cycle regulation, dephosphorylation of transcription factors, T-cell activation and deactivation, and signal transduction pathways. This type of protein phosphatase can be divided into three groups, termed PP2A, PP2B, and PP2C proteins (Mullis, 2008). PP2A is constitutively active, whereas PP2B and PP2C are activated by binding Ca$^{2+}$ and Mg$^{2+}$, respectively (Colbran, 2004).

The phosphorylation level of a target protein reflects the net effect of kinase–phosphatase competition (Purves et al., 2001). The phosphorylation sites on a protein are varied and thus may affect the activity of proteins differently. This increases the need for approaches that provide an accurate identification of these sites (McLachlin and Chait, 2001). Antibody detection methods are among the most popular approaches used to measure protein phosphorylation in tissue slices, fractionated material from organs and from whole organisms. Using this approach, proteins are separated by one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane and probed with a phospho-specific antibody to assess the phosphorylation state of proteins.
Toxins can disrupt protein kinases and/or phosphatases, leading to the disrupted phosphorylation of cellular proteins (Cohen, 2000). The alteration in the protein phosphorylation state due to metal exposure has been reported in numerous studies. For example, Pb$^{2+}$ caused a significant increase in the phosphorylation of the extracellular signal-regulated kinase 1/2 (ERK1/2) and mitogen-activated protein kinase P38 (MAPKs) in cerebellar slices of the *R. quelen* following both *in vivo* (3 h to 5 μM) and *in vitro* (48 h to 4.8 mM) exposure (Leal et al., 2006). Similarly, Kefaloyianni *et al.* (2005) found that 2 h exposure to Cu$^{2+}$ and Zn$^{2+}$ at sublethal concentrations (0.06 and 3.27 mg/L, respectively) produced a significant increase in the phosphorylation of P38 (MAPKs) in both the mantle and gill tissues of *M. galloprovincialis*. Burlando *et al.* (2003) examined the alteration in tyrosine kinase signalling in rainbow trout hepatoma cells (RTH 149) following 18 days caging at 4 sites contaminated with different Cu$^{2+}$ concentrations. They observed that the level of tyrosine phosphorylation in the treated cells was positively correlated with metal concentration and the period of exposure.

Studies regarding the utility of using the alteration in protein phosphorylation state in crustaceans as a stress marker are quite limited. For instance, exposure to the pesticide methoprene changed the pattern of protein phosphorylation in epithelial tissues of postmoult juvenile lobsters, in which the level of phosphorylation increased for some proteins and decreased for others (Walker, 2005).

The majority of available data regarding phosphoproteins is concentrated on mammalian systems. To the best of the researcher’s knowledge, this is the first study attempting to investigate the potential proteomic changes of *G. pulex* due to Pd$^{2+}$ exposure. Observed alterations in proteins may improve knowledge regarding Pd$^{2+}$ toxicity and indicate the potential of using such alterations as bioindicators of aquatic ecosystem quality. Therefore, the potential effect of Pd$^{2+}$ exposure on the phosphorylation level of protein bound serine, threonine
and tyrosine in soluble and particulate fractions of *G. pulex* was investigated employing SDS-PAGE and Western blotting analysis.

### 1.6 Aims and objectives of the study

The aims of the present study were to:

1. **Determine the 96 h LC$_{50}$ values of PGEs for *G. pulex*.** The tested organisms were exposed to several concentrations of Pd, Pt, Rh, Os, Ir or Rh. Survival was monitored every 24 h for 96 h. The data were then analysed to calculate the 96 h LC$_{50}$ concentrations of each tested element.

2. **Examine whether coexposure to PGE combinations affect the survival of *G. pulex* and whether there was any interaction between the uptake of Pd$^{2+}$ and Pt$^{4+}$ or Rh$^{3+}$ by *G. pulex*.** The test organisms were exposed to a combination of an acute Pd$^{2+}$ and a range of Pt or Rh concentrations to compare the mortality of the treated organisms under those different conditions. The uptake of examined PGEs by *G. pulex* were also tested following exposure to either single or combination solutions of these metals.

3. **Study the behavioural response (feeding and the vertical movement) of *G. pulex* to sub-lethal concentrations of Pd$^{2+}$ and examine whether these responses were associated with change in AChE activity.**

4. **Investigate the molecular basis of Pd$^{2+}$ toxicity with particular emphasis on osmoregulatory disturbance, oxidative damage, mitochondrial function, protein phosphorylation and the heat shock response.** The following approaches were employed to reach this objective:
   
   a) **measurement of Pd$^{2+}$-induced alteration in haemolymph cation concentrations (Na$^+$, K$^+$, Ca$^{2+}$ and Mg$^{2+}$),** which play key roles in osmoregulation.
b) examination of changes in several oxidative stress parameters (TBARS, GSH, GST and the level of free thiol groups).

c) application of the MTT assay to examine the effect of Pd exposure on metabolic activity in *G. pulex*.

d) Study of the induction of HSPs (HSP60, HSP70 and HSP90) due to Pd exposure.

e) examination of the effect of Pd exposure on levels of phosphoserine, phosphotyrosine and phosphothreonine in *G. pulex*.
2 Material and Methods

2.1 Safety considerations
All investigations in the current study were performed under the health and safety rules of Nottingham Trent University. These comply with the requirements of COSHH and include assessing the hazards of both chemicals and experimental procedures.

2.2 Chemicals
The details of all chemicals used are shown in Table 2.1.

Table 2.1 Source and Catalogue numbers of chemicals and solutions used in this thesis

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Catalogue number</th>
<th>Additional information</th>
</tr>
</thead>
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<tr>
<td>AccuGel™ 29:1 acrylamide</td>
<td>Geneflow</td>
<td>EC-852</td>
<td>(40% w/v)</td>
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<td>Acetylcholinesterase from <em>Electrophorus electricus</em> (<em>Electric Eel</em>)</td>
<td>Sigma-Aldrich</td>
<td>C2888</td>
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<td>Uptima-Interchim</td>
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<td>Ammonium persulphate (APS)</td>
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<td>UP95424</td>
<td>Contains bicinchoninic acid</td>
</tr>
<tr>
<td>BCA Assay kit reagent B</td>
<td>Uptima-Interchim</td>
<td>UP95425</td>
<td>Contains copper (II) sulphate</td>
</tr>
<tr>
<td>Blueye prestained protein ladder</td>
<td>GeneDirex</td>
<td>PM 007-0500</td>
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<td>Ethanol</td>
<td>Fisher Scientific</td>
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<tr>
<td>Ethylenediaminetetraacetic acid disodium salt (EDTA)</td>
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<td>Supplier</td>
<td>Code</td>
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<tr>
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<tr>
<td>Palladium atomic absorption standard solution</td>
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<tr>
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<td>78830</td>
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<tr>
<td>Platinum atomic absorption standard solution</td>
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<td>Potassium chloride (KCl)</td>
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<td>Bio-Rad</td>
<td>161-0374</td>
<td>500 μl, mixture of 10 recombinant proteins (10–250 kDa)</td>
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<tr>
<td>Protease inhibitor cocktail</td>
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<td>ProtoGel resolving buffer (4X)</td>
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<td>Protogel® stacking buffer</td>
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<td>Rhodium(III) chloride (RhCl₃)</td>
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<td>Sodium chloride (NaCl)</td>
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<td>Sodium dodecyl sulphate (SDS)</td>
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<td>Sodium hydroxide (NaOH)</td>
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<td>Sodium phosphate monobasic (NaH₂PO₄)</td>
<td>Sigma-Aldrich</td>
<td>S-8282</td>
<td>H₂O: soluble, 1 M, clear, colourless</td>
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<td>Sodium phosphate dibasic (Na₂HPO₄)</td>
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<td>1, 1, 3- Tetaethoxypropane (TEP)</td>
<td>Sigma-Aldrich</td>
<td>T9889</td>
<td>Density: 0.919 g/mL (25°C)</td>
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<td>2-Thiobarbituric acid (TBA)</td>
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<td>≥ 98%</td>
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Thiazolyl blue tetrazolium Bromide  | Sigma-Aldrich  | M5655  | Powder, ≥ 97.5% (HPLC)
---|---|---|---
Trichloroacetic acid (TCA)  | Analar Normapur®  | CAS: 76-03-9  | Density: 1.63 g/cm³ (20°C)
Tris base  | Sigma-Aldrich  | T-1503  |
Tris hydrochloride  | Sigma-Aldrich  | T-3253  | ≥ 99.9% (titration)
Tris/glycine (Electrophoresis transfer buffer)  | Geneflow  | EC880  | 10x concentrate
Tris/glycine/SDS (Electrophoresis running buffer)  | Geneflow  | EC870  | 10x concentrate
Triton X-100  | Sigma-Aldrich  | T8787  |
Tween 20  | P1379  |

### 2.3 Sample collection

A hand net was used to obtain adult *G. pulex* over the period of study, from a single population in Millwood Brook, a slow-flowing freshwater stream (approximate dimensions 3 m wide and 0.5 m deep), located near Creswell Crags, Derbyshire (Ordnance Survey grid reference SK533741). It is a Site of Special Scientific Interest (SSSI) that is a relatively unpolluted site with PGE concentration lower than the detection limit of inductively coupled plasma optical emission spectrometry (ICP-OES) (3, 6 and 6 µg/L for Pd, Pt and Rh, respectively). Each experiment in the current study was performed using organisms sampled simultaneously. This step allows a reduction in the potential seasonal variation of organismal responses within each test. *G. pulex* usually hide under stones on the stream bed and are also mobile on the surface of sediments. After placing a hand net next to the downstream side of stones, they were lifted up and organisms were then caught by the net. The animals were placed in 25 L food grade plastic containers with stream water and transferred to the laboratory. The duration of the journey from Creswell Crags to the laboratory is approximately 1 h. *G. pulex* that were visibly infected by acanthocephalan parasites were identified, as parasites appeared as intense orange dots through the cuticle, and then these infected *G. pulex* were removed. This step was necessary to avoid the potential of
parasite-induced change in the behaviour of *G. pulex*. Bakker et al. (1997) suggested that Acanthocephalan parasites could modify the behaviour of their intermediate amphipod hosts, increasing the probability of predation by the final vertebrate hosts. They showed that parasites changed the movement and escape behaviour of *G. pulex*. Pascoe *et al*. (1995) observed that the feeding rate of *G. pulex* was influenced by parasite load. Parasites can also induce changes in the heat shock protein response in *Gammarus* (Sures and Radszuweit, 2005). These authors also showed that parasites could also take up and accumulate metal such as palladium. Unpublished observations by Lloyd Mills (personal communication) indicate that less than 1% of *G. pulex* is parasitised by *Polymorphus minutes* in Creswell.

Undamaged active organisms were collected using a net and placed in plastic aquaria filled with continuously aerated dechlorinated tap water (pH 8.5; conductivity 561 micro-Siemens; hardness 231 mg/L CaCO₃) (50 animals in 6 L of water) under a 12 h light/dark photoperiod at 15°C. Aeration enables the provision of adequate oxygen for test organisms and can help to reduce ammonia levels in water (Kerri *et al*., 2006). The water in the tanks was renewed weekly to reduce accumulation of animal waste. To ensure removal of residual chlorine, tap water was aerated for no less than 48 h prior to use. Organisms were acclimated to laboratory conditions for a minimum of 7 days prior to their use in the experiments. Animals were fed to excess with wheat germ. To reduce metabolic activity and the consequential fouling of the test solutions with metabolic waste (e.g. faeces), experimental animals were starved for 24 prior to each test unless otherwise stated. Short periods of starvation can also decrease the organisms’ energy supply, reducing their movement and ventilation activity and thus reduce metal uptake (Alonso *et al*., 2010). To reduce the influence of body mass on stress responses of the examined organisms, all experiments in the current study were conducted using adult *G. pulex* of a specific body mass (around 25 mg wet weight). As the current study examines the impact of PGEs on
population-level responses, the sexes of the experimental *G. pulex* were not separated. Examination of the sex of *G. pulex* may also induce stress and/or damage to organisms.

### 2.4 Preparation of glass and plasticware

To reduce potential contamination, all glassware and plasticware were washed with detergent and then soaked in 5% v/v nitric acid for 24 h prior to each experiment. Dechlorinated tap water was used to rinse the glassware and plasticware for the *G. pulex* metal exposure and behaviour experiments. However, for experiments involving gel electrophoresis, the glassware and plasticware were washed using distilled water and 70% v/v ethanol in order to eliminate oil spots that can disturb the polymerisation of the gel.

### 2.5 Median lethal concentration (LC$_{50}$) assessment

A semi-static experiment, in which test organisms were subjected to periodic renewal of tank water with or without added metals, was designed to determine the 96 h LC$_{50}$ of PGEs (Pd$^{2+}$, Pt$^{4+}$, Rh$^{3+}$, Os$^{3+}$, Ru$^{3+}$, and Ir$^{3+}$) to *G. pulex*, in order to establish the concentrations of PGEs to be used in future tests. Due to the limited solubility of some PGEs, initial experiments used acid solubilised atomic absorption standards (AAS) to produce the experimental solutions of tested PGEs. Concentrations of PGEs used in LC$_{50}$ tests were selected according to their water solubility. Test solutions (0, 0.25, 0.5 and 1 mg/L Pd$^{2+}$; 0, 1, 5 and 10 mg/L Pt$^{4+}$; 0, 0.1, 0.25, 0.5 and 1 mg/L Os$^{3+}$; 0, 0.25, 0.5 and 1 mg/L Ru$^{3+}$; 0, 1, 5, 10 mg/L Ir$^{3+}$ and 0, 1, 5 and 10 mg/L Rh$^{3+}$) (nominal concentrations: control = 0 mg/L) were prepared using dechlorinated tap water. Sodium hydroxide was used to adjust the pH of the experimental solutions to that of the control solution. For the binary combinations of Pd$^{2+}$ and Pt$^{4+}$ or Rh$^{3+}$, the soluble Pt$^{4+}$ (PtCl$_4$) or Rh$^{3+}$ salts (RhCl$_3$) and the atomic absorption standard of Pd$^{2+}$ were used to produce the desired concentrations.
Treatments were performed using at least three replicates, each containing 10 randomly chosen adult intermoult *G. pulex* (both sexes; approximately 25 mg wet weight) giving a total of at least 30 individuals per concentration. Test organisms were placed in borosilicate glass beakers (600 mL) containing 500 mL of the respective test solutions. LC$_{50}$ tests were performed at 15°C under a 12 h light/dark photoperiod over a period of up to 96 h. Every 24 h the dead animals were counted then removed and the test solution renewed. A total lack of movement when stimulated was used to identify an organism’s mortality. The aim of the daily renewal of test solutions was mainly to reduce the potential variation in the nominal concentrations of the tested PGEs. This variation can result from chemical adsorption to the wall of containers, binding to organic matter, volatilisation or uptake of PGEs by exposed *G. pulex* (Thongra-ar *et al.*, 2003).

The majority of published investigations that examine the effect of metals on biota tend to represent metal toxicity on a mass basis, such as mg/L. In addition, similar units are used when producing or purchasing AAS. However, due to the wide variation in the molecular weight of metals, the use of such units when comparing the toxicity of different metals may be misleading. In addition, different forms of salts or complexes, which have different molecular masses, may be used as sources of metal exposure. Thus, different weights of these compounds will be required to obtain the same molar concentration of a certain metal. Molarity takes into the account the molecular weight of the tested metal or compound and is more relevant on a chemical stoichiometric basis. Therefore, the current study included molarity when comparing the effects of different metals on the 96 h survival of biota. This provides a better understanding with regard to the toxicity of different metals.

### 2.6 Metal uptake by *G. pulex*

The results of the 96 h LC$_{50}$ test showed that, amongst traffic related PGEs, only Pd$^{2+}$ affected the survival of *G. pulex* (Figure 3.5). Therefore,
further investigations were performed to measure the uptake of Pd\textsuperscript{2+} and its impacts on \textit{G. pulex}. The initial experiment investigated the required exposure period to get a sufficient concentration of Pd\textsuperscript{2+} into the \textit{G. pulex} tissues for detection. In this experiment, approximately 100 individual \textit{G. pulex} (both sexes) were exposed to 0.5 mg/L Pd\textsuperscript{2+} (nominal concentration). After 24, 48, 72 and 96 h, six replicates (each containing three organisms) of the treated organisms were prepared by rinsing the organisms with ultra-pure water (18MΩ) and then transferring them to a solution of 3 mM EDTA (pH 7.8) for 10 min to remove any metal loosely bound to the exoskeleton, as recommended by Geffard \textit{et al.} (2003). The organisms were then rinsed gently, dried with soft tissue and stored at -80°C in an Ultra-Low Temperature Freezer (New Brunswick Scientific) prior to further analyses. The frozen animals were then placed in an oven (Scientific Laboratory Supplies Ltd, UK) at 60°C for 24 h to determine the dry weight. Control organisms were similarly treated, but were kept in dechlorinated water and not exposed to Pd\textsuperscript{2+}.

Prior to ICP-OES measurement of the whole body PGE concentrations, the test organisms were subjected to an Aqua Regia (HCl-HNO\textsubscript{3}, 3:1 molar ratio) extraction procedure. HNO\textsubscript{3} is an oxidant agent that aids digestion of organic matter, while HCl provides a ready supply of chloride ions, which react with metal ions forming chloride salts. The latter reaction can help to remove metal ions from the solution and allow more oxidation of metals to take place, increasing the efficiency of metal extraction.

This method of extraction may be limited by incomplete destruction of organic matter, where the metal may be absorbed, and thus the determined metal concentrations may be less than the actual metal content in the organism tissue. Despite this, Aqua Regia tends to be preferred for PGE extraction as it more completely solubilises than other acid extraction procedures. The overnight heating can also improve the efficiency of the method employed to extract the tested metals. In the
current study, filtration with several washes with diluted nitric acid was carried out in an effort to improve the extraction and exclude the residual organic matter, which can interfere with the detection procedure.

For each condition, samples were digested by heating overnight at 80°C in 1 mL of Aqua Regia per 30 mg of dried *G. pulex*. The acid extracts were then filtered using Whatman (No. 4) filter paper (PerkinElmer), in combination with several washes using 0.1 M HNO₃. Extractions were transferred to 10 mL volumetric flasks and then made up to the mark with 0.1 M HNO₃. Pd²⁺ concentrations were measured precisely in the final volume using a PerkinElmer Optima 2100 DV and expressed as µg Pd/g dry weight of *G. pulex*.

The results showed that there was a general trend of increase in Pd content over time (Figure 2.1). Whilst 24 h exposure to 0.5 mg/L Pd²⁺ (nominal concentration) caused a significant, 16-fold increase in the Pd²⁺ content of *G. pulex* relative to the control organisms (24.6 and 1.5 µg/g dry weight, respectively), the result indicated that it took 72 h exposure to significantly increase the Pd content of *G. pulex* above the 48 h value (*p* < 0.001). Ninety-six hours of exposure to 0.5 mg/L Pd²⁺ (nominal concentration) did not significantly increase the content in *G. pulex* above that found at 72 h. It was therefore decided to conduct the Pd²⁺, Pt⁴⁺ and Rh³⁺ exposure trials over 72 h so that either a fall, or rise, in *G. pulex* Pd content could be detected by ICP-OES.
Although in natural environments metals are often present together, the majority of ecotoxicological studies have focused on the impacts of single metal exposures. The uptake of metals in combinations might have additive, synergistic or antagonistic influences and alter the toxicity of individual metals (Borgmann et al., 2008). Thus, it is important to investigate possible metal-metal interactions and predict the effect of metal combinations on exposed organisms. Interactions between metal toxicity might be the result of reducing metal uptake and/or enhancing the defence system in exposed organisms (Vellinger et al., 2013). Thus, the next experiment was designed to determine the accumulation of Pd, Rh and Pt in the body of G. pulex following exposure to either single or binary combinations of Pd$^{2+}$, Pt$^{4+}$, Rh$^{3+}$, Pd$^{2+}$/Pt$^{4+}$ or Pd$^{2+}$/Rh$^{3+}$. Based on the finding of the previous investigation, G. pulex (both sexes) were exposed to nominal concentrations of 0.25 or 0.5 mg/L Pd$^{2+}$ with coexposure to 0, 5, or 10 mg/L Pt$^{4+}$ or Rh$^{3+}$ for 72 h. The same Aqua Regia extraction procedure was applied to evaluate the Pd, Pt and Rh uptake by exposed G. pulex.
2.6.1 Metal partitioning in *G. pulex*

Toxicity studies that examine whole body metal content may not provide an accurate reflection of actual toxic risk. In particular, the physiological aspects of organisms that affect subcellular metal partitioning and compartmentalisation should be taken into account (Vijver *et al*., 2004; Péry *et al*., 2008; Geffard *et al*., 2010). Valuable information regarding metal toxicity, tolerance and the potential bioaccumulation of metals via the food chain can be obtained by examining the subcellular partitioning of accumulated metals in aquatic invertebrates (Wallace *et al*., 2003). Metals could cause damage when they accumulated in organelles and/or heat-sensitive protein fractions (Wallace *et al*., 2003). Geffard *et al*., (2010) showed that metal toxicity was dependent on the concentrations in metal-bound compounds with high (e.g. metal–ligand pools) or low-molecular weight (e.g. metallothioneins). Wallace *et al*., (2003) pointed out that that metal bound to non-soluble fractions (e.g. granules, cell walls and exoskeleton) of prey is less bioavailable to predators than metal bound to soluble fractions, such as cytosol and proteins including the metallothionein-like proteins (MTLP). The latter fractions can be broken down by the digestive processes of the predator (Marsden and Rainbow, 2004). Therefore, this part of the study investigated if subcellular partitioning of PGEs in *G. pulex* can be determined in order to examine the possible competition between Pd and Pt or Rh in subcellular storage locations.

The subcellular distribution of Pd$^{2+}$ within *G. pulex* was examined, as described by Wallace *et al*., (2003). *G. pulex* (both sexes; 15 individuals) were exposed to 0 or 0.5 mg/L Pd$^{2+}$ (nominal concentrations) for 72 h. Then, the living organisms in each group were rinsed in dechlorinated water and transferred to 3 mM EDTA (pH 7.4) for 10 min to remove metal loosely bound to the exoskeleton (Geffard *et al*., 2003). The test animals were then frozen at -80 °C until required for chemical analysis. The frozen *G. pulex* were firstly rinsed in deionised water, then four organisms from each group (in triplicate) were manually homogenised,
using a Potter Elvehjem homogeniser (to minimise disruption of cellular organelles). Homogenisation was performed in 4.5 mL of ice cold 0.1 M Tris-HCl buffer (pH 7.4) containing 5 mM β-mercaptoethanol and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) as a broad spectrum protease inhibitor. The procedure was carried out on ice to further reduce proteolytic activity. The tissue homogenates were transferred to ultracentrifuge tubes and subjected to subcellular fractionation, according to the methods described by Wallace et al. (2003) and Geffard et al. (2010), as shown in Figure 2.2. Firstly, the tissue homogenates were centrifuged at 1500 g for 15 min at 4°C to sediment exoskeleton, unbroken cells, membranes, nuclei and MRGs. The resultant pellet (P₁) was re-suspended in 500 µL of deionised water (DW) and heated at 100°C for 2 min. Next, 500 µL of 1 M NaOH were added and the sample incubated at 65 °C for 1 h. The sample was then centrifuged at 10,000 g using an Optima XP Ultracentrifuge (Beckman Coulter) for 20 min. This procedure aimed to isolate MRGs (P₂) and cellular debris (S₂). The supernatant from the initial 1500 g (S₁), containing the cytoplasm, was re-centrifuged at 100,000 g for 60 min at 4°C to obtain organelles including mitochondria, lysosomes and microsomes (P₃) and a cytosolic supernatant fraction (S₃). This fraction (S₃) was heated to 80°C for 10 min, cooled on ice for 60 min, and then centrifuged at 26,000 g for 15 min in order to obtain a pool of denatured heat sensitive proteins (P₄) and heat stable proteins (metallothionein and metallothionein-like proteins) (S₄). Unfortunately, the Pd content in tested fractions was lower than the detection limit of the ICP-OES (3 µg/L). Hence, it was not possible to continue with this approach. The fractions P₂, S₂, P₃, P₄ and S₄ were subjected to an Aqua Regia (HCl-HNO₃, 3:1 molar ratio) extraction procedure. Fractions were digested by heating overnight at 80°C in 0.5 mL of Aqua Regia. The acid extracts were filtered using Whatman (No. 4) filter paper, transferred to 2 mL volumetric flasks and then made up to the mark with 0.1 M HNO₃. Pd²⁺ concentrations were measured in the final 2 mL volume.
Figure 2.2 Procedure for determination of the subcellular partitioning of Pd\textsuperscript{2+} within \textit{Gammarus pulex}. Adapted from Wallace \textit{et al}. (2003) and Geffard \textit{et al}. (2010).

