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Bioaccumulation and mixture toxicity of aluminium and manganese in experimentally exposed woodlice, *Porcellio scaber* (Crustacea, Isopoda)

by

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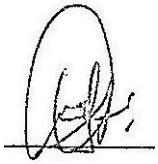
DECLARATION

I, Frederic Noel Kogoui Kamta, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed  **Date 16 March 2018**

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ABSTRACT

Soil ecosystems in urban, rural and agricultural environments receive chemical input from diverse sources of contamination, such as wastewater, industrial discharge, agricultural and urban runoff, fertilizers, vehicle leakages, landfill seepage, and animal waste overspill. Agricultural activities, transportation and industrial activities are suspected to be the highest sources of metal contamination in Cape Town. Although scientists generally have a good understanding of the toxicity of individual chemical pollutants, there is a great need to bridge the gap between our understanding of the toxic effects of exposure to individual contaminants and those effects from exposure to mixtures of chemicals. Woodlice and other soil detritivores have a particularly important ecosystem function in mineralising organic matter. Woodlice experience stress when exposed to toxic levels of metals in the diet, which can reduce feeding rates and may combine with natural stresses to reduce fitness and lower 'performance', thereby possibly resulting in these organisms being unable to completely fulfil their ecological function.

The objectives of this study were: to compare how aluminium and manganese are bioaccumulated in *Porcellio scaber* in terms of the contribution of the hepatopancreas in metal storage compared to the rest of the body; and to determine whether mixtures of aluminium and manganese affect each other's bioaccumulation and distribution in *Porcellio scaber*.

Woodlice collected from a clean field site (Kirstenbosch Botanical Garden) were experimentally exposed in the laboratory to a range of environmentally relevant aluminium and manganese concentrations. The woodlice were exposed to these metals in single and mixed metal experiments. Oak leaves, collected from a clean site, were contaminated with aluminium and manganese. Therefore, the woodlice were exposed via their food source. A control experiment, where oak leaves were not contaminated, was also prepared. At week 0 and after five weeks of exposure, a sample of the woodlice (5 per exposure group) were dissected to remove the hepatopancreas. Hepatopancreas and rest of the body samples were acid digested and analysed for the metals by means of the ICP-MS.

Contrary to the existing knowledge of metals accumulating in the hepatopancreas of woodlice when ingested, this study showed a higher bioaccumulation of aluminium in the rest of the body of woodlice after 5 weeks of exposure than in the hepatopancreas. This result was interpreted as a possible detoxification mechanism by woodlice through the use of the exoskeleton during the moult cycle. A similar result was found when woodlice were exposed to mixtures of aluminium and manganese. This translated to the fact that woodlice were unable to effectively deal with the toxicity caused by the mixture of aluminium and manganese. In the group of woodlice exposed to manganese alone, it was found that manganese concentrations in the rest of the body of woodlice exposed for 5 weeks were statistically higher than the manganese concentrations in the rest of the body of woodlice at the start of the exposure (week 0). However, in the hepatopancreas, there were no statistical differences between the manganese concentrations in week 0 woodlice and the manganese concentrations in week 5 woodlice. Furthermore, manganese concentrations in the rest of the body of week 5 woodlice were statistically higher than manganese concentrations in the hepatopancreas of week 5 woodlice. This was interpreted as further proof that woodlice would accumulate certain metals (aluminium and manganese in this case) in their exoskeleton so that elimination can follow during the moult cycle.

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DEDICATION

I dedicate this thesis to my beloved parents, Lucas and Jacqueline Kamta.

DEFINITION OF TERMS

ATSDR: Agency for Toxic Substances and Disease Registry

CCT: City of Cape Town

DEAT: Department of Environmental Affairs and Tourism

DME: Department of Minerals and Energy

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

SANBI: South African National Biodiversity Institute

ULSOP: University of London School of Pharmacy

USDA: United States Department of Agriculture

USEPA: United States Environmental Protection Agency

WHO: World Health Organisation

Bioaccumulation:

"Progressive increase in the amount of a substance in an organism or part of an organism that occurs, because the rate of intake from all contributing sources and by all possible routes exceeds the organisms ability to eliminate the substance from its body" (Rand, 1995).