2.7 \textbf{Pd}^{2+} \textbf{content in metal-rich granules (MRGs)}

\textit{G. pulex} were exposed to either 0 or 0.5 mg/L Pd\textsuperscript{2+} (nominal concentrations) for 72 h with daily renewal of the exposure solutions. The exposed organisms (1 organism from each treatment) were rinsed with deionised water, gently dried with tissue paper and immediately cryofixed by immersion in liquid nitrogen. Gammarid specimen sections of 20 µm were obtained using a Leica Cryostat CM1850 UV (Leica Biosystems Nussloch GmbH) at a temperature of $-20^\circ$C.
longitudinal sections that allowed observation of the entire internal anatomy of the tested organisms were mounted on carbon-covered aluminium stubs for analysis. A scanning electron microscope equipped with a solid-state back-scattered electron detector (JEOL JSM-840 A) was used to visualise the tested sections according to method of Khan et al. (2012). The element composition of the tested sections was investigated using energy-dispersive X-ray analysis (EDXa) at an accelerating voltage of 20 kV and a working distance of 15 mm.

2.8 Protein estimation

The protein content of samples was estimated using the bicinchoninic acid (BCA) protein assay kit from Sigma-Aldrich. In the BCA assay, which was introduced by Smith et al. (1985), Cu$^{2+}$ reacts with protein under alkaline conditions and is converted to Cu$^+$. The previous reaction is known as the Biuret reaction. The reduced Cu$^+$ then reacts with BCA forming an intense purple complex. Whilst the peptide bonds give rise to much of the signal, some amino acid residues, including cysteine, tyrosine, and tryptophan, in protein molecules also contribute to the signal. This assay is quick, compatible with detergents (e.g. Triton X-100 and SDS) and denaturing agents (e.g. urea) and is sensitive (detection threshold 5 µg of protein) (Smith et al., 1985; Rhem, 2006).

A stock solution of 2000 µg/mL bovine serum albumin (BSA) was prepared in the appropriate assay buffer. The assay buffer was used to dilute the stock solution to obtain a range of protein concentrations (0 to 2000 µg/ml). Twenty-five microliters of standard or tested sample (in triplicate) were loaded into individual wells of a 96-well microplate. A working solution was prepared by mixing 50 parts of reagent A (1% (w/v) bicinchoninic acid, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium tartrate and 0.95% (w/v) sodium bicarbonate in 0.1 M sodium hydroxide at pH 11.5) and 1 part of reagent B (4% (w/v) copper (II) sulphate pentahydrate). Then 200 µL of the working solution was added to each well. The 96-well microplate was covered and incubated for 30
min at 37°C in an LEED incubator (LSC 2933), then cooled to room temperature. Absorbance at 570 nm was measured for each sample using a Tecan SPECTRAFluor Plus microplate reader. The standards were freshly prepared and a new standard curve was constructed on each occasion.

2.9 Sublethal effects of Pd\textsuperscript{2+} on \textit{G. pulex}

To increase the sensitivity and reduce the cost and time of the toxicity investigations, the current study employed \textit{in vivo} exposure to investigate several sublethal effects of Pd\textsuperscript{2+} on \textit{G. pulex}. These tests were performed using four nominal concentrations of Pd\textsuperscript{2+} (0, 0.1, 0.25 and 0.5 mg/L). These concentrations were selected according to the results of the 96 h LC\textsubscript{50} test to obtain detectable responses within ≤ 96 h of Pd\textsuperscript{2+} exposure.

\textit{In vivo} investigation is a more environmentally relevant approach to examining the effect of pollutants on biochemical biomarkers. Although this approach is more time consuming than \textit{in vitro} assays, important factors that may affect the uptake of pollutants and their availability to an organism are taken into account (Ibrahim \textit{et al.}, 1998). For example, the \textit{in vivo} approach takes into account the metabolism of a toxic compound (Ozmen \textit{et al.}, 1999) as well as the uptake, excretion and physiological processes that may mediate its bioavailability and toxic impact in living organisms (Ibrahim \textit{et al.}, 1998). As a result, this technique may provide a better understanding of biomarker responses to contaminant exposure under natural conditions.

2.9.1 Vertical movement assay

The present study examined the effects of sublethal Pd\textsuperscript{2+} exposure on the vertical movement of \textit{G. pulex}. Vertical movement requiring more energy than other movement behaviours (Wallace and Estephan, 2004), thus it can provide a more sensitive marker of stress. This test was performed according to the method of Wallace and Estephan (2004). Twenty-five adult organisms (both sexes) were exposed to 0, 0.1, 0.25
or 0.5 mg/L Pd$^{2+}$ (nominal concentration) for 24 h under a 12 h light/dark photoperiod at 15°C. Twenty randomly selected individuals from each group were transferred to individual clear plastic boxes (dimensions 60 x 35 x 12 mm) containing a 2.5 cm layer of dechlorinated water. Due to the small size of the plastic boxes used in the current experiment, test organisms may be exposed to low concentration of dissolved oxygen. During hypoxia, which is generally defined as dissolved oxygen levels between 0 (anoxia) and 175 µmol/L (Diaz and Rosenberg, 1995), organisms may reduce their movement activity in order to reduce metabolic cost (Hervant et al., 1999). However, our investigation showed that *G. pulex* in a closed container consumed ≤ 25% of dissolved oxygen within an hour (data not shown). Therefore, it was assumed that, under the current study conditions (organism maintained for 45 min in approximately 10.5 mL of water with dissolved oxygen at 336 µmol/L in an open container), organisms would not be exposed to substantial hypoxia. This assumption was reinforced by the results of this test in which some organisms remained highly active. The movement of the organisms from the bottom to the top of the water column in the boxes was recorded using a Sony video camera (Sony DCR-HC37 Handycam) under infrared light (Scene®. IR, model: S-8030) for 40 min. The first 10 min were ignored in the subsequent analysis as it was considered to be the acclimation time for the test animals to adjust to the experimental conditions (Wallace and Estephan, 2004). A single movement event was defined as the full excursion from the bottom surface of the plastic box to the top of the water column (2.5 cm).

### 2.9.2 Feeding activity

Several feeding assays have been employed to investigate the impact of stresses on the feeding rate of gammarids. For example, a time response analysis of the consumption of *Artemia salina* eggs can be employed to assess the feeding activity of gammarids, and is a quick and non-destructive technique for the tested organisms (Pascoe et al.,
However, this method requires time-consuming observation of the number of eaten eggs. Alternatively, Maltby et al. (2002) examined the alteration in the feeding rate of *G. pulex* using the leaf weight loss between the dry weight of alder leaves initially supplied and the dry weight of leaf material remaining at the end of experiment. In such an approach, it is difficult to measure the feeding rate of the test organism at several time points due to the possibility of losing leaf material within the weighing, drying and rehydrating processes. This can be avoided by measuring the area instead of the weight of the leaf. This approach was used in the current study, in which feeding activity was measured employing the methods described by Geffard et al. (2009). However, measuring the area of the leaf material may not reflect the actual feeding activity of test organisms, as they may not consume the entire thickness of the leaf discs. Therefore, this technique might underestimate the consumed area.

The current study investigated the effect of sublethal Pd$^{2+}$ exposure on the feeding activity of *G. pulex*. Based on the 96 h LC$_{50}$ test results, a control, 0.1, 0.25 and 0.5 mg/L Pd$^{2+}$ (nominal concentration) were selected. Fresh alder (*Alnus glutinosa*) leaves were collected, during the autumn fall, at Nottingham Trent University’s Clifton Campus. To minimise differences in food quality, leaves were collected from the same tree on the same day. Alder leaves have been described as a high-quality food (Bloor, 2011), with high nitrogen and low phenolic content (Sousa et al., 1998). Nilsson (1974) offered *G. pulex* the choice between Willow (*Salix caprea*), Elm (*Ulmus glabra*), Beech (*Fagus sylvatica*), Ash (*Fraxinus excelsior*), Oak (*Quercus robur*) and alder. It was found that organisms had a higher consumption rate of alder leaves than other tested leaves (Nilsson, 1974). Therefore, alder leaves were used as food source in the current study.

A diet of conditioned leaf material was preferred by *G. pulex* over unconditioned leaves (Bloor, 2011). Conditioning refers to colonization
of leaves by microorganisms (e.g. fungi and bacteria). Microbial colonization can increase the nutritional quality of detritus via reducing carbon: nitrogen and carbon: phosphorus ratios and breaking down lignin and cellulose, which are probably indigestible by invertebrates’ enzymes into simpler compounds, such as proteins (Nelson, 2011). Using unconditioned leaves in feeding assays can reduce the metabolic demands of the tested organisms as they reduce their energy intake via reducing the respiration rate (Graça et al., 1993). However, oxygen consumption by microorganisms that colonize the leaves may reduce the oxygen concentration in exposure media. This reduction combined with oxygen consumption by the test organisms could produce hypoxic conditions, which would influence gammarid response (Wu and Or, 2005). In addition, Nelson (2011) reported that Gammarus could consume fungi. This may affect feeding measurements, especially at short exposure times (e.g. 24 h). Furthermore, microorganisms can accumulate high amounts of pollutants (Schaller et al., 2011). Therefore, unconditioned alder leaves were used in the feeding test in the current study. Unconditioned leaves are likely to be harder to process and digest. Hence the inhibitory effects of pollutants on these processes (e.g. inhibition of digestive enzymes) may be more apparent when using unconditioned rather than conditioned leaves.

The collected leaves were dried overnight at 60°C, and then stored until needed. Dried leaves were rehydrated for 24 h in dechlorinated Nottingham tap water. After rehydration, 2 cm diameter leaf discs were cut with a cork borer (avoiding the main veins). Image J software was employed to measure the area of the leaf discs. Prior to each experiment, the leaf discs were placed in a container of dechlorinated tap water for 24 h, while the test organisms were exposed to the Pd^{2+} test solutions. The latter step aims to acclimate experimental organisms to test conditions and thus reduce the variation in their responses (Agatz and Brown, 2014).
As the total amount of food consumed by one organism within a day is rather low, which increases the measuring uncertainty, three adult animals (both sexes), each approximately 25 mg wet weight, from each treated group (in triplicate) were transferred to a beaker containing 500 mL of test solution and a leaf disc was added. Three discs were also individually placed in three glass beakers containing only 500 mL of Nottingham tap water, to control for any alteration in leaf area not caused by feeding activity. To minimise the risk of cannibalism, which was observed in our preliminary experiments, a piece of plastic net was placed into each beaker. This step can provide both a resting surface and protective environment for the tests to reduce the confrontations between organisms (Geffard et al., 2009). Every 24 h of the experimental period, the leaf disc areas were measured using a Canon CanScan 8800F scanner, and the test solutions were renewed.

### 2.9.3 Acetylcholinesterase

This set of experiments investigated the response of AChE activity in *G. pulex* following exposure to sublethal concentrations of Pd$^{2+}$. The activity of AChE in *G. pulex* was measured using the colourimetric method based on Ellman *et al.* (1961), modified for microplates. This is a spectrophotometric approach which follows the development of a yellow colour that is formed due to reaction of thiocholine with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to measure enzyme activity. The thiocholine is produced from the hydrolysis of the substrate acetylthiocholine by the enzyme AChE. Ellman’s assay is quick, simple and inexpensive (Worek *et al.*, 2011).

All preparation procedures were carried out at 4°C. Twenty *G. pulex* (both sexes) were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd$^{2+}$ (nominal concentration) for 72 h. From each condition, 6 replicates, each consisting of three organisms, were manually homogenised in a Potter Elvehjem homogeniser. Homogenisation was performed on ice in 4 mL of ice-cold phosphate buffer (0.1 M; pH 7.8 unless otherwise stated).
containing 0.1% v/v Triton X-100 for 30 sec. To obtain a clear supernatant, reducing the effects of light scattering, the homogenate was centrifuged at 9000 g for 15 min at 4°C. The clear supernatant was carefully collected and placed into clean 1.5 mL microcentrifuge tubes and kept at 4°C until used in the assay.

For sample preparation, all substances were kept on ice to maintain their stability. Either 16 µL of the supernatant or homogenisation buffer were placed in the wells of a 96-well microplate. Then 285 µL of the reagent mix (0.1 M PBS pH 7.8, containing 0.2 mM DTNB) as chromogenic reagent, and 2 mM of acetylthiocholine iodide (ATCh) as a substrate was added. Two blanks, one without substrate and one without sample, were used to measure the reaction of thiol groups, and spontaneous substrate hydrolysis. The measurement of enzyme activity was carried out using a rate assay by following the rate of change of absorbance at 415 nm for 20 min (measurement once every 30 sec) at 25°C. This elevated temperature can provide a faster action, steeper line and less error than that can be obtained at 15°C. The enzyme activity was adjusted according to the protein content of the samples, which was measured using the BCA protein assay.

Initial experiments were conducted to investigate the effect of pH on AChE activity, using a pH range of 7.5 to 8.5, to obtain the optimal pH condition. AChE activity was not significantly affected by changing the pH (Figure 2.3). Thus, further experiments were performed at pH 7.8, as this pH is commonly used in the literature (e.g. Xuereb et al., 2009a, b). This allows easier comparison with previous studies. Next, the potential interaction between free thiol groups and Pd²⁺ was examined.
Figure 2.3 The effect of pH on AChE activity in *Gammarus pulex* as a percentage of that at pH 8.5 (means ± SEM, n=6).

### 2.9.3.1 Investigation of potential Pd$^{2+}$ interference with the Ellman assay

The utility of Ellman’s assay to measure the effect of metals on AChE activity may be limited. This is due to the interaction of some metals with the thiol groups in thiocoline (TCh), which could affect its ability to react with DTNB in the Ellman assay (Frasco et al., 2005). This would reduce the formation of the coloured complex, and thus overestimate the inhibition of AChE. Therefore, the current study investigated the potential interference of Pd$^{2+}$ with the Ellman assay.

Free thiocholine was produced enzymatically, following Frasco et al. (2005). For this purpose, 10 µL of electric eel AChE (10 units/ml) was incubated with 990 µL of 25 mM Tris buffer (pH 7) containing 1 mM ATCh, for 4 h at 25°C. The sample was then transferred to a water bath and heated at 98°C for 2 min to inactivate the AChE, then cooled and frozen at -20°C until required. The solution containing free thiocholine and inactivated AChE was then incubated with different concentrations
of Pd$^{2+}$ for at least 15 min. Thereafter, the interaction of these samples with 0.2 mM DTNB was investigated. This test was performed under the same conditions as the Ellman assay (Ellman et al., 1961). The interaction was then calculated as the percentage of change in absorbance in the solution pre-incubated with Pd$^{2+}$ relative to that without Pd$^{2+}$ (control).

The result of this study showed a significant interaction between the TCh group and Pd$^{2+}$ (Figure 4.3). Therefore, the current study employed dialysis in an effort to reduce the interaction of Pd$^{2+}$ with the products of Ellman’s assay. As a result, the potential overestimation of enzyme inhibition may be avoided. Dialysis is a method commonly used to separate different molecules based on the size of the molecules and diffusion patterns through a semipermeable membrane (Andrew et al., 2001). This technique has been extensively used to remove inorganic and other small molecules, including metals from a solution. Homogenates of three non-treated G. pulex (1 mL, 3 replicates) were in vitro exposed to Pd$^{2+}$ with a final concentration of 0 or 0.5 mg/L Pd, and then placed in a sealed dialysis membrane (15,000 Da-molecular weight cut off). Although the molecular weight of AChE in Gammarus has not been examined, Balerna et al. (1975) reported that the AChE molecular weight of axonal membranes of crustacean nerves is 27000 Da, thus it was assumed that AChE would be retained by the dialysis membrane.

Dialysis membranes were then immersed in 500 mL of phosphate buffer (0.1 M; pH 7.8) and incubated at 4°C. The substantial difference between the volume of sample in the dialysis membrane and that of dialysis buffer would increase the loss of unwanted small molecules. After 1 h, the dialysis buffer was renewed and dialysis continued for an additional hour. AChE activity in the part of the extract that remained inside the dialysis membrane was then measured using the Ellman assay (Ellman et al., 1961).
The results showed that dialysis caused a significant decrease in the activity of AChE activity in *G. pulex*. The activities of AChE in *G. pulex* following 2 h dialysis were approximately 25% lower than those of non-dialysed samples under all experimental conditions (0 and 5 mg/L Pd$^{2+}$) (Table 2.2). This suggests that dialysis did not reduce the interaction of Pd$^{2+}$ with the products of the Ellman assay. Therefore, dialysis was not employed in the further AChE tests. The reduction in AChE activity may be due to the non-specific binding of the enzyme to the dialysis membrane and/or inflows of the dialysis solution into the dialysis membrane.

Table 2.2 The effect of dialysis on AChE activity in *Gammarus pulex*. Means (% of non-dialysed control) ± SEM, n=3 (* means that activity is significantly different than that of the respective non-dialysed group *p*≤ 0.05)

<table>
<thead>
<tr>
<th>Control</th>
<th>Treated with 0.5 mg/L Pd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-dialysed</td>
<td>Dialysed</td>
</tr>
<tr>
<td>100 ± 10.11</td>
<td>74.17 ± 0.5*</td>
</tr>
<tr>
<td>Non-dialysed</td>
<td>Dialysed</td>
</tr>
<tr>
<td>89.1 ± 1.03</td>
<td>66.48 ± 0.98*</td>
</tr>
</tbody>
</table>

2.9.3.2 The effect of Pd$^{2+}$ exposure on AChE activity in *G. pulex*

The next experiment examined the effect of Pd$^{2+}$ exposure on AChE in the whole body extract of *G. pulex*. Thirty individual *G. pulex* were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd$^{2+}$ (nominal concentration) for 72 h. Then, 6 replicates consisting of three organisms from each condition were homogenised in 4 mL of PBS (0.1 M, pH 7.8) containing 0.1% v/v TritonX-100. Extracts were obtained using the procedure described previously in section 2.9.3.

AChE activity in the head of *G. pulex* is higher than that in the body (Xuereb *et al.*, 2007). Therefore, this part of the current study compared the activity of AChE in the head and the whole body of the tested organisms to investigate whether determination of AChE in the head would improve the sensitivity of the assay to examine the effect of Pd$^{2+}$ exposure on AChE in *G. pulex*. 

80
AChE activity in the head extract of *G. pulex* was higher than that in the whole body extract (see Chapter 4). Therefore, the effect of 72 h exposure to 0, 0.1, 0.25 or 0.5 mg/L Pd\(^{2+}\) (nominal concentration) on the AChE activity in the head of *G. pulex* was examined. Thirty individual *G. pulex* were exposed to each experimental condition. The heads of the test organisms were then cut using a small sharp scalpel. Six replicates consisting of three head capsules from each tested Pd\(^{2+}\) concentration group were than homogenised in 1 mL of PBS (0.1 M, pH 7.8) containing 0.1% v/v Triton X-100, while the whole bodies (6 replicates consisting of three whole animals, from each condition) were homogenised in 4 mL of PBS (0.1 M, pH 7.8) containing 0.1% v/v TritonX-100. The extraction and the measurement of AChE activity in both extracts (the whole body and head) were performed under the same conditions described previously.

### 2.9.4 Osmoregulation

The main purpose of this test was to examine the potential effect of *in vivo* Pd\(^{2+}\) exposure on levels of the main cations Na\(^+\), K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\) in the haemolymph of *G. pulex*. Organisms were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd\(^{2+}\) (nominal concentration) for 72 h. The times and concentrations of Pd\(^{2+}\) exposure were selected according to the 96 h LC\(_{50}\) test to obtain high stress conditions without mortality. Fine-tipped pipettes were produced by pulling glass Pasteur pipettes over a Bunsen burner. Exposed organisms were caught and then dried carefully with tissue paper to remove adhering surface water. A glass pipette was then inserted through the membranes between the second and fourth dorsal plates to extract the haemolymph by capillary action. The haemolymph samples were immediately transferred under a layer of mineral oil (Sigma) into 0.5 mL microcentrifuge tubes to reduce potential evaporation. The haemolymph samples were pooled from 3 to 5 individual *G. pulex* (3 replicates for each condition) and diluted with deionised water 1:3000 haemolymph samples (i.e. 1 µL in 3 mL of
deionised water). They were then analysed with an ICP-OES to measure Na\(^+\), K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\).

To determine the cation content in the haemolymph of the tested organisms, standard curves were produced from known concentrations of NaCl, KCl, MgCl\(_2\) and CaCl\(_2\) for Na\(^+\), K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\), respectively.

### 2.9.5 The MTT reduction assay

The effect of Pd\(^{2+}\) exposure on the metabolic activity assay of *G. pulex* was tested using the colourimetric MTT assay, which was introduced by Mossman (1983). This is a rapid quantitative colourimetric cell viability assay, which can be employed to investigate the cytotoxicity impact of toxic chemical exposure. MTT is taken up into cells and passes into the mitochondria, where it is converted to an insoluble, coloured (dark purple) formazan product (Fotakis and Timbrell, 2006). The water-insoluble purple formazan is then solubilised with dimethyl sulphoxide (DMSO), producing a purple solution, which is measured spectrophotometrically. MTT reduction is, at least partly, a result of the cleavage of MTT by the mitochondrial enzyme SDH, which is presented only in metabolically active cells (Fotakis and Timbrell, 2006). Hence, the MTT assay is mainly dependent on mitochondrial respiration and provides an indicator of the cellular energy capacity of the cell.

MTT can be also reduced outside the mitochondrial inner membrane (Berridge and Tan, 1992). Therefore, part of the present study examined the proportion of MTT reduction that was attributable to SDH using malonate as a competitive inhibitor of succinate-SDH complex. The similarity in the molecular structure of this inhibitor and that of succinate enables this dicarboxylate (malonate) to bind to the cationic amino acid residues in the active site of the enzyme. However, malonate cannot be dehydrogenated as the molecular structure of this compound does not include the CH\(_2\) - CH\(_2\) group (Hajjawi, 2011). Therefore, it simply occupies the active site, reducing its availability for succinate.
To examine the effect of in vivo Pd$^{2+}$ exposure on MTT reduction in G. pulex, 30 organisms were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd$^{2+}$ (nominal concentration) for 72 h. These exposure conditions were selected to maximise sublethal stresses. From each condition, 6 replicates consisting of 4 individual organisms, were homogenised in 2 mL of an ice-cold mitochondrial isolation buffer (225 mM mannitol, 125 mM sucrose, 1 mM EDTA, 5 mM HEPES, pH 7.4). The homogenate was then centrifuged at 1000 g for 10 min at 4°C and the clear supernatant was collected and used in the assay. Two hundred microliters of supernatant were incubated in the dark, with 20 µL of 5 mg/ml MTT for 60 min at 15°C. To solubilise the reduced dye (MTT-formazan crystals), 200 µL of DMSO were added and mixed with the incubated supernatant. Thereafter, the mixture was centrifuged at 9000 g for 10 min at 4°C, and the supernatant collected and remixed. Subsequently, 200 µL of the supernatant were added to the wells of a 96-well microplate and the reduction of MTT was measured by absorbance at 570 nm and corrected for the protein content in the samples.

To test the proportion of MTT reduction by SDH, sodium malonate was added to the extracts of the untreated organisms (6 replicates) with final concentrations of 0, 1, 10 or 100 µM. The samples were vortex-mixed and then incubated with MTT under the same conditions as previously mentioned. Next, the absorbance at 570 nm was measured to assess the effect of malonate on MTT reduction.

**2.9.6 Reduced Glutathione (GSH)**

The effects of Pd$^{2+}$ exposure on the amount of reduced GSH in G. pulex, as an oxidative stress marker, was measured using monochlorobimane (mCB) in an assay described by Kamencic et al. (2000). The free mBC is almost non-fluorescent. In this assay, glutathione-S-transferase (GST) catalyses the reaction of mBC with GSH, which produces a highly fluorescent complex. In the current study, 40 individual G. pulex were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd$^{2+}$ (nominal concentration) for 72
The period of exposure was selected to increase stress without causing mortality. Five organisms from each condition (6 replicates) were gently homogenised in 800 µL of ice cold 50 mM Tris buffer (pH 7.4) for 30 sec, followed by 1 min sonication to ensure that cells has been sufficiently homogenised. Next, samples were centrifuged at 1000 g for 10 min at 4°C. The clear supernatant was collected and immediately used for the assay. mCB was added to the supernatant at a final concentration of 0.1 M along with 1 unit/ml GST, and then the samples were incubated for 30 min at room temperature. GSH was measured in a Tecan BMG LABTECH FLUOstar OPTIMA microplate reader with excitation at 355 nm and emission measured at 460 nm. The sample GSH concentration was then calculated using a standard curve for a range of known GSH concentrations (0 to 0.2 mM) dissolved in 50 mM Tris buffer (pH 7.4), and then adjusted according to the protein content in the samples.

2.9.7 TBARS assay

TBARS is a commonly used assay to examine oxidative stress. In this assay, 2-thiobarbituric (TBA) acid reacts with malondialdehyde (MDA), which is a compound produced from the degradation of polyunsaturated fatty acid peroxidation in the cells, in acidic conditions and at high temperatures. This reaction produces a pink complex, the malondialdehyde tetrabutylammonium (MDA-TBA) adduct (Oakes et al., 2003). The final product has a strong absorbance maximum at 530 nm. In the current study, the TBARS assay was employed to measure the effects of Pd²⁺ exposure on lipid peroxidation in G. pulex, employing the method described by Camejo et al. (1998). Thirty individual adult G. pulex were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd²⁺ (nominal concentration) for 72 h, to maximise the sublethal stress. Then, 6 replicates consisting of three organisms from each group, were homogenised in 4 mL of phosphate buffered saline (PBS) containing 1 mM EDTA (PBS-EDTA) at pH 7.4. The homogenate was then centrifuged at 3000 rpm in an Eppendorf 5417R centrifuge for 5 min at 4°C. Next,
40 µL of homogenate were added to the wells of a 96-well microplate (4 replicates) containing 10 µL (0.001 M) of butylated hydroxytoluene (2,6-Di-O-tert-4-methylphenol, BHT), dissolved in absolute ethanol. The final volume was made up to 190 µL with PBS-EDTA (adjusted to pH 7.4). Thereafter, 50 µL of 50% w/v trichloroacetic acid (TCA) dissolved in PBS-EDTA and 75 µL of 1.3% w/v TBA (thiobarbituric acid) dissolved in 0.3% w/v NaOH were added.

TEP (1, 1, 3-tetraethoxypropane) was used as the standard. Firstly, 0.1 M TEP in absolute ethanol was prepared and then diluted in ethanol to a final concentration of 1 µM. This solution was used as a stock solution to prepare a range of TEP standards, as shown in Table 2.3. Then 40 µL of each dilution were added to separate wells of 96-well microplate that contained 10 µL of BHT (1 M) and the final volume was made up to 190 µL with PBS-EDTA. The 96-well plate containing both sample and TEP standards was incubated at 60°C for 60 min, and then placed on ice until cool. The absorbance was first measured at 540 nm and then at 620 nm with the value at 620 nm used to normalise samples for any turbidity.

Table 2.3. Preparation of standard concentrations of TBARS assay, range of 0 – 25 nM, by diluting 1 µM 1, 1, 3-tetraethoxypropane (TEP) in PBS-EDTA.

<table>
<thead>
<tr>
<th>TEP (1 µM) (µL)</th>
<th>PBS-EDTA (µL)</th>
<th>Standard concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>999.5</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>999</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>995</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>990</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>985</td>
<td>15</td>
</tr>
<tr>
<td>20</td>
<td>980</td>
<td>20</td>
</tr>
<tr>
<td>25</td>
<td>975</td>
<td>25</td>
</tr>
</tbody>
</table>

2.9.8 Glutathione-S-transferase (GST) activity
The alteration in GST in biota due to chemical exposure is commonly used as a biomarker of oxidative stress (Regoli et al., 2002). Thus, this
part of the present study used a GST assay to investigate the impact of Pd$^{2+}$ exposure on the oxidative state in *G. pulex*.

GST enzyme activity was assessed using the method described by Habig *et al.* (1974) and Warwick (1997), modified to a microplate format. In this assay, GST activity is evaluated by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. The reaction is associated with an increase in absorbance at 340 nm, which is directly related to the level of GST activity. To maximise the Pd$^{2+}$ stress, *G. pulex* were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd$^{2+}$ (nominal concentration) for 72 h. Next, 1 organism from each tested group (6 replicates) was manually homogenised for 30 sec in 0.1 mL of 0.02 M phosphate buffer (pH 6.5), containing 1.0% v/v Triton X-100 and 1 mM phenylmethyisulfonyl fluoride (PMSF) that was previously dissolved in 97% v/v ethanol. The homogenate was made up to 1 mL with 0.02 M phosphate buffer at pH 6.5 (containing 1 mM PMSF) and then centrifuged for 3 min at 4°C and 14000 g to remove debris. The clear supernatant was collected and 100 µL of it were diluted to 1 mL using 0.02 M phosphate buffer (pH of 6.5 containing 0.1% v/v Triton X-100 and 1 mM PMSF). This was then used to determine GST activity and protein content.

In the GST assay, CDNB was used as a substrate. The reagent mixture contained 20 mM glutathione dissolved in 0.02 M phosphate buffer (pH 6.5), containing 1 mM EDTA, and 40 mM 1-chloro-2,4-dinitrobenzene dissolved in 97% v/v ethanol in a volume ratio of 5: 9: 1 part, respectively. Fifty microlitres of homogenate were added to the wells of a 96-well microplate and then the final volume was made up to 200 µL with the reagent mix. The microplate was preincubated for 3 min at 30°C. GST activity was then assessed by measuring the absorbance at 340 nm using a rate assay over 10 min (measurement once every 30 sec) at 30°C. For each individual homogenate sample, GST activity was measured in triplicate. The enzyme activity was adjusted according to the protein content of the samples.
2.9.9 SDS Polyacrylamide Gel Electrophoresis

2.9.9.1 Preparation of samples
Ten individual *G. pulex* (at least three replicates) were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd$^{2+}$ (nominal concentration) for 24 h. For each condition, 8 organisms from each replicate were homogenised in 4 mL ice cold Tris-buffered saline (TBS; Tris 50 mM and 200 mM sodium chloride NaCl, pH 7.4) containing 0.1% (v/v) Protease Inhibitor Cocktail from Calbiochem (cat 539134), to prevent sample degradation, with an Ultra-Turrax T25 basic® homogeniser at 24,000 rpm for 5 sec. The homogenate was centrifuged (Eppendorf 5417 R centrifuges) at 1000 g at 4°C for 15 min. Then the supernatant was collected and centrifuged at 9000 g at 4°C for 15 min. Thereafter, the clear supernatant, which contains cytosol and microsomes, was collected and kept on ice until needed. The 9000 g pellets, which contain mitochondria, were collected, suspended in the same volume of TBS and then washed by a further centrifugation step at 9000 g for 15 min at 4°C. All samples (pellets and supernatant) were vortex-mixed with a final concentration of 0.5% (w/v) sodium dodecyl sulphate (SDS), a denaturing agent, and then incubated at 100°C for 5 min. This step denatures native proteins to separate polypeptides. Traces of DNA, which may interfere with PAGE separations, were removed using Uprep spin columns (Dutscher Scientific, Cat No 789068). The amount of protein in the samples was determined using the BCA assay (see section 2.8) to ensure that the same amount of protein was added to each well.

2.9.9.2 Preparation of polyacrylamide gels
The proteins were separated by SDS-PAGE according to the method described by Laemmli (1970). The procedure employed the Bio-Rad Mini-PROTEAN III™ electrophoresis cell, which was assembled according to the manufacturer's instructions.

All equipment was carefully wiped with 70% (v/v) ethanol. Approximately 8 mL of the resolving gel mixture plus TEMED (prepared
as shown in Table 2.4) were poured between two glass plates (1.5 mm spacers), held in the gel casting stand. Distilled water was immediately and gently added above the resolving gel mixture to obtain a smooth overlay. The gel mix was allowed to polymerise at room temperature for 30 min and then the distilled water overlay was removed. Next, 2 mL of the stacking gel mixture, which enhances the resolution of the protein bands made by electrophoresis, and 20 µL of TEMED (Table 2.4), were poured on top of the resolving gel. Ten individual wells were made by placing a 10-tooth comb in the stacking gel. After polymerisation of the stacking gel, the comb was carefully removed and the gel assembly was then transferred to an electrophoresis running chamber containing SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3).

Table 2.4 Preparation and reagents for resolving gel and stacking gel buffer

<table>
<thead>
<tr>
<th>Resolving gel (10%)</th>
<th>Volum</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccuGel 29:1 (40% w/v)</td>
<td>5 mL</td>
</tr>
<tr>
<td>Resolving buffer (1.5 M Tris–HCl, 0.4% (w/v) SDS, pH 8.8)</td>
<td>5 mL</td>
</tr>
<tr>
<td>Deionised water</td>
<td>9.78</td>
</tr>
<tr>
<td>10% w/v APS (ammonium persulphate (N₂H₆S₂O₈))</td>
<td>200 µL</td>
</tr>
<tr>
<td>TEMED (N, N, N’, N’-tetramethylethylenediamine) (C₆H₁₆N₂)</td>
<td>30 µL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stacking gel buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccuGel 29:1 (40% w/v)</td>
</tr>
<tr>
<td>ProtoGel stacking buffer (0.5 M Tris–HCl, 0.4 % SDS, pH 6.8)</td>
</tr>
<tr>
<td>Deionised water</td>
</tr>
<tr>
<td>10% APS</td>
</tr>
<tr>
<td>TEMED</td>
</tr>
</tbody>
</table>

2.9.9.3 One-dimensional gel electrophoresis (1D-PAGE) of samples

Samples were mixed with the Laemmli x4 electrophoresis buffer (see Table 2.5) in a 3:1 ratio, boiled at 100°C for 5 min and then allowed to cool down to room temperature prior to loading into the gel. The Laemmli buffer contains SDS, which denatures proteins by binding to the polypeptide giving the protein negative charge, β-mercaptoethanol to reduce disulphide bonds in proteins, glycerol to increase the sample
density to ensure the samples sink to the bottom of the sample well and bromophenol blue dye, which serves as a migration front to monitor to electrophoretic progression. Next, 20 µg protein from each sample were loaded into each well, to obtain even loading across the gel. Then, 2 µL of Blueye prestained protein ladder, (molecular weight range 11-245kDa) were added to one well. To allow samples to enter the stacking gel, the gel was firstly run at 50 volts for 5 min and the voltage was then increased to 150 volts until the dye front reached the bottom of the gel. The voltage was then switched off and the gels were carefully removed and used for the western blotting.

**Table 2.5 Preparation and reagents for Laemmli x4 SDS–PAGE sample buffer**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl (pH 6.8)</td>
<td>62.5 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>2% w/v</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10 v/v</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.01% w/v</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>5% v/v</td>
</tr>
</tbody>
</table>

**2.9.9.4 Western blotting**

Western blotting was performed using the method described by Towbin et al. (1979). After the separation of proteins by SDS-PAGE, gels were gently removed from the gel plates, stacking gels were cut off and discarded and the remaining resolving gel was used in Western blotting. The blotting papers (Whatman® filter paper, from Sigma-Aldrich) and nitrocellulose membrane filters (Hybond C, Amersham) were immersed in the transfer buffer (Table 2.6) for 5 min. A sandwich of sponges, filter papers, membrane and gel was arranged as shown in Figure 2.4. A glass roll was rolled across the components to remove air bubbles, which can disturb the transfer of proteins from the gel to the nitrocellulose membranes. The sandwich was then placed into a blotting chamber. An overnight procedure, using a Bio-Rad wet western blot system, was performed at room temperature. It was set at 30 volts and transferred proteins to nitrocellulose membranes using a transfer buffer.
Table 2.6 Preparation and reagents for the transfer buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Tris /Glycine (0.25 M Tris, 1.92 M Glycine)</td>
<td>100</td>
</tr>
<tr>
<td>Methanol</td>
<td>200</td>
</tr>
<tr>
<td>Distilled water</td>
<td>700</td>
</tr>
</tbody>
</table>

2.9.9.5 Immunodetection

Following protein transfer, the membranes were blocked in 3% (w/v) BSA in TBS containing 0.01% (w/v) sodium azide, which prevents microbial contamination. Blocking can eliminate non-specific protein binding to the membrane. Thereafter, an overnight incubation was performed at 4°C with a primary antibody (dilution 1:1000, from Sigma-Aldrich (Table 2.7)) against the primary protein of interest. This step allows the binding of specific antibodies to a protein of interest in the protein mixture. The membranes were then given six 10-min washes with TBS-Tween 20. To detect the binding of the primary antibody to the target protein, the membranes were incubated overnight at 4°C with a secondary antibody (horseradish peroxidise conjugated anti-mouse immunoglobulin, Catalogue No. PI-2000, from Vector) in TBS containing...
3% w/v BSA and 0.01% w/v sodium azide (dilution 1:1000). The membranes were then washed as previously with TBS-Tween 20, followed by a final wash in TBS. The enhanced chemiluminescence (ECL) reagent was used to develop the western blot in a dark box. The luminol \((\text{C}_8\text{H}_7\text{N}_3\text{O}_2)\) in ECL is oxidised producing light. This oxidation is catalysed by HPR, which is complexed with the protein of interest on the membrane. Therefore, the intensity and location of the produced light reflect the amount and molecular weight of the protein on the membrane. Advanced Image Data Analyser (AIDA) software (Fuji) was employed to measure the intensity of the protein bands.

**Table 2.7. Primary antibody application**

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Product number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HSP60</td>
<td>H3524</td>
</tr>
<tr>
<td>Anti-HSP70</td>
<td>H5147</td>
</tr>
<tr>
<td>Anti-HSP90</td>
<td>H1775</td>
</tr>
<tr>
<td>Anti-phosphoserine Clone:PSR-45</td>
<td>P-3430</td>
</tr>
<tr>
<td>Anti-phosphotyrosine Clone:PT66</td>
<td>P-3300</td>
</tr>
<tr>
<td>Anti phosphothreonine Clone:PTR-8</td>
<td>P-3555</td>
</tr>
</tbody>
</table>

**2.10 Long-term of Pd\(^{2+}\) exposure**

Part of the present study was conducted to investigate the effect of long term exposure to a low Pd\(^{2+}\) concentration (1 µg/L) on the survival and behavioural markers (oxygen consumption and phototactic behaviour) of *G. pulex*. However, the results of these investigations were not presented due to the high mortality in both the control and exposed organisms. This mortality might be a result of leaching of chemicals from the materials used (acrylic tubes, plastic mesh and plastic thread).

**2.11 Data analysis**

Data were represented as mean ± standard error (SEM), calculated using prism software. The Trimmed Spearman-Karber method
(Hamilton et al., 1977), which is a DOS-based non-parametric statistical procedure, was used to analyse the mortality data and thus estimate the 96 h LC$_{50}$ values together with their associated 95% confidence intervals (Hamilton et al., 1977; Haider and Inbaraj, 1986). Of particular importance is that this method is less subject to artefacts of the probit and logit models.

For all other experiments, data were analysed with the assistance of the Minitab 16 statistical software program. Data were firstly subjected to a Levene’s homogeneity of variance and a normality test. When data were normally distributed and variances were homogenous, they were analysed using one-way ANOVA. Where an overall significant difference between treatments was indicated, a post-hoc Tukey test was used to identify where these differences occurred using 95% confidence limits.

In the cases in where data were not normally distributed and/or variances were not homogenous, a non-parametric Kruskal-Wallis test was performed. If an overall significant effect of treatment was observed, critical differences between treatments were examined employing Dunn's multiple comparison test. Significance was invariable set at p< 0.05.

Data regarding the survival of G. pulex following 96 h exposure to a combination of PGEs were presented as percentages of surviving organisms at the end of the exposure period compared to that for the same treatment at the beginning of the experiment. Data were then subjected to one-way ANOVA and a post hoc Tukey test to identify significant differences between the survivals of the different examined groups.

PGE concentrations in G. pulex tissue were determined, adjusted to dry weight of examined tissue, and presented as μg/g dry weight. Data were then compared using one-way ANOVA followed by a post hoc Tukey’s
multiple comparison test. The bioaccumulation factor (BAF) of PGEs in *G. pulex* was calculated by dividing PGE concentrations in the tissue of exposed organisms by the nominal PGE concentrations in the exposure medium.

For the vertical movement assay, video records were reviewed and the number of surfacings were counted and then used in the data analyses. Due the wide variation between the activities of individual of each group, the vertical movement response of the tested organism was not normally distributed. Thus, data were analysed using the non-parametric Kruskal-Wallis test with Dunn's multiple comparison test.

Feeding activity data were presented as the percentage of consumed surface area relative to that consumed by the 72 h control group. Date were then analysed statically using ANOVA and a *post hoc* Tukey's test.

Data regarding the interference of Pd$^{2+}$ with the AChE assay were presented as the percentage of change in absorbance in the solution pre-incubated with Pd$^{2+}$ relative to that without Pd$^{2+}$. One-way ANOVA and a *post hoc* Tukey test were used to compare the sample means. These tests were also used to compare the AChE activity in the head of *G. pulex* to that in the whole body, after which the enzyme activity in the examined tissues was measured and then adjusted to protein content in the tested samples. Data in this test were presented as the percentages of the activity of AChE in the head of the exponential *G. pulex*. Similarly, the effect of Pd$^{2+}$ exposure on the AChE activity in the head and whole body of *G. pulex* was tested and then adjusted to protein content in the tested samples. The percentages of the AChE activity in the treated *G. pulex* relative to respective control group were then calculated and compared using one-way ANOVA. A *post hoc* Tukey’s multiple comparison test was used to identify significant differences between the AChE activities in the tested groups.
For the osmoregulation assay, data were presented as the haemolymph ion concentrations for Na\(^+\), K\(^+\), Ca\(^{2+}\) and Mg\(^{2+}\) in mM units. The Kruskal-Wallis test was performed to compare the haemolymph ion concentrations for Na\(^+\), K\(^+\) and Ca\(^{2+}\) among the experimental groups. The means of the haemolymph Mg\(^{2+}\) concentration in the tested groups were compared using the one-way ANOVA test.

The inhibitory effect of malonate on MTT reduction in *G. pulex* was presented as the percentage of change in absorbance in the solution pre-incubated with malonate relative to that without malonate. Data were then compared using using ANOVA and a post hoc Tukey’s multiple comparison test. These statistical tests were also employed to compare the effect of Pd\(^{2+}\) exposure on the level of MTT reduction by *G. pulex*, after which the MTT reduction in the examined groups was measured, adjusted to protein content in the tested samples and calculated as a percentage of the MTT reduction in the untreated control group.

The results of oxidative stress biomarkers were all adjusted to protein content in the tested samples and presented as a percentage of the activity from the untreated control group. The treatments were then compared using the non-parametric Kruskal-Wallis test. The result of GSH and GST assays were compared using Dunn's multiple comparison tests.

The results of Chapter 6 (HSPs and phosphoproteins) are expressed as percentages of the intensity of the protein bands for samples from the unexposed organisms. Statistical comparisons of these results were done by a one-way ANOVA with a post hoc Tukey test analysis.
3 Mortality studies and metal uptake

3.1 Introduction

This part of the present thesis examined the acute toxicity, the uptake and the partitioning of PGEs by *G. pulex*. The specific aims of this chapter were (1) to determine the 96 h LC50 values of PGEs (Pt, Pd, Rh, Os, Ir and Ru) for *G. pulex*, which would enable determination of PGE concentrations used in further sublethal investigations, (2) to examine the interaction of Pt and Rh with Pd toxicity and their uptake by the experimental organisms, and (3) to study the partitioning of PGE in *G. pulex* tissue. To achieve the first aim, *G. pulex* in a semi-static system were exposed to a range of Pt, Pd, Rh, Os, Ir or Ru concentrations for 96 h and the mortality of exposed organisms was assessed every 24 h. The effect of Pt or Rh on Pd toxicity to *G. pulex* was examined by investigation of the survival response of *G. pulex* following exposure to binary combinations of PGEs (1 mg/L Pd with coexposure to 0, 1, 5 or 10 mg/L Pt or Rh). The uptake of PGEs (Pd, Pt and Rh) under different exposure conditions (single metal or binary combinations of Pd, Pt, Rh, Pd/Pt or Pd/Rh) was then examined to investigate whether the uptake of Pt or Rh interacts with the uptake of Pd by *G. pulex*. In order to investigate Pd partitioning, *G. pulex* were exposed to either 0 or 0.5 mg/L Pd for 72 h. The exposed organisms were then subjected to a subcellular fractionation procedure. Pd content in MRG of *G. pulex* was investigated using a scanning electron microscope.