Biomarker:

"Biological response to an environmental chemical at the below-individual level, measured inside an organism or its products (urine, feces, hair, feathers, etc.), indicating a departure from the normal status and that cannot be detected in the intact organism" (Van Gestel and Van Brummelen, 1996).

Biomonitoring:

“Continuous or repeated measurement of any naturally occurring or synthetic chemical, including potentially toxic substances or their metabolites or biochemical effects in tissues, secreta, excreta, expired air, or any combination of these in order to evaluate occupational or environmental exposure and health risk by comparison with appropriate reference values based on knowledge of the probable relationship between ambient exposure and resultant adverse health effects” (Duffus et al., 2007).

Ecotoxicology:

“Study of the toxic effects of chemical and physical agents on all living organisms, especially on populations and communities within defined ecosystems; it includes transfer pathways of these agents and their interactions with the environment” (Duffus et al., 2007).

Emission:

“The production and discharge of something, especially gas or radiation” (Online oxford English living dictionary, 2017).

Exposure:

“Concentration, amount, or intensity of a particular physical or chemical agent or environmental agent that reaches the target population, organism, organ, tissue, or cell, usually expressed in numerical terms of concentration, duration, and frequency (for chemical agents and microorganisms) or intensity (for physical agents)” (Duffus et al., 2007).

Hazard:

“Set of inherent properties of a substance, mixture of substances, or a process involving substances that, under production, usage or disposal conditions, make it

capable of causing adverse effects to organisms or the environment, depending on the degree of exposure; in other words, it is a source of danger" (Duffus et al., 2007).

Metals:

"Metals may be defined by the physical properties of the elemental state as elements with metallic lustre, the capacity to lose electrons to form positive ions and the ability to conduct heat and electricity, but they are better identified by consideration of their chemical properties" (Duffus, 2002).

Mixture toxicity:

"The term mixture toxicity is understood as unwanted adverse effects of mixtures of chemicals" (ULSOP, 2009).

Pollution:

"Introduction of pollutants into a solid, liquid, or gaseous environmental medium, the presence of pollutants in a solid, liquid, or gaseous environmental medium, or any undesirable modification of the composition of a solid, liquid, or gaseous environmental medium" (Duffus et al., 2007).

Toxic:

"Able to cause injury to living organisms as a result of physicochemical interaction" (Duffus et al., 2007).

Uptake:

"Entry of a substance into the body, an organ, a tissue, a cell, or the body fluids by passage through a membrane or by other means" (Duffus et al., 2007).

Woodlice:

“Woodlice (also called sow bugs, pill bugs and slaters) are terrestrial isopods (Class Crustacea, Sub-Order Isopoda) of the Family Oniscidea, which have invaded terrestrial habitats from aquatic environments. Most species can still tolerate submersion in water saturated with O_2“ (Maurizio and Hassall, 1999).

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CHAPTER ONE: INTRODUCTION

1.1) Sources of metal contamination

Concentrations of metals in terrestrial environments have increased significantly over time as a direct result of human activities through emissions from industrial plants, thermal power stations, waste disposal, soil amendments and vehicle traffic/road infrastructure (Hughes et al., 1980). Metals reaching the soil remain present in the pedosphere for many years even after the removal of the pollution sources (Gal et al., 2008). This is due to the fact that metals and trace elements have a non-biodegradable nature and long biological half-lives (Rahman et al., 2012).

Statistics South Africa (2014) estimates that 6 116 300 people live in the Western Cape, which represents 11.3% of the total population of South Africa. Among the Western Cape population, the City of Cape Town (CCT) (2013) estimates that 3.7 million people reside within the CCT, which represents just about two thirds of the provincial population. The Department of Environmental Affairs (2010) reports that provincial traffic volumes are the highest within the CCT metropolitan area. This is a result of the CCT's high population level. High traffic density results in pollutant emissions from motor vehicles, containing amongst others, metals (Department of Environmental Affairs, 2010).