3.2 Results

3.2.1 Median lethal concentration (LC50) assessment

The solubility of ruthenium (Ru2+) was less than that of other PGEs, and Ru2+ did not caused a significant mortality (≤ 10 %) to *G. pulex* over 96 h at a concentration of ≤ 1 mg/L (Figure 3.1). The 96 h LC50 tests found no significant difference between the survival of *G. pulex* exposed to dechlorinated tap water (control), and *G. pulex* exposed to ≤ 10 mg/L Pt, Rh, or Ir (≤ 10% mortality for all test elements within 96 h) (Figures 3.2 to 3.4). Mortality data were employed to calculate the 96 h LC50 for
Pd$^{2+}$ and Os$^{3+}$ as they were the only PGEs to produce $> 50\%$ death within 96 h (Figures 3.5 and 3.6). The 96 h LC$_{50}$ values of Pd$^{2+}$ and Os$^{3+}$ to *G. pulex* were 0.52 mg/L (4.89 µM) and 0.41 mg/L (2.1 µM), respectively (Table 3.1).

The toxicity of Pd$^{2+}$ and Os$^{3+}$ to *G. pulex* was both concentration and time dependent (Figures 3.5 and 3.6). Exposure to $\leq 1$ mg/L Os$^{3+}$ or Pd$^{2+}$ caused little, if any, mortality over the first 24 h. At all tested Pd$^{2+}$ and Os$^{3+}$ conditions, the survival rates of exposed organisms were $> 74\%$ at the end of 48 h exposure. After 72 h of exposure, the survival rate of exposed organisms decreased to $\leq 62\%$ for both tested metals. However, if the exposure to 1 mg/L Os$^{3+}$ or Pd$^{2+}$ was continued for 96 h, $> 50\%$ mortality occurred (Figures 3.5 and 3.6).

![Figure 3.1](image.png)

*Figure 3.1 The time dependent survival of *Gammarus pulex* exposed to different concentrations of ruthenium. (Means, n= 30-70).*
Figure 3.2 The time dependent survival of *G. Gammarus pulex* exposed to different concentrations of iridium (means, n= 30-70).

Figure 3.3. The time dependent survival of *Gammarus pulex* exposed to different concentrations of platinum (means, n= 30-70).
Figure 3.4 The time dependent survival of *Gammarus pulex* exposed to different concentrations of rhodium (means, n= 30-70).

Figure 3.5 The time dependent survival of *Gammarus pulex* exposed to different concentrations of palladium (means, n= 30-70).
Figure 3.6 The time dependent survival of *Gammarus pulex* exposed to different concentrations of osmium (means, n= 30-70).

Table 3.1 Trimmed Spearman-Karber analysis of toxicity data (nominal concentrations)

<table>
<thead>
<tr>
<th>Metal</th>
<th>96 h LC₅₀</th>
<th>Lower 95% confidence limit</th>
<th>Upper 95% confidence limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Os³⁺</td>
<td>0.41 (2.1 µM)</td>
<td>0.27 (1.38 µM)</td>
<td>0.64 (3.28 µM)</td>
</tr>
<tr>
<td>Pd²⁺</td>
<td>0.52 (4.89 µM)</td>
<td>0.36 (3.44 µM)</td>
<td>0.73 (6.86 µM)</td>
</tr>
</tbody>
</table>

3.2.2 Effect of exposure to a combination of PGEs on the survival of *G. pulex*

The initial studies determined that only Pd²⁺ and Os³⁺ were toxic to *G. pulex* and Os³⁺ toxicity was significantly higher than that of Pd²⁺ on a molarity basis (as the 95% confidence limits for µM do not overlap) (Table 3.1). Since the project concerns the PGEs in catalytic converters, no further studies on osmium were undertaken. As catalytic converters usually contain a combination of PGEs, the toxicity of Pd²⁺/Pt⁴⁺ and Pd²⁺/Rh³⁺ combinations were also assessed.
The presence of Pt or Rh caused a significant decrease in Pd’s toxicity to *G. pulex*. The toxicity data showed that the percentage survival of *G. pulex* following 96 h exposure to a combination of Pt or Rh and 1 mg/L Pd\(^{2+}\) was positively associated with Pt and Rh concentrations (Figures 3.7 and 3.8).

**Figure 3.7** Survival of *Gammarus pulex* exposed to different concentrations of Pt\(^{4+}\) with coexposure to 1 mg/L Pd\(^{2+}\) for 96 h. Means ± SEM, n=3-5. Treatments that do not share a letter are significantly different.

**Figure 3.8** Survival of *Gammarus pulex* exposed to different concentrations of Rh\(^{3+}\) with coexposure to 1 mg/L Pd\(^{2+}\) for 96 h. Means ± SEM, n=3-5. Treatments that do not share a letter are significantly different.
3.2.3 PGE uptake by *G. pulex*

This part of the current study examined the accumulation of Pd, Rh and Pt in the body of *G. pulex* following exposure to either single or binary combinations of Pd, Pt, Rh, Pd/Pt or Pd/Rh. This determination might provide an explanation for how Pt and Rh reduce the effect of Pd on the survival of *G. pulex*. Initial experiments were conducted to establish the time dependence of Pd accumulation in *G. pulex*. The results showed that there was a general trend of increase in Pd content over time (Figure 2.1). Based on the results of this experiment (see section 2.6) it was decided to conduct the Pd$^{2+}$ and Pt$^{4+}$ or Rh$^{3+}$ exposure trials for 72 h.

In the Pd$^{2+}$/Pt$^{4+}$ coexposure experiment, the Pd content of exposed *G. pulex* was 25.4-33.5 and 27-84.1 µg/g dry weight for 0.25 and 0.5 mg/L Pd treated groups, respectively. Although Pt was added, up to a concentration of 10 mg/L, it did not cause a reduction in the uptake of Pd by *G. pulex* (Figure 3.9). Pd concentration in organisms exposed to 0.5 mg/L Pd was significantly higher than that of 0.25 mg/L group (Figure 3.9). When *G. pulex* was exposed to 0.5 mg/L Pd, only the presence of Pt at 10 mg/L Pt caused a significant increase in Pd content in comparison to 0 mg/L Pt group, reaching 69.4±7.7 µg/g dry weight (mean). Similarly, Pt uptake by *G. pulex* was not significantly reduced by the presence of Pd (Figure 3.10). The results also showed that, at the highest test Pt concentration (10 mg/L), a significant increase in Pt uptake by *G. pulex* occurred when organisms were coexposed to 0.5 mg/L Pd (Figure 3.10). No significant effect was observed under the other experimental conditions.
Figure 3.9 Palladium content of *Gammarus pulex* after coexposure to platinum. Means ± SEM, n = 6. Treatments that do not share a letter are significantly different.

Figure 3.10 Platinum content of *Gammarus pulex* after coexposure to palladium. Means ± SEM, n = 6. Treatments that do not share a letter are significantly different.
In the Pd\(^{2+}/\text{Rh}^{3+}\) coexposure experiment, the Pd content in the exposed organisms was 7.4-27.8 and 24.6-58.8 \(\mu\)g/g dry weight for 0.25 and 0.5 mg/L Pd treated groups, respectively. The Rh was added up to a concentration of 10 mg/L. The Pd content of organisms exposed to 0.25 mg/L Pd coexposed to Rh was significantly lower when Rh was present at a concentration of 5 mg/L than that of 0 and 10 mg/L Rh groups (Figure 3.11). The Pd content in \textit{G. pulex} following exposure to 0.5 mg/L Pd was significantly higher than that of organisms exposed to 0.25 mg/L Pd when coexposed to 0 or 5 mg/L Rh, but not at 10 mg/L Rh. The results also show that the Pd content in test organisms exposed to a combination of 0.5 mg/L Pd and 5 or 10 mg/L Rh were lower than that of organisms exposed to 0.5 mg/L Pd alone (Figure 3.11). However, this difference was only significant (\(p= 0.021\)) at 10 mg/L Rh.

No significant differences were observed in the Rh content of \textit{G. pulex} exposed to 5 mg/L Rh when Pd was presented at concentrations 0, 0.25 or 0.5 mg/L. In contrast, the Rh content of organisms exposed to 10 mg/L Rh was significantly reduced by the presence of Pd at concentrations of 0.5 mg/L (Figure 3.12).

![Figure 3.11 Palladium content of \textit{Gammarus pulex} after coexposure to rhodium. Means ± SEM, \(n =6\). Treatments that do not share a letter are significantly different.](image-url)
The present study showed that, following 72 h PGE (Pd, Pt and Rh) exposure, Pd had the highest bioaccumulation rate in *G. pulex* amongst the tested metals (Figures 3.9 to 3.12), with a bioaccumulation factor (BAF) (µg/kg in dry weight of *G. pulex* per µg/kg in exposure solution) of approximately 94.7 followed by Pt and Rh with BAFs around 19.1 and 11.4, respectively.

### 3.2.4 Pd$^{2+}$ content in metal-rich granules of *G. pulex*
Granules were visualised, using a scanning electron microscope, in a section of *G. pulex* exposed to Pd$^{2+}$ for 72 h. Observed granules were located dorsally below the exoskeleton of tested organisms. Pd$^{2+}$ was only detected in the tested sections of the 0.5 mg/L Pd$^{2+}$ treated group but not in the control group (Figures 3.13 and Table 3.2). Under all experimental conditions, oxygen (O) and silicon (Si) were the most abundant elements, with O more abundant (Figure 3.13). In the control sample, Na was present at a similar proportion to Ca. In contrast, in the Pd-treated sample, Na was 1.6-time as high as Ca (Table 3.2).
Figure 3.13 Energy dispersive x-ray microanalysis (EDX) of sections of *Gammarus pulex* after 72 h exposure to 0 (A) or 0.5 (B) mg/L Pd$^{2+}$ (n=1).

Table 3.2 The elements content in a tested section of *Gammarus pulex* exposed for 72 h to 0 and 0.5 mg/L Pd$^{2+}$, respectively. Measureable amounts of Pd$^{2+}$ were presented only on the granules of exposed organisms.

<table>
<thead>
<tr>
<th>Element</th>
<th>Control Weight%</th>
<th>Control Atomic%</th>
<th>0.5 mg/L Pd$^{2+}$ Weight%</th>
<th>0.5 mg/L Pd$^{2+}$ Atomic%</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>78.32</td>
<td>86.58</td>
<td>52.75</td>
<td>66.69</td>
</tr>
<tr>
<td>Na</td>
<td>4.53</td>
<td>3.48</td>
<td>8.56</td>
<td>7.35</td>
</tr>
<tr>
<td>Si</td>
<td>12.59</td>
<td>7.93</td>
<td>31.34</td>
<td>22.53</td>
</tr>
<tr>
<td>Ca</td>
<td>4.56</td>
<td>2.01</td>
<td>5.38</td>
<td>2.71</td>
</tr>
<tr>
<td>Br</td>
<td></td>
<td></td>
<td>1.86</td>
<td>0.47</td>
</tr>
<tr>
<td>Pd</td>
<td></td>
<td></td>
<td>0.12</td>
<td>0.2</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3 Discussion

3.3.1 Effect of PGEs exposure on the survival of *G. pulex*

Only Pd$^{2+}$ and Os$^{3+}$ caused a significant reduction in the survival of *G. pulex* (Figures 3.5 and 3.6) with a 96 h LC$_{50}$ of 0.52 mg/L (4.89 µM) and 0.41 mg/L (2.1 µM) for Pd$^{2+}$ and Os$^{3+}$, respectively (Table 3.1). The result presented in Table 3.1 shows that using molarity, the 96 h LC$_{50}$ test unambiguously determined that Os$^{3+}$ was more toxic than Pd$^{2+}$ to *G. pulex*. In contrast, on a mass basis, there was no significant difference between them, as their 96 h LC$_{50}$ 95% confidence limits overlapped (Table 3.1). The molarity-based results concur with those of Khangarot (1991) in which Os$^{3+}$ toxicity towards *T. tubifex* was higher than that of Pd$^{2+}$ with 96 h LC$_{50}$ values of 0.0067 mg/L (0.0013 µM) and 0.092 mg/L (0.87 µM), respectively.

In comparison with the results of the current study, published data have shown different sensitivity of aquatic animals to Pd$^{2+}$ exposure. For example, Borgmann *et al.* (2005) reported the 48 h LC$_{50}$ of Pd$^{2+}$ on *H. azteca* was 0.57 mg/L (5.4 µM). Their finding represents a higher toxicity than that observed in the present study: a survival rate higher than 74% when *G. pulex* was exposed to 1 mg/L Pd$^{2+}$ for 48 h. Similarly, the mortality of *G. pulex* due to Pd$^{2+}$ exposure was lower in the present study than that in *T. tubifex* (Khangarot, 1991).

Differences in the response of aquatic biota to metal exposure can be, at least partly, attributed to differences between sampling locations, season, size of containers and/or exposure conditions (Bat *et al.*, 2000). For instance, in the current study, LC$_{50}$ tests were performed at 15°C, while in the study of Borgmann *et al.* (2005), organisms were exposed to Pd at 25°C. This may partly explain the different survival of exposed organisms in both studies. Temperature may increase the bioavailability and thus the toxicity of metal (Bat *et al.*, 2000; Sokolova and Lannig, 2008). Elevated temperature can increase the concentrations of free metal ions, by increasing the solubility of metal compounds (Sokolova
and Lannig, 2008). However, these authors reported that metal speciation, particularly free ion activity, may not be significantly affected by the temperature within environmentally relevant ranges. This increases the need to consider the biological influences of temperature in order to understand its effect on the uptake and accumulation of metal by biota. Temperature-dependent alterations in metal uptake and/or excretion may be affected by numerous biological factors, such as metal transport, the permeability of cell membranes and systemic functions (e.g. ventilation or absorption from food) that can affect the degree of exposure to metals (Sokolova and Lannig, 2008). Active uptake, which is a major route of metal uptake in aquatic biota, is positively correlated with the environmental temperature. Thus, elevated temperature would increase metal uptake and, unless offset by a similar elevation in excretion rate, result in metal accumulation in the exposed organism. Metabolic rate and thus energy demand are increased at higher temperature. This increase may consequently lead to higher ventilation and/or feeding activity, which in turn may elevate metal uptake by the exposed organism due to greater contact with polluted water and/or higher consumption of metal polluted water or food (Sokolova and Lannig, 2008). Thus, the differences between the mortality response of *G. pulex* observed in the present thesis and that of *T. tubifex* reported by Khangarot (1991) may in part be due to differences between exposure conditions, in particular, water temperature, which can increase membrane permeability and metabolic rate and thus alter uptake, elimination and detoxification rates (Sokolova and Lannig, 2008; Vellinger et al., 2012). While the mortality test in the current study was conducted at a temperature of 15°C, the study of Khangarot (1991) was performed at a temperature of 30°C. This is supported by Veltz et al. (1996), who observed that Pt toxicity toward the annelid *Lumbriculus variegates* at 20°C was higher than that at 4°C.

Water hardness is another factor that can affect metal toxicity toward aquatic biota. For example, Kiyani *et al.* (2013) observed that an
increase in water hardness from 25 to 350 mg/L as CaCO$_3$ increased the 96 h LC$_{50}$ values of Zn$^{2+}$ and Cu$^{2+}$ to fish Gambusia holbrooki (Cyprinodontiformes: Poeciliidae) from 0.46 and 0.017 mg/L to 121.6 and 0.65 mg/L, respectively. Pascoe et al. (1986) reported that water hardness could reduce metal toxicity via two possible chemical mechanisms: decreased metal availability due to alteration of chemical speciation in the hard water, or due to physiological mechanisms. Ca$^{2+}$ and/or Mg$^{2+}$ ions in hard water may reduce the uptake and thus toxicity of metals towards aquatic biota by competing with these metal ions for the uptake sites in an organism (Kiyani et al., 2013). Hard water can also influence the permeability of cell membranes and thus control the access of metal ions to aquatic biota (Lockwood et al., 1982; Pascoe et al., 1986; Kiyani et al., 2013). Increased water hardness may cause stabilisation of cell membranes, altering their permeability (Penttinen et al., 1998). Tun (1975) reported that the half-time of exchange of tritiated water flux in the decapod crustacean Palaemonetes varians (Decapoda: Palaemonidae) in 2% seawater was 15.4, 19.5, 20.3 and 21.4 min when Ca$^{2+}$ was present at 0, 0.1, 1 and 10 µM (0, 0.004, 0.04 and 0.4 mg/L, respectively). Hard water can saturate the binding sites in the gill surfaces with Ca$^{2+}$ (Kiyani et al., 2013). Therefore, it can be suggested that the differences between the mortality response of G. pulex observed in the present thesis and that of H. azteca observed by Borgmann et al. (2005) were partly due to differences in water hardness (231 and 124 mg/L CaCO$_3$, respectively).

By comparing the 96 h LC$_{50}$ value of Pd$^{2+}$ observed in the current study and that of other toxic metals reported in previous studies, Pd$^{2+}$ had a lower effect on the survival of G. pulex than Cu$^{2+}$ (Guven et al., 1999; Bat et al., 2000) and Cd$^{2+}$ (Felten et al., 2008). In contrast, Bat et al. (2000) reported lower toxicity of Pb$^{2+}$ to G. pulex, with a 96 h LC$_{50}$ range between 54.1 to 112 µM (11.2 to 23.2 mg/L), than that of Pd$^{2+}$ observed in the current study. These differences in the sensitivity of G. pulex to metal exposure may in part be due to different bioavailability or subcellular partitioning and compartmentalization of these metals.
(Wallace et al., 2003; Khan et al., 2012). Although metals are often non-toxic when they accumulate in the cell, toxic impacts of metals may occur when metals accumulate in organelles (e.g. lysosomes and mitochondria) and cytosolic heat-denaturable protein fractions (Khan et al., 2012). In addition, the toxicity of metals, particularly essential metals, may be influenced by the ability of *G. pulex* to regulate their tissue levels. This regulation is mainly achieved via increasing the excretion rate of a metal to match its uptake rate (Guven et al., 1999). Guven et al. (1999) reported that exposure to high concentrations of essential metals may affect the ability of aquatic organisms to maintain the body concentration of these metals below toxic levels. Detoxification of some metals may also be due to their binding to metallothioneins (Guven et al., 1999; Cheung and Wang, 2005) or MRGs (Wallace et al., 2003). Aquatic organisms can also adapt to environmental stresses. For example, Stuhlbacher and Maltby (1991) reported that pre-exposure to low concentrations of Cd or Zn enhanced the tolerance of *G. pulex* to acute Cd stress. They reported that pre-exposure to these metals increased the level of metallothionein in the exposed organisms. Similarly, Fraser (1980) observed that the Freshwater isopod *Asellus aquaticus* can acclimate to lead under both laboratory and natural conditions.

The influence of the period of exposure and metal concentration on metal toxicity is well documented. Several studies have shown a positive relationship between PGE toxicity and their concentrations and the exposure time (Khangarot, 1991; Farago and Parsons, 1994; Veltz et al., 1996). Ferreira Jr and Wolke (1979) reported that the 24, 48, and 96 h LC$_{50}$ values of Pt$^{4+}$ on *O. kisutch* were 15.2, 5.2 and 2.5 mg/L, respectively. Similarly, the present study shows that the mortality response of *G. pulex* to Pd$^{2+}$ and Os$^{3+}$ exposure was both time and concentration dependent. The mortality of exposed *G. pulex* increased with increasing Pd$^{2+}$ and Os$^{3+}$ concentration and/or period of exposure. After 96 h of exposure to 0.5 or 1 mg/L Pd$^{2+}$ or Os$^{3+}$, treated organisms
had survival rates substantially lower than that of untreated control groups for the same period of exposure.

In the current study, it was unexpectedly found that both Pt\textsuperscript{4+} and Rh\textsuperscript{3+} significantly reduced the impact of Pd\textsuperscript{2+} on the survival of \textit{G. pulex} (Figures 3.7 and 3.8). After 96 h of exposure, the mortality of \textit{G. pulex} exposed to 1 mg/L Pd in the presence of Pt or Rd was ≤ 50% that of the group exposed to only 1 mg/L Pd, suggesting an antagonistic interaction between Pd and Pt or Rh. The antagonistic interaction of metal toxicity has previously been reported by Charles \textit{et al.} (2014), who observed that low concentrations of Ni decreased Cu toxicity towards \textit{G. pulex}. Similarly, Bat \textit{et al.} (1998) found that Zn reduced the effect of Cd on the survival of the amphipod crustacean \textit{Corophium volutator} (Amphipoda: Corophiidae).

There is a lack of data regarding combined toxicities of PGE. The reasons behind the reduction of Pd toxicity to \textit{G. pulex} in the presence of Pt and/or Rh are still not clearly understood. This reduction may be due to a decrease in the uptake rate, increase in excretion, change of distribution, and/or activation of detoxification (Mohan \textit{et al.}, 1986). Uptake of a metal may be reduced due to its competition with other metals for transmembrane transport. Such competitive inhibition was reported by Mandal \textit{et al.} (2006), who observed that, in lobster (Decapoda: Nephropidae), Zn uptake into hepatopancreatic lysosomal membrane vesicles was inhibited by the present of Cd and Cu. In another study, it was demonstrated that Cd reduced the uptake of Pb by the gills of the freshwater rainbow trout, \textit{O. mykiss} (Rogers and Wood, 2004). The latter researchers found that Cd and Pb competed for entry into the cell via Ca\textsuperscript{2+} channels. The results of the current study, however, showed that the protective effect of Pt and Rh against Pd toxicity to \textit{G. pulex} was often not accompanied by a reduction of Pd uptake in exposed organisms.
The alteration in a metal’s toxicity in the presence of other metals can also be attributed to a competition between metal ions for cellular binding sites (Morley et al., 2005). They also reported that such protective effects may be associated with the probable induction of metallothionein, or metallothionein-like, protein synthesis. It might be that Pt increases the level of metallothionein, which plays an important role in the detoxification of several non-essential xenobiotic metals, whilst Pd has a significantly lower effect on metallothionein induction. Under these circumstances, it might be anticipated that exposure of Pt reduces the toxicity of Pd even if Pd uptake is unaffected.

Another possible mechanism of reducing Pd toxicity by Pt and Rd could be that Pt and Rh have the ability to stimulate the antioxidant system, and thus reduce ROS in living organisms. It has been demonstrated that Pt, particularly platinum nanoparticles, can act as antioxidants that protect cells against oxidative damage. Onizawa et al. (2009) reported that the presence of PAA-Pt (platinum nanoparticles and polyacrylate as a stabiliser) enhances antioxidant capacity in the lungs of mice exposed to cigarette smoke for 72 h. Similarly, Kim et al. (2008) reported that exposure to 0.05 g/L platinum nanoparticles extended the lifespan of Caenorhabditis elegans. These authors reported that such effects were due to antioxidant activities. To the best of the author’s knowledge, such effects have not been reported for Pd.

Although the results of the current study show that, under certain conditions, Pt or Rh may cause significant alterations in Pd\textsuperscript{2+} uptake by G. pulex (Figures 3.9 and 3.11), no clear relationship was observed between these changes and the reduction in the mortality of G. pulex when coexposed to Pd and Pt or Rh. Therefore, further investigations, using a technique with a lower detection limit, such as inductively coupled plasma mass spectrometry (ICP-MS) (0.1 µg/L for Pd, Pt and Rh), are needed in order to clarify the mechanism by which Pt or Rd reduce the toxicity of Pd towards G. pulex. These investigations could include metallothionein induction and subcellular partitioning of Pd in G.
pulex following single and combination exposure to PGEs (Pd, Rh and Pt).