Emissions from airports are another contributor to atmospheric pollution (DEA&DP, 2013). The CCT is home to one of South Africa's major airports, Cape Town International Airport. An aircraft engine emits a range of contaminants, including metals (DEA&DP, 2013). Emissions are released at different rates depending upon the phases of operation such as take-off, landing, idling, climbing and taxiing (Schlenke and Walker, 2011). Emissions also have different impacts depending on the height at which they are released (Schlenke and Walker, 2011). Significant levels of these contaminants are deposited on soil where it may exert toxic effects. Other sources of metal contamination in Cape Town include leakage from landfill sites, use

of fertilizers and pesticides in the agricultural sector, motor vehicle emissions, industrial emissions as well as mining activities (DEA&DP, 2013).

Soil is a crucial component of all terrestrial environments. Mining, manufacturing and the use of synthetic products (e.g. pesticides, paints, batteries, industrial waste and land application of industrial or domestic sludge) can result in metal contamination of urban and agricultural soils (USDA, 2000). Potentially contaminated soils may occur at old landfill sites (particularly those that accepted industrial waste), old orchards that used insecticides containing arsenic as an active ingredient, fields that had past applications of waste water or municipal sludge, areas in or around mining waste piles and tailings, industrial areas where chemicals may have been dumped on the ground, or in areas downwind from industrial sites (USDA, 2000). The use of fungicides and insecticides is also known to be a major source of metals in soil (Wuana and Okieimen, 2011). Gimeni-Garcia et al. (1995) determined the metal incidence in the application of pesticides to rice farming soils. The study revealed for example that, pesticides such as Antracol, saturn-G and Ordram contained concentrations of iron, nickel, cobalt, cadmium, lead, zinc and manganese. Gimeni-Garcia et al. (1995) estimated that the application of metal-containing pesticides translated in the presence of the detected metals in agricultural soils at concentrations ranging from 1.10 $g^{-1}ha^{-1}year^{-1}$ to up to 1100 $g^{-1}ha^{-1}year^{-1}$.

The potential for contamination is increased when mining exposes metal-bearing ores and when mined ores are dumped on the earth's surface in manual dressing processes. Through rivers and streams, the metals are transported as either dissolved species in water or as an integral part of suspended sediments (dissolved species in water have the greatest potential of causing the most deleterious effects). They may then be stored in river bed sediments or seep into the underground water thereby contaminating water from underground sources, particularly wells. The extent of contamination of water sources will depend on the proximity of water wells to sites of contamination. Wells located near sites of contamination have been reported to contain metals at levels that exceed drinking water criteria (Duruibe et al., 2007). Metal contaminated water may also result in the contamination of soil if it is used for irrigation purposes (Wuana and Okieimen, 2011).

1.2) Metals in the environment

1.2.1) Aluminium in the environment

Aluminium is a ubiquitous element and the third most abundant element in the earth's crust, comprising approximately 8% of the earth's crust, exceeded only by oxygen (47%) and silicon (28%). The ubiquitous presence of this element has so heavily contaminated the environment that exposure to it is virtually inescapable (Buraimoh et al., 2011). It is a major component of almost all common inorganic soil particles with the exceptions of quartz sand, chert fragments, and ferromanganiferous concretions. The typical range of aluminium in soils is from 1% to 30% (10,000 to 300,000 $mg.kg^{-1}$) (Lindsay, 1979) with naturally occurring concentrations variable over several orders of magnitude. As a result of natural weathering processes, aluminium becomes enriched in soils as stable secondary mineral forms such as impure aluminium silicates or clays, aluminium hydroxides or bauxite alumina (as trihydrate gibbsite $Al(OH)_3$ or the monohydrate boehmite AlO(OH)). Other aluminium-containing minerals including feldspar, mica, amphibole, gernet, cryolite, zeolite, alunite, dowsonite and corundum may also be present in soils (Butcher, 1988). Aluminium sulphate compounds, called alum as a group, are introduced commonly to water supplies for the removal (by flocculation) of suspended solids, colour bodies, microorganisms, pH adjustment and for dechlorination purposes. Alum has also been used to precipitate phosphorus in highly eutrophic lakes (Butcher, 1988).

The aluminium ion bonds through oxygen to form a wide variety of functional groups. In igneous rocks, aluminium is largely bonded to oxygen ions in tetrahedral coordination. As the rocks weather, aluminium progressively acquires more octahedral bonding. The weathering release of aluminium from 2:1 layer silicates in soils is enhanced by inputs of acids from the natural decomposition of organic matter and minerals and from pollution (McBride, 1994). Acids as weak as dilute H_2CO_3 have been shown to decompose the silicate and montmorillonite layers, facilitating the release of aluminium (Jackson, 1963).

Aluminium metal is used as a structural material in the construction, automotive, and aircraft industries, in the production of metal alloys, in the electric industry, in cooking utensils and in food packaging (WHO, 1998). Aluminium compounds are used as antacids, antiperspirants and food additives. Aluminium salts are also widely used in water treatment as coagulants to reduce organic matter, colour, turbidity and microorganism levels (WHO, 1998).

The ubiquitous presence of aluminium in soil, water, food and pharmaceuticals makes exposure to this metal unavoidable for most species. There has been some concern about transfer of aluminium along the food chain. It has been suggested that elevated levels of aluminium in invertebrates could affect wild birds feeding in or near aluminium-laden waters (Miyasaka et al., 2007). Female birds have been reported to have elevated bone aluminium levels and laid deformed eggs with soft shells leading to dehydration and reduced hatchability. Other concerns were with bone growth and body weight gain in growing chicks since aluminium in the diet at a level of 1000 $mg.kg^{-1}$ has been shown to inhibit phosphate absorption, reduce feed intake and accumulate in bone (Miyasaka et al., 2007). It was observed that the presence of aluminium caused the death of stoneflies and caddis larvae (Burton and Allan, 1986). However, Burton and Allan (1986) observed a reduced mortality whenever the organic content of the water was high. In fish, after hatch, aluminium is more likely to affect the gill where the ion and gas exchange takes place. Aluminium was also found to cause loss of plasma ions (Na^+ and Cl^+), reduced osmolality and increased haematocrit (Muniz and Leivestad, 1980; Rosseland and Skogheim, 1982). Rosseland et al. (1990) stated that the routes and the degree of accumulation of Al along food chains have not yet been fully investigated. The findings of Nyholm (1981;1982) indicated that increased aluminium concentrations in water bodies may cause aluminium accumulation in terrestrial animals eating prey originated from contaminated water.

1.2.2) Manganese in the environment

Manganese is ubiquitous in the environment. It comprises about 0.1% of the Earth's crust. Manganese does not occur naturally as a base metal but is a component of

more than 100 minerals, including various sulfides, oxides, carbonates, silicates, phosphates and borates (WHO, 2004). The most commonly occurring manganese-bearing minerals include pyrolusite (manganese dioxide), rhodocrosite (manganese carbonate [$MnCO_3$]), rhodonite (manganese silicate) and hausmannite (manganese tetroxide [Mn_3O_4]) (WHO, 2004; Howe et al., 2005). The major anthropogenic sources of environmental manganese include municipal wastewater discharges, sewage sludge, mining and mineral processing (particularly nickel), emissions from alloy, steel and iron production, combustion of fossil fuels, and, to a much lesser extent, emissions from the combustion of fuel additives (WHO, 2004). Manganese is an essential nutrient important for normal processes in the body, though adverse health effects have been noted at higher doses. Excessive manganese exposure, predominantly reported in adults exposed occupationally via inhalation, has been associated with adverse central nervous system effects. “Manganism” refers to a set of symptoms associated with relatively high levels of exposure to manganese, reported in adult occupational exposure studies and includes muscle stiffness, lack of coordination, tremors, difficulty with breathing or swallowing and other neuromuscular problems (USEPA, 2007).