### 3.3.2 Uptake of PGEs (Pd, Pt and Rh) by G. pulex

The uptake of Pd by aquatic biota has been previously shown (Rauch and Morrison, 1999; Moldovan et al., 2001; Sures et al., 2002b; Sures et al., 2005; Haus et al., 2007, Sures and Zimmermann, 2007). Similarly, the initial exposure study (Figure 2.1) clearly demonstrated the uptake and bioaccumulation of Pd by G. pulex. Pd content in the whole tissue of organisms exposed to 0.5 mg/L Pd was time-dependent, with an increase in the whole body Pd concentration in G. pulex by increasing the period of exposure reaching a peak at the end of 96 h (Figure 2.1). In addition, the Pd content in G. pulex was dose-dependent (Figures 3.9 and 3.11), with a higher Pd content in organisms exposed to 0.5 mg/L than that of 0.25 mg/L group. Metal uptake by G. pulex might occur via several pathways (Rainbow, 1997). Some metals, particularly essential metals, can be transferred across the membranes into the cell via binding to a membrane carrier protein. Metal ions may also bind to a membrane protein and then move through ion pumps. This type of active metal transport allows for accumulation of metal ions against a concentration gradient (Rainbow, 1997). Passive diffusion is a possible transport route of non-polar metal forms (e.g. alkyl-metal compounds). These forms are soluble in the lipid phase and can cross the phospholipid bilayer to enter the cell interior. Metals can also be carried into cells by endocytosis, by which membrane invagination is stimulated, engulfing a metal liferous particle and transferring it into an intracellular vesicle (Rainbow, 1997). However, active transport and membrane carrier protein present the main routes of trace metal uptake from solutions by aquatic crustaceans (Rainbow, 1997).

Following 72 h PGE (Pd, Pt and Rh) exposure, Pd had the highest bioaccumulation rate in G. pulex amongst the tested metals (Figures 3.9 to 3.12). This trend in bioaccumulation reflects the relative
bioavailability of PGEs. This finding is consistent with the results of Sures et al. (2002b), who reported that the bioavailability of PGEs to *D. polymorpha* was in the following order: Pd > Pt > Rh. Similarly, Moldovan et al. (2001) demonstrated that the BAFs of PGEs in *A. aquaticus* following 24 h exposure to PGE standard solutions were 150, 85, and 7 for Pd, Pt and Rh, respectively. However, a few other investigations have shown a higher bioaccumulation of Pt than that of Pd. Such observations were reported by Singer et al. (2005) in *D. polymorpha*; these authors observed the following trend of bioaccumulation Pt > Pd > Rh. These differences, between the findings of studies compared the bioavailability of PGEs, may be attributed to the metal speciation, which influences the water solubility and hence the bioavailability.

The results of the present study suggested no significant interaction between the uptake of Pd and Pt or Rh, when organisms were exposed to 0.25 mg/L Pd with all tested Pt and Rh concentrations (Figures 3.9 to 3.12). In general, the observed bioaccumulation of Pt and Pd by *G. pulex* was similar when organisms were exposed to either a single solution or a binary combination of these metals. This finding proposes that Pt does not interact with Pd when they are both present together and are taken up by *G. pulex*. Similar results were observed by Moldovan et al. (2001), who reported that the uptake of PGEs by *A. aquaticus* exposed to PGE standard solutions for 24 h was similar whether the test organisms were exposed to mixed or to individual standard solutions. When *G. pulex* were exposed to a combination of the highest tested concentrations of Pd and Pt (0.5 and 10 mg/L, respectively), the concentrations of both metals in the tested organisms were higher than those observed under other experimental conditions (Figure 3.9 and 3.10). This suggests that, at these high concentrations, Pd and Pt stimulated the accumulation of each other. In line with this finding, enhancing the uptake of a metal by the presence of another metal in aquatic crustacea has been previously reported. For example, Cu accumulation in *H. azteca* exposed to Cu and Cd combination was more than 3-fold higher than that of organisms
exposed to Cu alone (Shuhaimi-Othman and Pascoe, 2007). They suggested that the presence of Cd may reduce the elimination of Cu by \textit{H. azteca}. Another possible explanation for the higher uptake Pd and Rh by \textit{G. pulex}, when metals were present at their higher tested concentration, is that these concentrations induce gill damage and thus increase membrane permeability (Vellinger \textit{et al.}, 2012).

The Pd/Rh coexposure test, in the present study, showed that these metals only interacted at the highest tested concentrations (0.5 mg/L Pd$^{2+}$ and 10 mg/L Rh) (Figures 3.11 and 3.12). This interaction caused a significant reduction in the content of both tested metals in exposed organisms. This reduction might be the result of a competition between Pd and Rh for a common uptake route (e.g. metal binding protein or ion channels) (Holwerda, 1991), or stimulation of defence or metal elimination mechanisms in the exposed organisms (Shuhaimi-Othman and Pascoe, 2007).

\subsection*{3.3.3 Subcellular partitioning of PGEs in \textit{G. pulex}}

The result presented in Figure 3.13 indicated that \textit{G. pulex} exposed to 0.5 mg/L Pd$^{2+}$ for 72 h partitioned Pd to MRGs. This result agrees with that of Khan \textit{et al.} (2012), who reported that MRGs may act as detoxification pools in aquatic invertebrates, and thus reduce metal-induced toxicity. MRGs, which are found in the majority of macro-invertebrate phyla, may represent biomarkers of trace metal toxicity, they can be classified into three types (Khan \textit{et al.}, 2012). Type A contains calcium in different forms. When this type of granule contains Ca$^{2+}$ in calcium phosphate form they provide stores for trace metals, which are often associated with detoxification. These granules serve as temporary Ca$^{2+}$ stores. For example, during moulting, large fluxes in Ca$^{2+}$ concentration occur. By temporarily storing calcium removed from the exoskeleton prior to its shedding, the calcium can be returned as the new exoskeleton hardens (Meyran \textit{et al.}, 1984). These granules contain Ca$^{2+}$ (and some Mg$^{2+}$) in the form of carbonates, sulphates or...
phosphates. Type B granules contain mixtures of sulphur with copper or zinc, and type C, which is more crystalline, contains iron as the main component (e.g. ferritin). In crustaceans, granules mainly belong to types A and B (Khan et al., 2012). The role of MRGs in the storage, sequestration and excretion of accumulated metals has been previously shown (Voets et al., 2009). Excess metals can be bound in MRGs, which can be eliminated in faeces via discharge from digestive gland cells by exocytosis during the normal digestive cycle (Deba and Fukushima, 1999). This will enable organisms to decrease the total metal load. The current study attempted to investigate the role of MRGs in G. pulex as a detoxification store of Pd²⁺.

The data in Table 3.2 show that tested sections contained O, Si, Na and Ca. This observation agrees with a previous study report that MRGs of G. pulex contain O, Na and Ca (Khan et al., 2012). They, however, reported that MRGs of G. pulex contain P, which was not observed in the present study. In addition, they did not report the presence of Si in the tested sections of G. pulex. The differences in the composition of the tested sections in the current study and that reported by Khan et al. (2012) might be because they examined the elemental composition of MRGs, while the current study tested the elemental composition of sections containing granules. These granules were located dorsally below the exoskeleton of the tested organisms. This observation concurs with previous reports that showed that metal granules found in gammarids were mainly located dorsally, between the exoskeleton and the digestive tract (Khan et al. 2012).

Despite the very small sample size used in the current study, it was indicated that Pd can be portioned to MRGs in G. pulex (Figure 3.13). More investigations are needed to improve knowledge of the potential role of MRGs in G. pulex in Pd detoxification and the effect of Pd exposure on granule formation. Such investigations would require a larger sample size and wider ranges of Pd concentrations than used in the current study.
To conclude, the current chapter showed that, amongst traffic-related PGEs, only Pd affected the survival of *G. pulex* and had the highest BAF. The presence of Pt or Rh reduced the toxicity but not the uptake of Pd by *G. pulex*. It was also found that Pd concentration in subcellular fractions was lower than the detection limit of the ICP-OES, and thus future studies should employ a more sensitive technique, such as ICP-MS. Following 72 h exposure, some Pd was partitioned to MRGs.
4 Behavioural markers and acetylcholinesterase

4.1 Introduction

This chapter examines the sensitivity of some behavioural and biochemical endpoints as markers of Pd toxicity in *G. pulex*. It also investigates the possible relationship between these tested biomarkers. The main aims of this part of the present study were (1) to investigate the effects of Pd on the vertical movement activity of *G. pulex*, (2) to examine the alteration in feeding activity of *G. pulex* following sublethal Pd exposure and (3) to study the change in AChE activity (in the head and the whole body) induced by Pd exposure in *G. pulex*. To achieve these aims, *G. pulex* were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd (nominal concentrations) and the alteration in the examined biomarkers was examined after 24 h for movement activity, after 24, 48 and after 72 h for feeding activity and 72 h for AChE activity.

4.2 Results

4.2.1 Vertical movement assay

Figure 4.1 shows the impact of 24 h Pd$^{2+}$ exposure on the vertical swimming activity of *G. pulex*. Only the highest tested concentration of Pd$^{2+}$ (0.5 mg/L) caused a significant reduction in the vertical movement of *G. pulex*. Although the mean values of all tested doses were lower than that of the control group, this reduction increase was only significantly different from that of the control group at the highest tested Pd$^{2+}$ concentration (0.5 mg/L).
Figure 4.1 The effect of palladium on vertical movement of *Gammarus pulex* after 24 h exposure. Means ± SEM, n=20. Treatments that do not share a letter are significantly different (p< 0.04).

### 4.2.2 Feeding activity

The effect Pd\(^{2+}\) exposure on the feeding activity of *G. pulex* is shown in Figure 4.2. The feeding response to Pd\(^{2+}\) exposure was both time and concentration-dependent. Exposure for 24 h to Pd\(^{2+}\) decreased the feeding activity of the exposed organisms. However, this reduction was only statistically significant at the highest tested Pd\(^{2+}\) concentration (0.5 mg/L). This may be due to the low number of replicates employed in the current study (N= 3). Similarly, at the end of 48 h of Pd\(^{2+}\) exposure, the reduction in feeding activity for the 0.1 and 0.25 mg/L Pd\(^{2+}\) groups was not significantly lower than that of the untreated control group. At the end of 72 h of Pd\(^{2+}\) exposure, the feeding activity of exposed *G. pulex* was significantly depressed under all the conditions tested. The food consumption of organisms exposed to 0.1 or 0.25 mg/L Pd\(^{2+}\) was reduced by 49±7 % of that of the untreated control group. This reduction was nearly doubled (95% of the untreated control group) when *G. pulex* were exposed to 0.5 mg/L Pd\(^{2+}\).
4.2.3 Acetylcholinesterase

4.2.3.1 Pd$^{2+}$ interference with the Ellman assay

In the first experiment, the possible interference of Pd$^{2+}$ with thiocholine (TCh) in the Ellman assay was examined. Following 15 minutes pre-incubation of free TCh with final Pd$^{2+}$ concentrations of 0, 0.1, 0.25 and 0.5 mg/L (0, 0.94, 2.35 and 4.7 µM, respectively), Pd$^{2+}$ did interfere with the reaction between TCh and DTNB. As shown in Figure 4.3, a strong negative relationship was observed between Pd$^{2+}$ concentration and the ability of TCh to react with DTNB to form a coloured product. TCh reactivity was 69.1, 61.2 and 48.8% of that of the untreated control group at 0.1, 0.25 and 0.5 mg/L Pd$^{2+}$, respectively.
4.2.3.2 The effect of Pd$^{2+}$ exposure on AChE activity of G. pulex
The effects of Pd$^{2+}$ exposure on the whole body AChE activity was. The AChE activity of the exposed organisms increased with increasing Pd$^{2+}$ concentration reaching 111.2, 135.8 and 140.6% of that of the untreated control group at 0.1, 0.25 and 0.5 mg/L Pd$^{2+}$, respectively (Figure 4.4). However, this increase was only significantly different from that of the control group at the highest tested Pd$^{2+}$ concentration (0.5 mg/L).
Figure 4.4 Effects of Pd$^{2+}$ on AChE activity on whole body *G. pulex* extract after 72 h exposure. Means ± SEM, n=6. Treatments that do not share a symbol are significantly different (p < 0.05).

To examine whether measuring the effect of Pd$^{2+}$ exposure on AChE activity in the head of *G. pulex* will provide a clearer response than that observed using the whole body, the enzyme activity in the head of the tested organism was compared to that of the whole body. As shown in Table 4.1, a significantly higher AChE activity was observed in the head of *G. pulex* in comparison to that detected in the rest of the animal, when corrected for protein content.

Table 4.1 AChE activity in the head and body of *Gammarus pulex* (% of the enzyme activity of the head normalised to protein content) (* means that activity is significantly different than that of the control p < 0.025).

<table>
<thead>
<tr>
<th>Part of organism</th>
<th>AChE activity (% of that in head)</th>
<th>SEM</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head</td>
<td>100</td>
<td>15.24</td>
<td>3</td>
</tr>
<tr>
<td>Body</td>
<td>44.7*</td>
<td>3.7</td>
<td>3</td>
</tr>
</tbody>
</table>

Subsequently, the alteration in AChE activity in the head of *G. pulex* following 72 h of Pd$^{2+}$ exposure was investigated. Pd$^{2+}$ did not cause significant alterations in AChE activity in the head of treated *G. pulex* (Figure 4.5). AChE activity in the head of the treated organisms was
95.1, 92.3 and 98.6% of that of the control organisms, at 0.1, 0.25 and 0.5 mg/L Pd\(^{2+}\), respectively (Figure 4.5).

![Figure 4.5 Effects of Pd\(^{2+}\) exposure on AChE activity in the head of Gammarus pulex after 72 h exposure. Means ± SEM, n=6.](image)

4.3 Discussion

4.3.1 The effect of Pd\(^{2+}\) exposure on behavioural markers (vertical movement and feeding) of G. pulex

In this study, the vertical swimming (number of surfacings) assay was used to examine the effect of Pd\(^{2+}\) exposure on G. pulex. Under the current study conditions, 24 h of Pd\(^{2+}\) exposure led to ≤ 60% reduction in the vertical movement activity of the tested organisms (Figure 4.1). However, due to the high variation between the activities of individuals within each tested group, this decrease was only significantly different from the control group at the highest tested Pd\(^{2+}\) concentration (0.5 mg/L). This variation may be reduced by increasing the sample size. Variation in the vertical swimming activity of the tested organisms may also indicate that test organisms need > 10 min to acclimate to the experimental conditions. Variation in movement response between individuals due to the differences in their response to handling stress and/or time needed to acclimate to the test conditions has been previously observed. Peeters et al. (2009) used the Multispecies
Freshwater Biomonitor (MFB), which is an online continuous biomonitor uses impedance conversion to record behaviours of vertebrates and invertebrates (Alonso et al., 2009), to monitor the movement activity of *G. pulex* under laboratory conditions. These authors reported that 2 h was required for the tested organisms to acclimate to their experimental conditions. Similar studies, however, reported different acclimation periods for *G. pulex* to test conditions, varying from 20 min (Gerhardt et al., 1994) to 7 h (Gerhardt et al., 2003). Long acclimation periods may reduce the concentration of dissolved oxygen in the exposure medium and thus affect the response of the experimental organisms. Therefore, in the current study, test organism were acclimated to the test conditions for only 10 min as suggested by Wallace and Estephan (2004).

Variation in the movement activity of aquatic crustaceans may be due differences in gender, age, the presence of predators / parasites, the light conditions, temperature, feeding state, molting stage, water level and/or water quality (Hultin, 1971; Peeters et al., 2009). In the present study, the vertical movement assay was performed, in the absence of predators, employing 20 replicates of organisms that had a similar weight (approximately 25 mg) and did not show symptoms of parasite infection. In addition, conditions of temperature, light, feeding state, water level and water quality were standardised. This was done in an effort to minimise statistical variation. Consequently, individual variance in movement activity of examined organisms may be, at least, partially related to sex, reproductive cycle and/or molting state.

To the author’s knowledge, the effect of PGEs on the movement activity of aquatic biota has not been reported in the literature. Thus, Pd$^{2+}$ effect will be compared with the effect of other metals on *Gammarus* spp movement. The finding of this study agrees with that of Wallace and Estephan (2004), who observed a significant reduction in the vertical movement of *G. lawrencianus* following 72 h exposure to 0.55 µM (62 µg/L) Cd$^{2+}$. This concentration was approximately 8-times lower than
the Pd\(^{2+}\) concentration required to induce significant reduction in the vertical movement of \textit{G. pulex} in the current study (4.69 µM = 0.5 mg/L). However, the exposure period employed in the current study was shorter than that indicated by Wallace and Estephan (24 and 72 h, respectively). This may suggest that the toxic effects of metal build up over time. Gerhardt (1995) and Lloyd Mills \textit{et al.} (2006) also reported that metals such as Pb\(^{2+}\) and Cu\(^{2+}\) can inhibit the movement activity of \textit{G. pulex}. However, the latter two studies only investigated aspects of horizontal movement, which requires less energy than vertical movement, and thus may be less sensitive (Wallace and Estephan, 2004).

As movement activity requires the assistance of the nervous system, alterations in this activity may be attributed not only the influence of stresses (e.g. metal exposure) on the muscular systems but also on the nervous and muscular systems (Untersteiner \textit{et al.}, 2003). For example, metal exposure may affect Ca\(^{2+}\) signalling mechanisms in synapses and lead to an alteration in neurotransmitter release. Thus, toxicity of divalent metals, such as Pd\(^{2+}\), may at least be partly a consequence of the competition of divalent metal ions with calcium at the synaptic Ca\(^{2+}\) channels (Gerhardt, 1995).

Another suggested explanation for the reduction in movement activity by metals is that metal exposure may increase the energetic cost of the protection and/or adapational mechanisms. This could lead to higher metabolic rates in some non-muscular tissue, while spontaneous muscular activity becomes low (Untersteiner \textit{et al.}, 2003). A significant amount of energy is consumed in muscular activity during the movement behaviour of an organism (Baillieul and Blus, 1999). They reported that movement activity of permanently swimming organisms is strongly correlated with their energy metabolism and external environmental conditions. Alteration in the conditions of surrounding media (e.g. higher toxic concentration or the appearance of predators) can disturb the normal function and consequently cause stress. Thus, a
part of the metabolic energy of stressed organisms may be spent in the stress response (Baillieul and Blus, 1999). As a result, under stressful conditions, such as Pd\(^{2+}\) exposure, the movement activity of organisms may be lowered to reduce consumption of energy and oxygen (Vellinger et al., 2012). It was previously shown that reduction in the movement behaviour of \textit{G. pulex} and \textit{G. duebeni} following metal exposure might be a consequence of redirecting of a part of the energy used for these behaviours into maintenance mechanisms (Lawrence and Poulter, 1998; Felten et al., 2008; Vellinger et al., 2012). These mechanisms include osmoregulation, detoxification (e.g. metallothionein synthesis) toxicant elimination, or cellular repair mechanisms.

Locomotion is a highly ecologically relevant behaviour, due to the roles of this behaviour in food acquisition, predator avoidance and reproduction. Thus, interaction of contaminants, such as Pd\(^{2+}\), with movement behaviour can impact the fitness of the tested organisms and could result in ‘ecological death’. This is when impacted organisms cannot function in an ecological context due to alteration in their normal behaviour (Felten et al., 2008).

Several studies have observed an inhibition in the feeding behaviour of \textit{G. pulex} as a result of exposure to various pollutants, such as lindane (Malbouisson et al., 1995) and cadmium (Felten et al., 2008; Alonso et al., 2009). Similarly, in the present study, feeding activity presented a sensitive marker of Pd\(^{2+}\) exposure being able to differentiate between the control and exposed organisms. The feeding activity of Pd-treated organisms was significantly lower than that of the control group following 24 h or longer exposure to 0.5 mg/L Pd\(^{2+}\) or 72 h exposure to lower tested Pd\(^{2+}\) concentrations (0.1, 0.25 mg/L) (Figure 4.2). This finding supports the findings of previous laboratory (Naylor et al., 1989; Maltby et al., 1996; Felten et al., 2008) and field (Maltby and Crane, 1994; Forrow and Maltby, 2000; Maltby et al., 2002; Dedourge-Geffard et al., 2009) studies that suggested the feeding activity of \textit{Gammarus} spp as a sensitive indicator of environmental stressors. The impact of
environmental stresses on the feeding activity of individuals can be extrapolated to impacts on population or even vital ecosystem functions (Forrow and Maltby, 2000). Maltby et al. (2002) suggested that, in polluted environments, inhibition in feeding activity of G. pulex can negatively affect its abundance, detritus processing and macroinvertebrate diversity. They also reported that a 25 to 30% reduction in the feeding rate of G. pulex could lead to a significant inhibition in its reproduction and individual growth rate (Maltby et al., 2002). That reduction in feeding activity is considerably lower than that observed in the current study (50%), when organisms were exposed to the lowest tested concentration of Pd$^{2+}$ (0.1 mg/L) for 72 h. This suggests that exposure to 0.1 mg/L Pd$^{2+}$ would have a severe negative impact on G. pulex. Environmental pollutants, such as Pd$^{2+}$, may inhibit feeding activity either directly, by reducing the quality of available food, or indirectly, by impacting digestive enzymes (Maltby et al., 2002; Geffard et al., 2009) and/or the nervous system of exposed organisms (Xuereb et al., 2009a). These impacts can reduce the feeding rate and/or search effectiveness of exposed organisms. Energetic reallocation from behavioural responses into maintenance function is another possible explanation for feeding inhibition by Pd (Felten et al., 2008).

Based on the data discussed above, it can be argued that investigation of the physiological effects of Pd$^{2+}$ (e.g. ATP production, MT synthesis, induction of HSPs) on G. pulex would improve knowledge of the potential mechanisms of the inhibitory effect of this metal on the examined behavioural endpoints.

**4.3.2 The effect of Pd$^{2+}$ exposure on AChE activity in G. pulex**

The high affinity of thiols (SH) for metal is well known (Arduini et al., 2005). In the current study, the potential complexation of ThC with Pd$^{2+}$ was examined to evaluate the utility of the Ellman assay to measure the effect of Pd$^{2+}$ on AChE in G. pulex. A significant negative dose-dependent
relationship between the measured absorbance, which reflects the binding of TCh to DTNB, and Pd$^{2+}$ concentrations was observed (Figure 4.3). This finding concurs with that reported by Frasco et al. (2005) and Arduini et al. (2005), who observed a clear interaction between the TCh group and a number of metals including Cu$^{2+}$, Cd$^{2+}$, Zn$^{2+}$, Ni$^+$, Ag$^+$, and Hg$^{2+}$. The affinity of TCh for a metal and the stoichiometry of the TCh-metal complex control the level of the reaction of metals with TCh (Arduini et al., 2005).

Following 72 h of in vivo exposure to $\leq 0.5$ mg/L Pd$^{2+}$, AChE activity in whole body extract of G. pulex increased with increasing Pd$^{2+}$ concentration (Figure 4.4). However, under the present study conditions, the increase in AChE activity was only significant at the highest tested Pd$^{2+}$ concentration (0.5 mg/L), when compared to the untreated groups (Figure 4.4). The low statistical significance of the differences between treatments may be due to the high variation between replicates within each experimental condition and/or the low number of replicates. Increasing the sample size and/or number of replicates would bring results (e.g. means and variation) closer to the true values of the whole population and increase the power of the test (Cumming et al., 2007).

In line with the findings of this study, earlier studies presented similar results, in which AChE of aquatic organisms was increased following exposure to other metals. For example, Jemec et al. (2007) observed a positive dose-dependent increase in AChE activity in D. magna following in vivo Cd$^{2+}$ exposure. Likewise, Cunha et al. (2007) observed a significant increase in ChE activity of N. lapillus following in vivo exposure to Cd$^{2+}$.

Metal could enhance the formation of AChE-ACh complex, leading to a higher enzyme activity. However, Lima et al. (2013) reported that metal can affect AChE by reducing the enzyme affinity to ACh, complicating the interpretation of the results (Lima et al., 2013). Other hypotheses
can be suggested to explain the stimulatory effect of metals, such as Pd$^{2+}$ on AChE activity. For instance, increase in AChE activity following metal exposure might be a consequence of metal interaction with acetylcholine receptors. This interaction can block the binding of ACh to its receptors, leading to ACh accumulation. This would increase AChE synthesis to degrade the elevated levels of ACh (Bainy et al., 2006). However, further studies, such as ligand binding, radiolabelled PCh quantitative PCR and western blotting of AChE, are needed to examine this hypothesis. An alternative possibility is that the activation of AChE arises via the initial inhibition of the enzyme by metals, which then leads to an up-regulation of the expression of AChE gene and protein synthesis (Bainy et al., 2006). The binding of metals to enzymes can also stimulate the catalytic function of the enzymes (Najimi et al., 1997). Due to the small number of replicates employed in the current study, more investigations with a higher number of replicates are needed before AChE activity can be suggested as a sublethal indicator of Pd$^{2+}$ exposure. In addition, further investigations are needed to clarify the mechanism(s) involved in AChE activation by in vivo Pd$^{2+}$ exposure.

In contrast to these findings, inhibitory effect of other metals on AChE activity have been previously reported. Quintaneiro et al. (2014) found that AChE activity in *Echinogammarus meridionalis* (Crustacea: Amphipoda) and *Atyaephyra desmarestii* (Crustacea: Decapoda) was reduced as a response to zinc exposure. Similarly, Shirvani et al. (2013) reported that in vivo silver exposure caused a significant reduction in the AChE activity of *Gammarus pseudosyriacus* (Crustacea: Amphipoda).

Reduction in AChE activity may disturb the central and peripheral nervous system, leading to behavioural influences, such as hyperactivity, asphyxia, and eventually death (Roex et al., 2003; Xuereb et al., 2009a). Reduction in AChE activity following metal exposure might be a consequence of metal binding to thiol residues in the enzyme structure (Lionetto et al., 2010). The inhibitory effect of metal exposure
on AChE may be also a result of a strong ionic interaction between the metal and the enzyme. Metals at high concentrations can relocate important ionic bonds in the enzyme structure, altering enzyme conformation (Lima et al., 2013). Variation in AChE response to metal exposure can be attributed to several factors, such as the period of exposure, tested concentration (Najimi et al., 1997), exposed organisms (Brown et al., 2004) and metal species (Cunha et al., 2007).

The present study showed that AChE activity in the head of G. pulex was more than 2-times higher than that in the whole body (Table 4.1). This result is in agreement with the findings of Xuereb et al. (2007). Higher AChE activity in the head is because the head has a higher density of cholinergic neurons that the rest of the body of G. pulex. This finding suggested that investigation of Pd$^{2+}$ effect on AChE activity in the head of G. pulex may provide a more sensitive response than that shown by using the whole body extracts.

The alteration in AChE activity in the head of G. pulex following 72 h Pd$^{2+}$ exposure is shown in Figure 4.5. Exposure to ≤ 0.5 mg/L Pd$^{2+}$ had no significant effect in AChE activity. The utility of AChE inhibition as a marker of metal exposure will depend on the concentration needed to induce a toxic effect. The results of this thesis suggest that AChE in the head of G. pulex is not a sensitive marker of Pd$^{2+}$ toxicity but whole body activity may be. However, further studies with different Pd$^{2+}$ concentrations and exposure periods may provide a more reliable conclusion regarding the usefulness of AChE as a sensitive biomarker of Pd$^{2+}$ toxicity.

It has been documented that the inhibition of AChE can affect the survival of G. pulex. According to Crane et al. (1999) and Xuereb et al. (2007), G. pulex would be in a life-threatening situation when AChE is inhibited by more than 50%. This reduction is approximately 10-times higher than that in the head of G. pulex observed in the present study.
(<5%) (Figure 4.5). In addition, the current study showed a stimulatory effect of Pd\(^{2+}\) exposure on the whole body of exposed organisms. Thus, it can be argued that the mortality of *G. pulex* observed following Pd\(^{2+}\) exposure (Chapter 3) was not a consequence of a reduction in AChE activity.

**4.3.3 The relationship between AChE and behavioural biomarkers**

According to the results of the present chapter, it can be argued that the sensitivity of tested behavioural markers (feeding and vertical movement) to Pd\(^{2+}\) exposure is higher than that of AChE. Significant behavioural alterations were observed in the tested organisms at lower Pd\(^{2+}\) concentration and/or for shorter periods of exposure than that required to cause a significant change in AChE. However, further investigations (e.g. time course investigation of the effect of Pd\(^{2+}\) on AChE activity) are needed to support this argument.

No similarity between the trends of behavioural alteration (movement and/or feeding) and that of AChE in tested organisms was observed in the current study. This suggests that the observed reduction in tested behaviour is not related to alteration in AChE activity or that Pd\(^{2+}\) affect the enzyme in a specific tissue or organelle and this effect was not detected due to the biological dilution of the sample. Alternatively, it might be that the higher activity of AChE led to lower concentration of ACh, reducing the activity of muscles and consequently reducing locomotor activity.

The results of this thesis, suggest that behavioural alterations, such as vertical movement and feeding, can be used to examine the effect of Pd\(^{2+}\) on other aquatic animals. However, more studies are necessary to examine the utility of these behaviour markers in more relatively environmental conditions (e.g. longer term exposure to low Pd\(^{2+}\) concentrations).
5 Mechanisms of Pd$^{2+}$ toxicity

5.1 Introduction

This part of the current study investigated the molecular basis of Pd$^{2+}$ toxicity towards *G. pulex*. The main aim of this chapter was to investigate the response of three cellular toxicity indicators, namely haemolymph ion concentrations, metabolic activity and oxidative stress in *G. pulex* exposed to Pd. To achieve this aim, experimental organisms were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd$^{2+}$ (nominal concentrations) for 72 h, and alteration in the examined markers were examined. The alteration on haemolymph ion concentrations for Na$^+$, K$^+$, Ca$^{2+}$ and Mg$^{2+}$ was employed as a marker of osmoregulatory dysfunction. The effect of Pd$^{2+}$ exposure in metabolic activity in *G. pulex* was examined using the MTT reduction assay. In addition, Pd$^{2+}$-induced injury in *G. pulex* was monitored employing assays of TBARS, GSH, GST and free thiol groups as biomarkers of oxidative stress.

5.2 Results

5.2.1 The effects of Pd$^{2+}$ exposure on haemolymph ion concentrations in *G. pulex*

*In vivo* exposure to $\leq$ 0.5 mg/L Pd$^{2+}$ for 72 h had no significant effect on the on the haemolymph ion concentrations for Na$^+$, K$^+$, Ca$^{2+}$ and Mg$^{2+}$ (Figure 5.1). Na$^+$ concentration was 119±4, 116±16, 121±10 and 126±16 mM at 0, 0.1 0.25 and 0.5 mg/L Pd$^{2+}$, respectively. Similar concentrations of K$^+$ and Ca$^{2+}$ were observed in the haemolymph samples of all tested groups (12±2, 10±2, 11.2±2 and 11.7±2.2 mM for K$^+$ and 10.9±2, 11±2.6, 10.1±2 and 12.4±2.4 mM for Ca$^{2+}$ at 0, 0.1 0.25 and 0.5 mg/L Pd$^{2+}$, respectively) (Figure 5.1). The haemolymph Mg$^{2+}$ concentration was 2.3±0.4, 1.1±0.6, 1.3±0.7 and 1.7±0.7 mM at 0, 0.1 0.25 and 0.5 mg/L Pd$^{2+}$, respectively (Figure 5.1).
5.2.2 The inhibitory effect of malonate on MTT reduction in *G. pulex*

In order to determine the proportion of MTT reduction attributable to mitochondrial SDH activity, the effect of a competitive succinate dehydrogenase inhibitor, sodium malonate, on succinate dehydrogenase activity in *G. pulex* was investigated.

MTT reduction had a significant negative relationship with the concentration of the added malonate (Figure 5.2). Treated samples had 79±3, 72±1.8 and 38±4% of the MTT reduction value of the control group at 1, 10 and 100 µM malonate, respectively. From the data in Figure 5.2, the IC$_{50}$ (half maximal inhibitory concentration) of malonate on MTT reduction in *G. pulex* was determined to be approximately 68 µM.

Figure 5.1 Cation concentrations in the haemolymph of *Gammarus pulex* following 72 h of Pd$^{2+}$ exposure. Means ± SEM, n=3.
5.2.3 The impact of Pd$^{2+}$ exposure on MTT reduction in *G. pulex*.

The effect of 72 h Pd$^{2+}$ exposure on MTT reduction in *G. pulex* was examined. Pd$^{2+}$ exposure caused a significant decrease in MTT reduction by *G. pulex* (Figure 5.3). This reduction was not dose-dependent. The amount of MTT reduction by treated organisms were 61±2, 73±3.4 and 60±4.5% of the unexposed control group for 0.1, 0.25 and 0.5 mg/L Pd$^{2+}$ respectively.
Figure 5.3 The effect of 72 h Pd\textsuperscript{2+} exposure on MTT activity in *Gammarus pulex*. Means ± SEM, n=6. Treatments that do not share a letter are significantly different (p< 0.02).

### 5.2.4 The effect of Pd\textsuperscript{2+} exposure on oxidative stress.

**Reduced glutathione**

Alteration in reduced GSH content was employed as an indicator of oxidative stress in *G. pulex* exposed to Pd\textsuperscript{2+} for 72 h. Pd\textsuperscript{2+} exposure increased the GSH content in the treated organisms (Figure 5.4). The GSH content of Pd\textsuperscript{2+}-treated *G. pulex* was higher than that of the control group (153±11%, 13±7 and 122±4% of control values, at 0.1, 0.25 and 0.5 mg/L Pd\textsuperscript{2+}, respectively). Statistical analysis indicated that this increase was significantly different at 0.1 and 0.5 mg/L, but not at 0.25 mg/L Pd\textsuperscript{2+}(Figure 5.4).
Figure 5.4 The effect of 72 h Pd$^{2+}$ exposure on GSH in *Gammarus pulex*. Means ± SEM, n=6. Treatments that do not share a letter are significantly different (p< 0.001).

**TBARS**

The impact of 72 h Pd$^{2+}$ exposure on oxidative stress in *G. pulex* was also investigated by employing a TBARS assay. In comparison to the untreated control group, Pd$^{2+}$-exposed organisms had TBARS levels around 84±11, 85±11 and 64±7% for 0.1, 0.25 and 0.5 mg/L Pd$^{2+}$ (Figure 5.5). However, this reduction was not significantly different from untreated control *G. pulex*.

**Free thiol content**

The assay of Ellman *et al.* (1961) was used to examine the impact of Pd$^{2+}$ on the availability of free thiol groups in *G. pulex*. Exposure for 72 h to 0.5 mg/L or lower Pd$^{2+}$ concentrations caused a slight but non-significant increase in the free thiol group content of test organisms (Figure 5.6). The concentration of the free thiol group in the treated organisms was 111±8, 107±3.5, 104±10% of that of the control organisms, at 0.1, 0.25 and 0.5 mg/L Pd$^{2+}$, respectively (Figure 5.6).
Figure 5.5 The effect of 72 h Pd$^{2+}$ exposure on TBARS in *Gammarus pulex*. Means ± SEM, n=6.

Figure 5.6 Free thiol group content in *Gammarus pulex* following 72 h of Pd$^{2+}$ exposure. Means ± SEM, n=6.
**GST activity**

GST activity in Pd-treated *G. pulex* was lower than that of the unexposed group (Figure 5.7). The enzyme activity was 89.5±5.7, 71±0.6 and 67±3.5% at 0.1, 0.25 and 0.5 mg/L of that of the control organisms, at 0.1, 0.25 and 0.5 mg/L Pd$^{2+}$, respectively (Figure 5.7). However, this reduction in GST activity was only significantly different from the control group at 0.25 mg/L or higher tested Pd$^{2+}$ concentrations.

![Graph showing GST activity vs Pd concentration](image)

*Figure 5.7 The effect of 72 h Pd$^{2+}$ exposure on GST activity in *Gammarus pulex*. Means ± SEM, n=6. Treatments that do not share a letter are significantly different (p< 0.001).*

**5.3 Discussion**

**5.3.1 Effect of Pd$^{2+}$ exposure on haemolymph ion concentrations**

The current study examined the possibility that the change in the haemolymph ion concentration might serve as a marker of Pd$^{2+}$ influence on osmoregulation in *G. pulex*. Exposure for 72 h to ≤ 0.5 mg/L Pd$^{2+}$ did not cause a significant alteration in the haemolymph ion concentrations for Na$^+$, K$^+$, Ca$^{2+}$ and Mg$^{2+}$ in *G. pulex* (Figure 5.1). The mean of haemolymph Na$^+$, which performs a significant role in the
osmotic pressure of haemolymph in G. pulex, and K\(^+\) concentration in all tested groups were similar to literature values for non-metal impacted G. pulex (120 mM) (Sutcliffe, 1971; Spicer et al., 1998). To reduce the variation between samples, the haemolymph samples in the current study were pooled from 3 to 5 individual G. pulex. However, despite the pooling of samples, some results showed high variation in ion levels between replicates. This variation may be due to the low number of replicates.

Metals can block ion channels in the gill membrane, disturbing ion regulation (Gerhardt, 1995). Metal may also damage the structural integrity of the gills, increasing the loss of haemolymph ions (Brooks and Lloyd Mills, 2003). This loss may be counteracted by increasing the ventilation rate in an attempt to provide the energy required for active ion uptake (Gerhardt, 1995). A higher ventilation rate, however, involves increasing energy expenditure, leading to even higher ventilation in order to obtain enough oxygen. Higher energy expenditure may affect the fitness of an organism, if not counteracted by a higher energy intake (Gerhardt, 1995).

Although the effect of Pd\(^{2+}\) on ionoregulation in aquatic biota has not been documented, these results are in accordance with those of Felten et al. (2008) and Vellinger et al. (2012), who found no significant effect on G. pulex haemolymph Na\(^+\) concentration following Cd\(^{2+}\) (15 µg/L for 168 h) and As\(^{3+}\) (1.7 mg/L for 96 h) exposure, respectively. Those studies, however, observed that exposure to the same metals led to a significant reduction in haemolymph Ca\(^{2+}\) concentration in the test organisms (Felten et al., 2008; Vellinger et al., 2012), which did not occur in this study.

Several hypotheses may explain the absence of a significant Pd\(^{2+}\) impact on haemolymph Na\(^+\), K\(^+\), Ca\(^{2+}\) and Mg\(^2\) concentrations. First, it might be that the uptake route of Pd\(^{2+}\) by G. pulex is different to that of the
haemolymph cations examined in the present study. Although no study has investigated the routes of entry of Pd$^{2+}$ in *G. pulex*, it has been reported that routes other than gills, such as drinking water or diet, may present the major entrance routes of some metals (e.g. As$^{3+}$) to *G. pulex* (Vellinger *et al.*, 2012). In this scenario, metals would have less impact on the structural integrity of the gills and iono-osmoregulation processes. Secondly, Pd$^{2+}$ may not be able to compete with tested haemolymph cations for the same binding sites on cotransporters and/or is less able to move through ion channels. Thirdly, Pd$^{2+}$ may not be able to bind to and thus inhibit Na$^+$/K$^+$ ATPase, which provides an important part of the driving force for active Na$^+$ uptake (Brooks and Lloyd Mills, 2003).

An alternative explanation is that exposed organisms relocated energy from other behaviours (e.g. feeding and locomotion) into osmoregulatory compensation mechanisms, such as Na$^+$/K$^+$ ATPase, thereby increasing the uptake of Na$^+$ from external media (Vellinger *et al.*, 2012). If this were the case in the current study, it can be hypothesised that increasing Na$^+$ losses by Pd$^{2+}$ exposure was counteracted by the increase in Na$^+$/K$^+$ ATPase activity. This hypothesis is in agreement with the finding of Felten *et al.* (2008), who observed a 71% increase in Na$^+$/K$^+$ ATPase activity in *G. pulex* as a response to Cd$^{2+}$ exposure.

The combination of high variability in the haemolymph ion concentrations (Na$^+$, K$^+$, Ca$^{2+}$ and Mg$^{2+}$) and the lack of statistically significant differences in ion concentrations makes the use of haemolymph ion concentrations questionable as a biomarker of Pd$^{2+}$ exposure. However, a larger sample size than the one used in the current study may be required to confirm such a conclusion. Moreover, further investigations, such as on the effects of Pd$^{2+}$ exposure on Na$^+$/K$^+$ ATPase activity and Na$^+$ influx and efflux, would improve knowledge with
respect to the potential for osmoregulatory disturbance due to Pd\textsuperscript{2+} exposure.

5.3.2 The inhibitory effect of malonate on succinate dehydrogenase activity in *G. pulex*

Malonate possesses two COOH groups and has a very similar structure to that of succinate, the normal substrate of SDH. Hence, malonate is a strong inhibitor of SDH (Potter *et al.*, 1943). Previous work has shown that this inhibition is competitive, that is it depends on the ratio of succinate/malonate rather than the total concentration of malonate, if both are present (Potter and DuBois, 1943). The present study also examined the inhibitory effect of malonate on SDH, in order to determine the proportion of mitochondrial MTT reduction.

As predicted, a negative relationship was observed between the concentration of malonate and MTT reduction in *G. pulex* (Figure 5.2). In comparison to the untreated control group, the inhibition of MTT reduction was approximately 60% at 100 µM malonate. This suggests that approximately 60% of MTT reduction in *G. pulex* was due to the activity of SDH. The rest of the measured MTT reduction (approximately 40%) probably occurred outside the mitochondria, for example, in the cytoplasm, and non-mitochondrial membranes including the endosome/lysosome compartment and the plasma membrane via trans-plasma membrane electron transport (Berridge *et al.*, 2005). Berridge and Tan, (1993) found that, in whole cultured cell homogenates, NADH dependent dehydrogenases had the highest MTT reduction activity, while less than 10% of this reduction was caused by succinate dehydrogenases (Berridge and Tan, 1993). Differences between the source material and experimental conditions used may account for the difference in proportion in SDH inhabitable MTT reduction between the current study and Berridge and Tan (1993). In the current study, MTT reduction was examined in a partly mitochondria-enriched fraction from *G. pulex* (1000 g supernatant), from where only cell debris and cuticle
were removed, where as Berridge and Tan (1993) were working with a bone marrow-derived cell line.

5.3.3 The impact of Pd$^{2+}$ exposure on MTT reduction in *G. pulex*

The reduction of MTT by viable cells has been widely suggested to examine the cytotoxicity of chemicals, test proliferation rates and to investigate cell activity, as discussed by Tominaga *et al.* (1993). These authors reported that cell viability assays can be used to select anticancer drugs and their doses. Despite the variability of the subcellular localisation of MTT reduction, it is presumed that MTT reduction is strongly correlated with the number of respiring cells (Jaszczyszyn and Gąsiorowski, 2008). Thus, the MTT reduction assay has been widely employed to examine the effect of toxins on cellular metabolism, in particular on the activity of dehydrogenases such as SDH (Kostoryz *et al.*, 2000; Schmid *et al.*, 2007). The decline of SDH activity indicates impairment of aerobic metabolism, and thus reduction in its energy generation (Moorthikumar and Muthulingam, 2011). Consequently, the rate of anaerobic metabolism may increase to meet energy needs. Chemicals, such as metal compounds, can bind to SDH and/or block the enzyme’s synthesis, reducing the enzyme activity (Moorthikumar and Muthulingam, 2011). In the current study, the effect of Pd$^{2+}$ exposure on MTT reduction in *G. pulex* was investigated.

Following 72 h exposure to up to 0.5 mg/L Pd$^{2+}$, the level of MTT reduction in treated *G. pulex* was ≤ 72% of the value of the unexposed control group (Figure 5.3). This inhibition was not dose-dependent but significantly different from the control in all treated groups. The effect of Pd$^{2+}$ exposure on MTT reduction and/or SDH activity is not reported in the literature. However, this result concurs with that of a number of previous investigations which recorded the inhibitory effect of metal exposure on SDH and MTT reduction. For example, Ordoñez *et al.* (2011) reported that the activity of SDH in the oligochaete *Limnodrilus hoffmeisteri* (Haplotaxida: Tubificidae) was significantly reduced after
exposure to Cd$^{2+}$, Fe$^{2+}$ or Zn$^{2+}$. In another study, Goswami et al. (2013) reported a significant decrease in the activity of SDH in a fish cell line (RF) by Cd$^{2+}$ and Zn$^2$. Similarly, Ananth et al. (2014) found that SDH activity in the gill, kidney and liver of freshwater fish grass carp *Ctenopharyngodon idella* (Cypriniformes: Cyprinidae) was significantly reduced by arsenic (As).

Metals can modify the structure of SDH; Ordoñez et al. (2011) reported that metals could displace iron, which plays an important role in SDH function, and thus inhibit the enzyme activity. Another possible explanation of the inhibition of SDH activity by Pd$^{2+}$ is the interaction of metal with free thiol groups in cysteine residues of SDH. The results in Chapter 4 showed that thiol groups have high affinity toward Pd$^{2+}$ (Figure 4.3). Alterations in SDH and other mitochondrial enzymes (e.g. NADH-dehydrogenase) can impact energy metabolism and cell respiration, thus reducing the ATP production by mitochondria (Ordoñez *et al.*, 2011).

Further investigations, such as the measurement of ROS and ATP generation by mitochondria of *G. pulex*, are needed to improve knowledge regarding the effect of Pd$^{2+}$ on organisms. It is well known that the main function of mitochondria is to generate ATP through oxidative phosphorylation and oxygen consumption. Mitochondrial dysfunctions, such as reduction in ATP generation and increase in ROS production, may result from toxicant exposure. Oxidative damage and cell death can result when ROS production overwhelms the enzymatic and non-enzymatic antioxidant defence system (Fariss *et al.*, 2005; Murphy, 2009).

### 5.3.4 The effect of Pd$^{2+}$ exposure on oxidative stress in *G. pulex*

Metal toxicity may, at least in part, be attributed to its ability to generate elevated levels of ROS and induce oxidative stress (e.g. lipid peroxidation) (Kunz *et al.*, 2010). Previous studies have reported induction of oxidative stress as a consequence of metal exposure in the
amphipods *G. locusta* (Correira *et al.*, 2002), *G. roeseli* and *Dikerogammarus villosus* (Sroda and Cossu-Leguille 2011) and *G. pulex* (Khan *et al.*, 2011). Disturbance of the balanced state between the production of ROS and the antioxidant defence system could cause oxidative damage to important molecules in the cells. Chemical exposure can not only increase the level of ROS in the cell by stimulation of ROS production, but also by reducing the activity of antioxidant enzymes (Schmid *et al.*, 2007). Several parameters, such as the level of natural antioxidants (glutathione; superoxide dismutases; catalase, etc.), oxidative changes in lipids and enzymatic activities (e.g. GST and glutathione peroxidase), have been employed as markers of oxidative stress. For this reason, the current study examined the possibility of Pd$^{2+}$ induced oxidative stress in *G. pulex* by monitoring several biomarkers, including GSH, TBARS, GST and the level of free thiol groups.

The concentration of reduced GSH, which is an example of a non-enzymatic antioxidant defence system, in Pd$^{2+}$-treated *G. pulex* was higher than that of unexposed organisms (Figure 5.4). However, this increase was significantly different from the control group when organisms were exposed to 0.1 or 0.5 mg/L, but not at 0.25 mg/L Pd$^{2+}$. The absence of significant changes in the GSH in the 0.25 mg/L Pd$^{2+}$ group may be due to the high variation amongst the replicates, which can be explained by the low number of replicates and/or the differences between individual responses due to differences in their sex and/or reproductive status (Correia *et al.*, 2003). Increased concentration of reduced GSH by Pd$^{2+}$ may indicate higher production of GSH as a stress response to Pd$^{2+}$ in *G. pulex*. GSH can play an important role in the detoxification process of metals, either as a substrate of antioxidant enzymes, such as GST (Canesi *et al.*, 1999), or via binding to toxic metals which have high affinity for its thiol (-SH) group (Singhal *et al.*, 1987; Gismondi *et al.*, 2012). The thiolate sulphur atom is the major element of the antioxidant capacity of -SH compounds, as this atom is able to accommodate the loss of a single electron (Flora, 2009). The
apparent reduction in GSH back to control levels at higher Pd$^{2+}$ concentrations could have been due to metal binding to its thiol groups.

The ability of Pd$^{2+}$ to stimulate elevated levels of GSH in the current study agrees with the findings of Thomas and Wofford (1984) and Thomas and Juedes (1992), who observed an induction of GSH following metal (Cd$^{2+}$, Hg$^{2+}$ and Pb$^{2+}$) exposure in fish tissue. Increasing GSH content may reflect the attempts of cells to reduce the oxidative damage (Kovačević et al., 2008). Lange et al. (2002) observed that Zn$^{2+}$ exposure increased hepatic GSH level in O. mykiss. The same study, however, found that the GSH content in tested tissue was not affected by Cd$^{2+}$.

In contrast to the result of this study, MacFarlane et al. (2006), reported that the uptake of a number of metals (Pb, Cu, Cr, Zn, Cd, As, and Se) by the crab Parasesarma erythodactyla (Crustacea: Decapoda) did not affect the GSH content in the tested organisms. The same study found the activity of the antioxidant enzyme GPx, was positively correlated with these metals content in the exposed organisms. This may suggest that GSH content, which is important for the maintenance of GPx activity, was maintained to combat metal-induced oxidative stress. Wang et al. (2008) reported that, following Cd$^{2+}$ exposure, the GSH content in the freshwater crab Sinopotamon yangtsekiense (Crustacea: Decapoda) had a negative relationship with Cd$^{2+}$ concentration. This reduction in the GSH content may be a consequence of GSH binding and/or oxidation by metals (Canesi et al., 1999) and/or consuming GSH in antioxidant defence (Wang et al., 2008). GSH can form water soluble metal-GS complexes, stimulating metal extrusion from the cell (as they can be transported via membrane proteins). GSH can also bind to metals producing a stable metal–SG complexes and thus preventing metal interaction with essential cell components (Balshaw et al., 2001; Bánhegyi et al., 2003).
The rise in reduced GSH content of *G. pulex* exposed to Pd\(^{2+}\) (Figure 5.4) suggests that this metal can interfere with GSH metabolism, increasing the concentration of GSH in treated organisms (Canesi *et al*., 1999). Such an increase may be due to reduction in the activity of γ-glutamyl cysteine synthetase (GCS), which is involved in GSH metabolism (Canesi *et al*., 1999).

Alterations in TBARS levels in Crustacea due to metal exposure have been reported in *G. locusta* exposed to Cu\(^{2+}\) (Correia *et al*., 2002a, b), *D. magna* exposed to Cd\(^{2+}\) (Barata *et al*., 2005) and in *G. pulex* exposed to Cu\(^{2+}\) and Zn\(^{2+}\) (Khan *et al*., 2011). As this suggests that metal exposure can affect TBARS levels, the impact of Pd\(^{2+}\) exposure on the TBARS level in *G. pulex* was investigated in this thesis. A small, but not significant, reduction in the TBARS level in *G. pulex* was observed following 72 h *in vivo* exposure to ≤ 0.5 mg/L Pd\(^{2+}\) (Figure 5.5).

In agreement with the results of the current study, there are several reports of the non-significant effects of metal exposure on TBARS levels in aquatic biota. For example, Correia *et al.* (2002b) reported that 4 days exposure to up to 10 µg/L Cd\(^{2+}\) did not cause a significant change in the TBARS concentration in *G. locusta*. Likewise, Bouskill *et al.* (2006) found that 7 days exposure to 80 µg/L As\(^{3+}\) did not affect the TBARS level in *A. aquaticus* and *D. polymorpha*.

In the current study, the absence of significant changes in the TBARS level in the tested *G. pulex* groups may also be due to the high variation amongst the replicates of each treatment. This variation can be explained by the low number of replicates and/or the differences between individual responses due to differences in their sex and/or reproductive status (Correia *et al*., 2003). Further investigation, with a larger sample size and more extensive exposure duration and Pd\(^{2+}\) concentrations than those used in the current study, would help to confirm whether TBARS can be used as a marker of Pd\(^{2+}\) toxicity.

Under the existing study’s conditions, Pd\(^{2+}\) exposure caused a slight but non-significant increase in the concentration of the free thiols in *G. pulex*.
The absence of significant alteration in the free thiol content and the TBARS level in *G. pulex* may indicate that, under the experimental conditions used, Pd\(^{2+}\) exposure did not induce oxidative damage. It might be suggested that exposed organisms were able to adapt to Pd-induced stress and improve their regulatory and metabolic mechanisms to protect protein thiols against this metal.

The GST activity had a negative dose-dependent relationship with Pd\(^{2+}\) concentration (Figure 5.7). However, reduction in GST activity was only significantly different from the untreated control group when organisms were exposed to 0.25 mg/L or higher Pd\(^{2+}\) concentrations. This finding may be an indicative of further reduction in GST on longer periods of exposure and/or higher Pd\(^{2+}\) concentrations. Reduction in GST activity at higher Pd\(^{2+}\) concentrations suggests that these concentrations enhance other mechanisms for metal detoxification (e.g. metallothionein synthesis) in exposed organisms (Kuroshima, 1995; Géret *et al.*, 2002; Kovářová and Svobodová, 2009). Binding of Pd\(^{2+}\) to MT would reduce its free ion concentrations, which is likely to reduce Pd-induced oxidative stress. Another potential interpretation of decreased levels of GST is that high Pd\(^{2+}\) concentrations activate antioxidant enzymes, for example, catalase, superoxide dismutase and peroxidase (Vinodhini and Narayanan, 2009), leading to a decrease in GST induction in exposed organisms.

Another possible hypothesis to explain the mode of GST response is that higher tested Pd\(^{2+}\) concentrations start to influence basic cell metabolism, decreasing the production of ROS by normal mitochondrial activity (Schmid *et al.*, 2007). They demonstrated that reduction in ROS can be an indicator of a reduction in cell metabolism. This hypothesis agrees with the result of the MTT assay that showed an inhibitory effect of Pd\(^{2+}\) on MTT reduction (Figure 5.3).

The results of the GST assay suggest that high Pd\(^{2+}\) concentrations are required to disturb the balanced state between the induction and inhibition of the enzyme or impact the ability of *G. pulex* to regulate GST.
activity (Figure 5.7). However, as it has been demonstrated that GST in some *Gammarus* species, particularly *G. italicus*, has different isoforms (Aceto et al., 1991). Investigation of the effects of pollutant exposure on individual isoforms of GST may provide a more sensitive response than that of total GST (Pérez-López et al., 2002).

The result of this thesis agrees with that of previous studies reported a decrease in the GST activity in aquatic species in response to exposure to different chemicals. Akcha et al. (2000) found that GST activity in the gills of *M. galloprovincialis* was significantly reduced following 28 days dietary exposure to 0.5 g/kg dry weight mussel benzo[a]pyrene. Likewise, Awoyemi et al. (2014) showed that sublethal metal exposure reduced GST activity in 2 species of freshwater fish. They reported a significant reduction in GST activity in the liver of *C. gariepinus* following 30 days exposure to either 1.78 mg/L Pb$^{2+}$ or 1.67 mg/L Zn$^{2+}$. The same study observed that 30 days exposure to 1 mg/L Zn$^{2+}$ caused a significant decrease in GST activity in the liver of *O. niloticus*. However, McLoughlin et al. (2000) found that 48 h exposure to 0.4 mg/L Zn$^{2+}$ did not cause significant change in GST activity in *G. pulex*. Similarly, Yin et al. (2001) observed no significant alteration in GST activity in *G. pulex* following 48 h of exposure 0.27 mg/L Zn$^{2+}$. Different GST responses may be attributed to differences in the chemical applied, difference in the concentration and/or duration of the chemical stress employed and/or differences in tested species.

The results of the current study suggest that the level of lipid peroxidation and free thiol group in *G pulex* are not sensitive biochemical markers of Pd$^{2+}$ toxicity but induction of antioxidants (GSH and GST) may be. However, further investigations with a higher number of replicates may help to confirm the trends of observed response. These investigations could include Pd-induced oxidative stress in specific tissues or organelles (e.g. mitochondria) and/or organs (e.g. gills) of *G. pulex*. Such studies would improve knowledge regarding the utility of oxidative stress parameters as sensitive markers of Pd$^{2+}$ toxicity.
Despite the low number of replicates, the results of the current study indicate that disturbance of cation regulation may not represent sensitive responses of Pd\(^{2+}\) toxicity to *G. pulex*. However, data from MTT and antioxidants (GSH and GST) assays suggest that Pd\(^{2+}\) toxicity to tested organisms was, at least partly, attributable to its effect on mitochondrial functions. This finding indicates the need for further studies on the effect of Pd\(^{2+}\) exposure on the mitochondria of exposed organisms in order to improve knowledge regarding mechanisms of Pd\(^{2+}\) toxicity. This could include examining the effect of exposure on mitochondrial oxygen consumption and ATP production.
6  Heat shock proteins and phosphoproteins as potential molecular markers of Pd\textsuperscript{2+} toxicity

6.1 Introduction

The aim of the work in this chapter was to investigate whether induction of the HSPs (HSP60, HSP70 and/or HSP90), and/or the change in the phosphorylation state of protein bound, serine, threonine and tyrosine, which are the most common sites of protein phosphorylation, could be employed to monitor exposure of \textit{G. pulex} to Pd\textsuperscript{2+}. This aim was achieved by SDS-PAGE and Western blotting analysis (section 2.9.9). Test organisms were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd\textsuperscript{2+} (nominal concentrations) for 24 h. Next, both mitochondrial (pellet) and cytosolic (supernatant) fraction of the exposed \textit{G. pulex} were probed with antibodies against the proteins of interest. The intensity of the protein bands on probed blots was then measured densitometrically.

6.2 Results

6.2.1 HSPs as potential molecular markers of Pd\textsuperscript{2+} toxicity

6.2.1.1  HSP60

In the current study, HSP60 was detected only in the pellet but not in the supernatant with its known. The migration of the observed anti-HSP60 reactive band was consistent with the known molecular weight of this HSP (i.e. between 58 to 65 kDa) (Lindquist, 1992). Exposure for 24 h to 0.1 mg/L Pd\textsuperscript{2+} or higher concentrations significantly increased the relative level of HSP60 expression when compared to unexposed control extracts (Figure 6.1). Increasing Pd\textsuperscript{2+} concentrations elevated HSP60 concentrations to reach a peak (343% of the control group) at 0.25 mg/L and was subsequently reduced to 277% of control values in the 0.5 mg/L group (Figure 6.1).
Figure 6.1 HSP60 content in the 9000 g pellet of *Gammarus pulex* after 24 h Pd\(^{2+}\) exposure. A: shows a representative probed blot of extracts from gammarids exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd\(^{2+}\) for 24 h; B: densitometric analysis. Means ± SEM, n=4. Treatments that do not share a symbol are significantly different (p< 0.001).

### 6.2.1.2 HSP70

Following 24 h Pd\(^{2+}\) exposure, the content of HSP70 in the pellet and decreased in the supernatant extracts in exposed *G. pulex* was higher than that observed in the untreated control group (Figure 6.2). However, this increase was not statistically significant in either fraction at any of the tested Pd\(^{2+}\) concentrations.
Figure 6.2 HSP70 content in the 9000 g fraction of *Gammarus pulex* after 24 h Pd$^{2+}$ exposure. A: shows a representative probed blot of extracts from gammarids that were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd$^{2+}$ for 24 h; B: densitometric analysis (Means ± SEM, n=6).

### 6.2.1.3 HSP90

In the current study, the molecular weight of the observed HSP90 band in both pellet and decreased in the supernatant fractions was consistent with its known molecular weight (82 to 96 kDa) (Lindquist, 1992). HSP90 content in the supernatant samples of *G. pulex* had a non-significant dose-dependent relationship with Pd$^{2+}$ concentrations (Figure 6.3). Increasing Pd$^{2+}$ concentrations led to a slight, but not significant, decrease in HSP90 content in supernatant samples of the exposed animals (Figure 6.3). In contrast, the level of HSP90 in the pellet samples showed a non-significant positive relationship with Pd$^{2+}$ concentration (Figure 6.3). Under the current study conditions, HSP90 content in the pellet of exposed organism was ≥ 130% of that of unstressed control samples (Figure 6.3).
6.2.2 Phosphoproteins as potential molecular markers of toxicity

6.2.2.1 Phosphoserylne

In the pellet extracts, the reactivity of antibodies with polypeptide bands corresponding to 245 kDa increased by Pd$^{2+}$ exposure. However, this increase was only significant at 0.5 mg/L but not at lower tested Pd$^{2+}$ concentrations (Figure 6.4). No significant alteration was observed in the rest of the major detected polypeptide bands (63 and 100 kDa). The levels
of these polypeptide bands in treated samples were 100 ± 6% of that of the control group.

In the supernatant samples, 24 h Pd$^{2+}$ exposure caused a non-significant increase in the phosphoserine content in the major polypeptide bands at 25 and 100 kDa (Figure 6.5). No significant change was detected in reactivity of antibodies with polypeptide bands corresponding to 63 kDa in exposed organisms. The level of this polypeptide band was 76 to 103% of the control group.

![Protein bound phosphoserine content in the 9000 g pellet of *Gammarus pulex*.](image)

Figure 6.4 Protein bound phosphoserine content in the 9000 g pellet of *Gammarus pulex*. (A) A representative probed blot of pellet extracts from gammarids that were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd$^{2+}$ for 24 h. The position of the 245 kDa, 100 kDa and 63 kDa bands are indicated by horizontal arrows. B densitometric analysis of selected proteins in the pellet samples (Mean ± SEM, n= 4). Treatments that do not share a letter are significantly different.
Figure 6.5 Protein bound phosphoserine content in the 9000 g supernatant of *Gammarus pulex*. Panel A shows a representative probed blots of supernatant extracts from gammarids that were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd^{2+} for 24 h. The position of the 100 kDa, 63 kDa and 25 kDa bands are indicated by horizontal arrows. B densitometric analysis of selected proteins in the supernatant samples (Mean ± SEM, n= 4).

### 6.2.2.2 Phosphothreonine
In the pellet samples, the reactivity of antibodies with polypeptide bands corresponding to 63 kDa in treated organisms was slightly, but not significantly, higher than that of the untreated control group (Figure 6.6). In contrast, Pd^{2+} exposure caused a non-significant reduction in the level of reactivity of phosphothreonine antibody with polypeptide bands corresponding to 100 kDa (Figure 6.6).

In the supernatant extracts, the level of phosphothreonine antibody reactivity with a 100 kDa polypeptide band in exposed *G. pulex* was not
significantly affected by Pd$^{2+}$ (Figure 6.7). The reactivity of antibodies with polypeptide bands corresponding to 30 kDa in treated organisms was relatively higher than that observed in the unexposed control group (132, 122 and 125% at 0.1, 0.25 and 0.5 mg/L Pd$^{2+}$, respectively). However, this increase was only statistically significant compared to the control group at 0.1 mg/L Pd$^{2+}$ (Figure 6.7).

![Figure 6.6](image)

Figure 6.6 Protein bond phosphothreonine content in the 9000 g pellet of *Gammarus pulex*. Panel A shows representative probed blot of pellet extracts from gammarids that were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd$^{2+}$ for 24 h. The position of the 100 kDa and 63 kDa bands are indicated by horizontal arrows. Panel B shows densitometric analysis of selected proteins in the pellet samples (Mean ± SEM, n= 4).
6.2.2.3 Phosphotyrosine

The level of anti-phosphotyrosine antibody reactivity with a 63 kDa polypeptide band in pellet extracts increased to up to 200% of the control group (Figure 6.8). However, due to the high variation, this increase was not statistically significant.

In the supernatant extracts, the western blot analyses (Figure 6.9) showed Pd\(^{2+}\) exposure caused a non-significant increase in the protein bound phosphotyrosine level in tested organisms with polypeptide bands corresponding to 70 kDa.
Figure 6.8 Protein bound phosphotyrosine content in the 9000 g pellet of *Gammarus pulex*. Panel A shows a representative probed blot of pellet extracts from gammarids that were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd\(^{2+}\) for 24 h. The position of the 60 kDa bands is indicated by a horizontal arrow. Panel B shows densitometric analysis of selected proteins in the pellet samples (Mean ± SEM, n= 4).
Figure 6.9 Protein bound phosphotyrosine content in the 9000 g supernatant of *Gammarus pulex*. Panel A shows a representative probed blots of supernatant extracts from gammarids that were exposed to 0, 0.1, 0.25 or 0.5 mg/L Pd\(^{2+}\) for 24 h. The position of the 70 kDa bands is indicated by a horizontal arrow. Panel B shows densitometric analysis of selected proteins in the supernatant samples (Mean ± SEM, n= 4).

### 6.3 Discussion

#### 6.3.1 HSPs

Since exposure of biota to sublethal concentrations of environmental contaminants has been shown to induce HSPs, the induction of these proteins has been extensively used as a biochemical marker in ecotoxicological studies (Werner and Nagel, 1997; Schill et al., 2003). In the present study the effect of Pd\(^{2+}\) exposure on the levels of HSP60, HSP70 and HSP90 in the 9000 g fractions (pellet and supernatant) of *G. pulex* was examined.
6.3.1.1 HSP60

HSP60 is a mitochondrial heat shock protein family that plays important roles in the folding and assembly of proteins (Paranko et al., 1996; Clayton et al., 2000). It was reported that HSP60 can be employed as a specific marker for mitochondrial injury (Ivanina et al., 2008). Under the current study conditions, HSP60 was continuously detected in the 9000 g pellet fraction of control samples, which indicates that mitochondria in G. pulex have a high content of constitutive HSP60. This finding agrees with earlier studies showing that HSP60 was constitutively expressed in the mitochondria of eukaryotes (Belles et al., 1999). Background levels of HSP60 in the pellet fraction have previously been detected in several aquatic amphipods, such as H. azteca, A. abdita and R. abronius (Werner and Nagel, 1997). They, however, reported detectable concentrations of HSP60 in the supernatant fraction, which was not the case in the current study. This may be due to different loaded protein concentrations (20 and 50 µg protein in the current study and in the study of Werner and Nagel, respectively). Differences between the findings of Werner and Nagel (1997) and the findings of this study may also be a consequence of differences in the antibodies used, samples preparation and/or sensitivity of the experimental organisms. It could also be that more HSP60 is expressed in the cytoplasm of H. azteca, A. abdita and R. abronius than that expressed in the cytoplasm of G. pulex.

The result of the present study demonstrated that Pd$^{2+}$ was taken up by G. pulex and caused a stress response observed as an increase in the HSP60 content of the 9000 g pellet extracts of the exposed organisms (Figure 6.1). As HSP60 plays an important chaperone role in mitochondria, these observations suggest that the mitochondrial matrix proteins may represent major targets of Pd$^{2+}$. Although the effect of PGE exposure on the expression of HSP60 has not been reported in the literature, the result of the current study is in accordance with numerous previous reports that observed induction of HSP60 as a response to metal exposure (Sanders et al., 1991; Werner and Nagel, 1997; Kammenga et al., 1998; Clayton et al., 2000).
The role of mitochondria in cell energy production is well known (Dimmer et al., 2002; McBride et al., 2006). Mitochondria are also involved in several other cell processes such as generation and detoxification of ROS, the process of programmed cell death and the regulation of Ca\(^{2+}\) in the cytoplasm and in the mitochondrial matrix (Brand and Nicholls, 2011). Thus, several harmful impacts, such as reduction in cellular energy production and/or abnormal death of the cell may result from mitochondrial dysfunction. For example, in mitochondria, metal exposure (e.g. Pd\(^{2+}\)) can disrupt the electron transport chain and ATP production, increase ROS induction and damage enzymes (e.g. ATPases and kinases) (Ivanina et al., 2008). ROS can attack proteins leading to denaturation or form cross-linkages and subsequent protein dysfunction (Hansen et al., 2008). These impacts may lead to an induction of HSP60 expression as an early mitochondrial protective mechanism (Ivanina et al., 2008).

The result of the HSP60 test suggests this protein as a promising biomarker of Pd\(^{2+}\) effects on G. pulex. However, more investigations involving the effect of long-term exposure to low Pd\(^{2+}\) concentration are needed to demonstrate whether HSP60 is a sensitive biomarker of Pd\(^{2+}\) toxicity at exposure levels more likely to be found in the natural environment.

6.3.1.2 HSP70

HSP70 was detected in both pellet and supernatant 9000 g fractions (Figure 6.2). This finding may reflect the abundance of this protein in both mitochondrial and post-mitochondrial fractions. It has been shown that HSP70 is abundant in mitochondria, cytosol and endoplasmic reticulum (Gupta et al., 2010).

Following 24 h exposure to up to 0.5 mg/L Pd\(^{2+}\), the HSP70 content increased in both the pellet and supernatant fractions. However, due to the high variation, which may be at least partly due to the low number of replicates, this increase was not statistically significant. High variation in the HSP70 response may also reflect differences of HSP expression between individuals as proposed by Vedel and Depledge (1995) and Werner and Nagel (1997). Variation in HSP70 concentration might also suggest that factors other than Pd\(^{2+}\) toxicity affect the content of HSP70 in
tested organisms. An alternative explanation for the absence of significant alteration in HSP70 content is that the Pd\(^{2+}\) concentration in *G. pulex* tissue remained lower than the threshold level for the induction of HSP70 synthesis. Consequently, it can be argued that significant alteration in the HSP70 content of *G. pulex* requires higher Pd\(^{2+}\) concentrations, a longer period of exposure and/or higher number of samples than used in the current study. In agreement with the finding of this study, HSP70 was not a sensitive marker of metal exposure in *H. azteca*, *A. abdita* and *R. abronius* (Werner and Nagel, 1997) or in the gill tissue of *C. maenas* (Vedel and Depledge, 1995).

In contrast to the results of the present study, significant alterations in HSP70 level in *Gammarus* spp. as a response to exposure to other metals have been previously reported. Schill *et al.* (2003) observed significant modifications in the HSP70 concentration in *G. fossarum* following Cd\(^{2+}\) exposure. Their findings indicated an increase in the content of HSP70 in gammarids exposed to 0.008 mg/L Cd\(^{2+}\), whereas higher Cd\(^{2+}\) concentrations (2 mg/L) reduced the level of HSP70 in exposed *G. fossarum*. This reduction in the content of HSP70 might indicate high Cd\(^{2+}\) concentrations inhibited the HSP70 synthesis (Schill *et al.*, 2003). However, these authors used a longer exposure period than the one used in the current study (120 and 24 h, respectively). In another study, Shatilina *et al.* (2010), showed a time and dose-dependent modification in HSP70 content in *G. lacustris* following Cd\(^{2+}\) exposure.

However, only a few studies have examined the effect of Pd\(^{2+}\) exposure on the induction of HSP70 in aquatic biota. Singer *et al.* (2005) employed the level of HSP70 as an indicator of PGE toxicity towards *D. polymorpha*. These authors observed increased HSP70 induction in organisms exposed to 0.5 mg/L Pd\(^{2+}\). However, the induction only occurred after at least 24 days of exposure, which is much longer than the period of exposure in the current study (24 h). In another study, 24 day exposure to 0.01 mg/L Pd\(^{2+}\) resulted in increased HSP70 levels in *G. roeseli* (Sures and Radszuweit, 2007). The same study however, could not detect HSP70 in untreated control groups. The differences between the findings of those studies might
be due to different sensitivity of tested organisms to \( \text{Pd}^{2+} \), period of exposure, the loaded protein concentration and/or the sensitivity of the techniques employed. Therefore, although not significantly affected by \( \text{Pd}^{2+} \) in the present study, HSP70 may still prove a promising biomarkers to examine the impact of \( \text{Pd}^{2+} \) or other chemicals in other aquatic organisms or different experimental conditions (e.g. longer term of exposure).

6.3.1.3 HSP90
In the cell, HSP90 is present in the cytosol as well as in mitochondria (Kang et al., 2007). Indeed, in agreement with this, as shown in Figure 6.3, HSP90 was detected in the both pellet and supernatant fractions under all experimental conditions.

After 24 h \( \text{Pd}^{2+} \) exposure, the content of HSP90 in the exposed \( \text{G. pulex} \) increased in the pellet and decreased in the supernatant (Figure 6.3). However, the alterations in both fractions were not statistically significant. This result suggests that \( \text{Pd}^{2+} \) exposure may increase HSP90 synthesis and/or stability in the mitochondria enriched pellet of \( \text{G. pulex} \). A higher \( \text{Pd}^{2+} \) concentration, a longer exposure period and/or a higher number of samples may be needed to confirm whether statistically significant changes can occur. In contrast, the decrease in the level of HSP90 in the post mitochondrial supernatant, although not statistically significant, may be due to disruption of protein synthesis and/or elevated protein degradation under such stressful conditions. Another possible interpretation of the HSP90 data is that cytosolic HSP90 forms complexes with newly-synthesised proteins and assists their import to the mitochondria (Faou and Hoogenraad, 2012). Consequently, the concentration of this chaperone protein would increase in mitochondria and decrease in cytosol.

In agreement with the results of this study, Ivanina et al. (2008) reported different patterns of HSP induction between tissues. They reported that following 4 h exposure to \( \leq 0.22 \text{ mg/L Cd}^{2+} \), the relative level of HSP60 and HSP70 significantly increased in the gills of the eastern oyster \( \text{Crassostrea virginica} \) (Ostreoida: Ostreidae), whilst no alteration in those stress proteins content was observed in the hepatopancreas of this
organism. Similarly, Eder et al. (2004) reported that the HSP90 response differed between the muscles and gills tissues of juvenile Chinook salmon Oncorhynchus tshawytscha (Chordata: Salmonidae) exposed to either 1.2 µg/L chlorpyrifos or 0.1 µg /L esfenvalerate for 96 h. In muscles, the expression of HSP90 was significantly increased, whereas there was no significant effects in HSP90 on the gills of the exposed organisms (Eder et al., 2004). The different pattern in HSP response may be linked to different induction of other protection mechanisms such as MT and GSH (Ivanina et al., 2008).

The results of this thesis concur with those of Planelló et al. (2010), who found no significant alteration in HSP90 concentration in the larvae of the aquatic midge Chironomus riparius following 24 h exposure to Cd\(^{2+}\). In contrast, a significant alteration in the induction of HSP90 in a range of aquatic biota has been observed following exposure to metals such as Cd\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\) (Choi et al., 2008; Taylor et al., 2013; Wang et al., 2013a).

The current study showed that the alteration on HSPs was only significant for HSP60 and not HSP70 or HSP90. This was mainly due to the high variation in observed responses. Variation in HSP response in gammarids can also reflect differences in sex, temperature, reproductive cycle, age, feeding state, photoperiod and water conditions (Schirling et al., 2004). As this study was conducted under controlled conditions using similar sized animals, variation in HSP response may be attributed to differences in the sex or reproductive cycle. However, the effect of such factors might be reduced as each of the samples, in the current study, was prepared by homogenising 8 animals together. It might be useful to monitor the alteration in HSP induction in different tissues (e.g. gills, which represent an important route of metal uptake) of G. pulex. However, such investigation may require using a large number of organisms to obtain detectable concentrations of HSPs.

The different HSP response mentioned in the previous paragraphs might reflect the different sensitivity of test organisms to test metals, metal doses and/or exposure conditions. Thus, investigation of the induction of
HSP90 over different ranges of Pd\textsuperscript{2+} concentrations and/or duration of exposure is necessary to determine whether the induction of HSP90 can be used as a marker of Pd\textsuperscript{2+} toxicity in \textit{G. pulex}.

6.3.2 Phosphoproteins
The effect of Pd\textsuperscript{2+} exposure on protein phosphorylation in \textit{G. pulex} has not been reported in the literature. However, the effects of pollutant exposure (e.g. metals and pesticides) have been previously observed. Thus, the current thesis investigated the effect of sublethal Pd\textsuperscript{2+} exposure on the phosphorylation state of protein bound serine, threonine and tyrosine in \textit{G. pulex}. The results showed that the use of anti-phosphoserine, phosphothreonine and phosphotyrosine antibodies in the western blot technique enabled detection of alterations in the phosphorylation of individual phosphoproteins in the 9000 g pellet and supernatant fractions of \textit{G. pulex}.

6.3.2.1 Phosphoserine
Data from western blot analysis indicates that exposure to 0.5 mg/L Pd\textsuperscript{2+} was associated with significantly increased serine phosphorylation on a 245 kDa in the pellet extract (Figure 6.4). This finding suggests that only 0.5 mg/L Pd\textsuperscript{2+}, but not lower concentrations, was capable of activating serine kinases and/or inactivating serine phosphatases. However, lower Pd\textsuperscript{2+} concentration with longer exposure time than those used in the current study may also modify the phosphorylation state of serine. There is a lack of studies regarding the effect of environmental pollutants on protein-bound phosphoserine in aquatic biota. However, the result of this thesis is in accordance with that of published literature showing dysregulation of protein serine phosphorylation in mammalian systems due to exposure to environmental pollutants. For instance, Tampio \textit{et al.} (2008) reported 24 h \textit{in vitro} exposure to 0.252 mg/L benzo[a]pyrene increased the level of protein bands phosphoserine in human MCF-7 breast adenocarcinoma cells. Similarly, Rahman \textit{et al.} (2011) reported that 72 h \textit{in vitro} exposure to 5 µg/L Pb\textsuperscript{2+} elevated phosphorylation of serine proteins in primary cultures of human neurons.
6.3.2.2 Phosphothreonine
Exposure to 0.1 mg/L Pd\(^{2+}\), but not higher concentrations, caused a significant increase in the level of anti-phosphothreonine antibody reactivity with a 30 kDa band in 9000 g supernatant extracts (Figure 6.7). This significant increase at low Pd\(^{2+}\) concentration may suggest the induction of threonine phosphorylation of certain proteins as an initial stress response to Pd\(^{2+}\) toxicity in exposed organisms. It may be speculated that higher Pd\(^{2+}\) concentrations activated some cellular defence mechanisms (e.g. MT), and consequently reduced the effect of the metal on the activity of threonine kinases and/or threonine phosphatases. To confirm this suggestion, further investigations (e.g. the effect of Pd\(^{2+}\) exposure on MT synthesis), with a bigger sample size than that used in the current study, are needed. In agreement with the finding of this study, several previous studies reported modifications in the level of threonine phosphorylation of proteins following metal exposure. For example, Rani and Sivaraj (2010) reported that, following 28 days exposure to 14 pg/L chromium, the level of phosphothreonine in the tissues of the freshwater fish *Clarias batrachus* (Linn.) was elevated in the gills, kidneys, muscle and brain and decreased in the liver. In mammalian cells, Rahman *et al.* (2011) found that 72 h *in vitro* exposure to 40 µg/L Pb\(^{2+}\) increased the level of protein band phosphothreonine in primary cultures of human neurons.

6.3.2.3 Phosphotyrosine
Exposure for 24 h to up to 0.5 mg/L Pd\(^{2+}\) did not cause significant alterations in tyrosine phosphorylation on bands corresponding to 63 kDa and 70 kDa in the pellet and supernatant extracts, respectively (Figures 6.8 and 6.9). This lack of significant differences may be, at least partly, due to the low number of replicates and/or variation in response between individuals as a result of differences in sex or reproduction state. Another possible explanation of the non-significant effect of Pd\(^{2+}\) on the phosphotyrosine content is that Pd\(^{2+}\) does not affect the delicate coordinated balance between tyrosine kinases and tyrosine phosphatases in *G. pulex* or that the tested concentrations and/or exposure time used did not reach the threshold required for the induction of tyrosine phosphorylation. In line with this finding, Burlando *et al.* (2006) reported
that copper did not modify the protein bound phosphotyrosine level in the digestive gland of *M. edulis*. They however, observed that copper induced a significant increase in the phosphorylation of tyrosine residues of proteins in the gills and mantle of tested organisms. Similarly, Burlando *et al.* (2003) reported that the level of tyrosine phosphorylated in RTH 149 trout hepatoma cells was increased following exposure to some metals, such as Hg$^{2+}$ and Cu$^{2+}$ (Burlando *et al.*, 2003). Different tyrosine phosphorylation responses may be due to the different exposure conditions, tested organisms, tissues and/or different metals examined.

The results in this chapter have shown that, at least under certain conditions, Pd$^{2+}$ can disrupt protein phosphorylation in *G. pulex*. Given that Pd$^{2+}$ is an important environmental pollutant, this finding concurs with previous studies, which reported modifications in protein phosphorylation in Crustacea as a response to other environmental pollutants. For example, Walker *et al.* (2005), using the Pro-Q Diamond stain, showed that 4 h exposure to 50 µg/L methoprene changed the pattern of protein phosphorylation in epithelial tissues of the American lobster *Homarus americanus* (Crustacea: Decapoda). In another study, it was shown that an *in vitro* exposure to a 0.5 mg/L concentration of the pyrethroid ester insecticide deltamethrin inhibited the activity of protein kinases in the nervous system of *H. americanus* (Miyazawa and Matsumura, 1990). These authors identified a 260 kDa protein as one of those showing increased phosphorylation following deltamethrin exposure. Similarly, in the current study, 24 h exposure to 0.5 mg/L elevated the level of protein-bound serine phosphorylation reactivity with a high molecular weight (245 kDa) in the mitochondrial extract. These findings together may suggest high molecular weight bands as a sensitive target of environmental pollutants.

The current study suggests that HSP60 in *G. pulex* as a sensitive biomarker of Pd$^{2+}$ exposure. However, further investigations are needed to examine the utility of other HSPs as indicators of Pd$^{2+}$ stress in such organisms. Although the current work shows that Pd$^{2+}$ exposure could lead to significant alterations in the content of numerous phosphoproteins, testing this response in test organisms in different exposure conditions (e.g.
longer period and/or lower concentration) would improve knowledge regarding the usefulness of employing the alteration in the phosphorylation states of certain proteins as a marker of Pd\textsuperscript{2+} exposure.

As only phosphoproteins that are relatively abundant are visible on western blots, a phosphoprotein enrichment strategy (e.g. immobilized metal affinity chromatography, Phos-Tag chromatography, immunoprecipitation, and phosphopeptide precipitation) prior to western blotting would also be useful as this might help to reveal other phosphoproteins not detected in a total protein extract (Delom and Chevet, 2006; Thingholm \textit{et al.}, 2009; Fíla and Honys, 2012).

The current study has demonstrated that Pd\textsuperscript{2+} exposure can change the phosphorylation state of proteins in mitochondrial and post mitochondrial extracts of \textit{G. pulex}. However, more research is needed to identify those proteins. This can be achieved by the combination of two-dimensional gel electrophoresis (2D-PAGE), in which proteins are separated according to two independent properties: the pH and the molecular weight (Marko-Varga, 2004), with mass spectrometric (MS) protein identification. This is known as a powerful, mature and sensitive proteome analysis method.
7 General discussion

PGEs (Pt, Pd and Rh) are metals of increasing environmental concern. However, at the start of the current study little was known about the acute toxicity and sublethal effects of these metals in aquatic biota. Thus, this study aimed to narrow this knowledge gap using the freshwater gammarid *G. pulex* as a toxicity test species.

Mortality studies (Chapter 3) showed that Pd$^{2+}$ was the only traffic-related PGE that caused a significant reduction in the survival of *G. pulex* and that both Rh$^{3+}$ and Pt$^{4+}$ reduced the effects of Pd$^{2+}$ on the survival of the exposed organisms (Figure 7.1). Such findings have not been previously reported, and could therefore encourage further investigations in order to determine the mechanism(s) behind these protective effects of Pt and Rh against Pd toxicity.

Although the tested Pd$^{2+}$ concentrations were high and outside the normal environmental range, the current study was conducted using only short-term exposures (≤ 96 h). In addition, there is evidence that environmental Pd$^{2+}$ concentrations are increasing and that they can accumulate in biota. This was supported by the findings of the current study in which Pd$^{2+}$ was taken up and accumulated by *G. pulex* with a BAF of 94.7. This suggests that Pd$^{2+}$ may reach the threshold required for the induction the toxic effect on *G. pulex* following long-term exposure to low concentrations. In addition, PGE concentrations in the environment are expected to continue to increase as a consequence of the rapid growth in the number of vehicles equipped with catalytic converters worldwide (Zereini and Wiseman, 2015).

In the current study, tested organisms were kept under well-controlled conditions (e.g. temperature, food, oxygen etc.) to optimise performance in the control treatment and isolate the influences of PGEs in question. However, this may not reflect the natural environmental conditions, where organisms are often exposed to a combination of stresses. These added environmental stresses can often increase the effect of toxicant, such as Pd, on organisms (Holmstrup *et al*., 2010). For example, interactions of heat stress with metal toxicity has been previously observed. Heugens *et
al. (2003) reported that Cd toxicity to *D. magna* was positively correlated with temperature. Likewise, Bat *et al.* (2000) observed that the 96 h LC_{50} values of Cu to *G. pulex* were 0.08 and 0.028 mg/L at 15°C and 25°C, respectively. Elevated temperature may increase contaminant uptake via increased diffusion and/or increase ventilation rate in response to an increased metabolic rate and reduction in oxygen concentration (Cairns *et al*., 1975; Schiedek *et al*., 2007). Thus it is possible that increased water temperature due to global warming will enhance Pd toxicity to aquatic biota.

Toxicity of metals to aquatic biota may also be affected by the level of dissolved oxygen in aquatic ecosystems. Ferreira *et al.* (2008) observed that the effect of Cd on the survival of *D. magna* was increased by reducing the oxygen concentration in the medium. This may be due to higher ROS formation on exposure to a combination of hypoxia and Cd. In another study, Kienle *et al.* (2008) reported that oxygen depletion increased Ni toxicity to *D. rerio*. This suggests that additional stresses can enhance metal toxicity.

Other stress factors, such as nutritional conditions (Holmstrup *et al*., 2010), the presence of other metals (the present study), can also modify metal toxicity to aquatic biota. Thus, it may be useful to investigate the effect of toxic metals (e.g. Pd) in aquatic biota in the presence of other stressors. Such data could improve present risk assessment strategies significantly.

The response of *G. pulex* to Pd^{2+} in this thesis was examined following short-term of exposures to relatively high Pd^{2+} concentrations (0.1, 0.25 and 0.5 mg/L). Like many other organisms, *G. pulex* can adapt to environmental stresses (Khan *et al*., 2011). In the case of metals, adaptation may be a consequence of exposure to a metal concentration higher than the normal environmental concentration of that metal. Long-term exposure to environmental stresses, such as metals, may increase resistance or tolerance of organisms to the action of these stresses (Calow, 1991). This author reported that organisms could resist chemical stresses in different ways; these include elimination, sequestration (by binding to defensive proteins), excretion and/or repairing damage resulting from
chemical exposure. However, these resistance mechanisms are metabolically costly (Calow, 1991; Pook et al., 2009). This may reduce the fitness of resistant organisms under unstressed conditions in comparison to non-resistant organisms. For example, in a laboratory study by Pook et al. (2009), it was found that the scope for growth of a metal-resistant population of harbour ragworms *Nereis diversicolor* (Phyllodocida: Nereidae), was significantly lower (46–62%) than that of organisms from two non-resistant reference populations.

The present study showed that the molecular and behavioural responses of *G. pulex* were significantly impacted by Pd\(^{2+}\) exposure at concentrations markedly below the 96 h LC\(_{50}\). Behavioural investigations (Chapter 4) demonstrated that a significant reduction in the vertical movement and feeding activity occurred following 24 h exposure to 0.5 mg/L Pd\(^{2+}\). This exposure condition decreased the survival of *G. pulex* by < 6% (Chapter 3). This suggests that behavioural markers can be utilised to examine chemicals in the aquatic environment and their impact on health, fitness and consequently the survival of biota. Behavioural alteration could reduce the ability of the amphipods to survive, grow and reproduce, which can lead to subsequent influences on the population in aquatic ecosystems (Felten et al., 2008). Under the present study’s conditions, HSP60 was an even a more sensitive marker than the other examined behavioural markers. After 24 h of exposure to \(\leq 0.1\) mg/L Pd\(^{2+}\), the concentration of HSP60 in the mitochondrial fraction of exposed organisms was significantly higher than that of the untreated control animals (Chapter 6). This suggests that HSP60 expression might be developed as a general biomarker of stresses in *G. pulex*.

The number of replicates used in the feeding assay was considerably lower than that used in the vertical movement assay (3 and 20 replicates, respectively). However, the variation in feeding behaviour was substantially lower than that observed in the vertical movement assay. This might be because the feeding assay was conducted with three individual organisms of a similar weight, per replicate. This approach can
reduce the effects of intra-specific variability and increase the statistical power of feeding assays (Agatz and Brown, 2014).

Although the direct impact of Pd$^{2+}$ cannot be excluded, this reduction in behavioural activities may be due to a decrease or reallocation of available energy from these behaviours to detoxification and/or adaption mechanisms (e.g. metallothionein, osmoregulation and/or stress proteins), as suggested by Lawrence and Poulter (1998). This suggestion agreed with the results of Chapter 6, in which Pd$^{2+}$ increased the protein level of the mitochondrial chaperone protein HSP60 in the mitochondrial fraction of exposed G. pulex (Figures 6.1 and 7.1). Taken together, the observed increase in HSP60 expression and decrease in MTT reduction in G. pulex, suggest that mitochondria represent an initial target for Pd$^{2+}$ toxicity. (Figure 7.1). The argument is further supported by the result of oxidative stress investigations (Chapter 5) which showed a reduction in GST activity and an increase in GSH concentration in the whole body extract of G. pulex exposed to ≤0.25 mg/L Pd$^{2+}$. It could therefore be suggested that the deterioration in behavioural markers was, at least partly, a consequence of reduction in mitochondrial energy generation (Figure 7.1).

Biomarker detection is considered an advantageous approach to assessing a contaminant’s impact, as investigation of the concentrations of chemicals in environmental samples alone may not provide an appropriate reflection of the effect of pollutants on biota. However, more studies are required in order to determine the optimal biomarkers that can be employed to examine the effects of a certain stress. The vast majority of biomarkers currently known are not specific. As previously discussed, effects on movement and feeding behaviours, on HSPs, on oxidative stress markers, on AChE activity, MTT reduction, and other biomarkers have been observed as responses to several environmental stressors (e.g. metals, pesticides, extreme temperature). Therefore, biological and/or chemical measurements are still needed to determine sources of stress that induces the observed response.
One of the limitations of the current study was the low number of replicates that led to high variation in the examined responses of exposed organisms in some experiments. A large sample size would provide more reliable results and increase the power of a statistical analysis (Cumming et al., 2007). However, increasing sample size would increase the cost of the study (e.g. using higher amounts of chemicals and number of organisms).

The positive relationship between uptake of Pd$^{2+}$ by exposed organisms and both the dose and length of exposure suggested that *G. pulex* were unable to regulate Pd$^{2+}$, and/or eliminate it rapidly. Accumulation of the metal via food chains in this way could increase the potential risk for other organisms. *G. pulex* represents an important food source for macroinvertebrates, fish, bird and amphibian species (Felten et al., 2008). The risk of biomagnification will increase substantially if Pd can be converted to organic forms. This has already been demonstrated for Hg, where some bacteria could form methylmercury (Ridley et al., 1977). Although Pd was able to demethylate methylcobalamin *in vitro* (Taylor, 1976), it is not known whether methylation of Pd occurs *in situ* in aquatic ecosystems.

In conclusion, the current study provides original data on the effects of PGEs toward the freshwater shrimp *G. pulex*. The results of this thesis suggest the mitochondrial matrix as an early target of Pd$^{2+}$ (see Figure 7.1). However, mitochondrial dysfunction due to Pd exposure needs further investigation.
Figure 7.1 A summary of the main investigations, findings and the conclusion of the current thesis
**Future work**

Based on the findings of the current study, the following investigations would be of value:

- Investigation of the intracellular storage locations of Pd$^{2+}$ in *G. pulex* using techniques of higher sensitivity than those employed in the current study (e.g. ICP-MS). A suitable Pd$^{2+}$ radioisotope would have allowed a metal partitioning and flux studies. These could help to explain the potential mechanism of reduced Pd$^{2+}$ toxicity in the presence of Pt or Rh.

- Determination of the effect of long-term exposure to low Pd$^{2+}$ concentrations on behavioural and molecular markers. This would help to obtain more environmentally relevant responses and establish environmental quality standards for Pd.

- Examination of the potential link between the behavioural and molecular changes obtained in the current study. This could include an investigation of the influence of Pd$^{2+}$ exposure on ATP production.

- An examination of the ability of the presence of Pt$^{4+}$ or Rh$^{3+}$ to prevent the behavioural and/or molecular alterations induced by Pd$^{2+}$ would be useful. This may provide information that is more environmentally relevant, as nowadays the majority of modern catalytic converters contain Pd and Rh or Pd, Rh and Pt.

- Investigation of proteomic changes using 2D-PAGE and mass spectrometry to quantify proteins showing changes in expression and phosphorylation status would be worthwhile. Purification of mitochondria would allow a more in depth analysis of the mitochondrial proteome using these approaches.
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