The manganese-containing fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT) was introduced to motor vehicle fuel formulae as an octane boosting and ‘’anti-knock’’ agent to replace the lead in petrol (Health Canada, 2003). MMT was introduced in South Africa in 2000 (DEAT and DME, 2003).

Bordean et al. (2014) reported that manganese can pose serious threats to both aquatic and terrestrial ecosystems. As environmental problems become global in scope, manganese contamination originating from escalating mining, manufacturing, agricultural and industrial activities, especially in the less developed countries, has raised serious concerns about its putative ecological side-effects. As a result, manganese is currently regarded as a new emerging contaminant in the environment, and its toxicity has attracted considerable scientific interest from ecotoxicologists and environmental chemists (Bordean et al., 2014).

As with other elements, manganese cannot break down in the environment. It can only change its form or become attached or separated from particles (ATSDR, 2008). The chemical state of manganese and the type of soil determine how fast it

moves through the soil and how much is retained in the soil. In water, most of the manganese tends to attach to particles in the water or settle into the sediment (ATSDR, 2008). In most studies, the accumulation of manganese in animals is reported to occur via food uptake (ATSDR, 2008). Manganese uptake has also been addressed as a direct uptake from soil by cutaneous contact and/or by soil ingestion. Manganese is present in soil as a result of mineral weathering and atmospheric deposition, originating from both natural and anthropogenic sources. There are three possible oxidation states of manganese in soil: Mn(II), Mn(III) and Mn(IV). The divalent ion is the only form that is stable in soil solution, while Mn(III) and Mn(IV) are only stable in the solid phase of soil (Nadask et al., 2010). In most cases, earthworms, nematodes, or collembolans have been used as invertebrate study system (Kuperman et al., 2004, Tatara et al., 1998).

It can only change its form or become attached or separated from particles. The chemical state of manganese and the type of soil determine how fast it moves through the soil and how much is retained in the soil. In water, most of the manganese tends to attach to particles in the water or settle into sediment (Williams et al., 2012).

Freshwater molluscs and crustaceans appear to be the most manganese-sensitive freshwater invertebrates followed by oligochaetes (Howe et al., 2005). Sea stars (*Asteria rubens*) showed no mortality at 10 and 25 mg manganese/litre (as manganese chloride). But when exposed to 50, 100 and 200 mg/litre manganese, they showed median survival times of 72, 18 and 14.4 hours, respectively (Hansen and Bjerregaard, 1995). MacDonald et al. (1988) reported a significant reduction in survival and hatching of yellow crab (*Cancer anthonyi*) embryos at 0.1 mg manganese/litre (as manganese chloride) in 7-day seawater tests. Significant adverse effects on growth and behaviour of herring gull (*Larus argentatus*) chicks were observed following a single intraperitoneal injection of manganese acetate (25 mg body weight) (Burger and Gochfeld, 1995).

Williams et al. (2012) suggested that extremely high levels of manganese exposure may produce undesirable effects on brain development, including changes in behaviour and decreases in the ability to learn and remember. In some cases, manganese exposure has been suspected of causing severe symptoms of

manganism disease (including difficulty with speech and walking). It is not known for certain that these changes are caused by manganese alone, neither is it known if these changes are temporary or permanent.

1.2.3) Metal mixtures in the environment

Since the 1990's, mixture toxicology has undergone a remarkable and productive development. All organisms are typically exposed to chemical mixtures, present in the surrounding environmental media (water, air and soil) and food. However, with a few exceptions, chemical risk assessment considers the effects of single substances in isolation (ULSOP, 2009). The toxicity of metals is also suspected to differ when they are in combination. In a study by Ince et al. (1999), a battery of two bioassays (Microtox and duckweed) was used to generate data for predicting the interactive effects of metals in binary mixtures by a novel method based on statistical testing of additive toxicity as a null hypothesis. It was found that the total fraction of antagonistic responses in the battery was 66%, implying that suppression of toxic effects is highly probable when metals are combined in binary mixtures.

In 2001, Lock and Janssen studied the chronic toxicity of mixtures of zinc, cadmium, copper, and lead to the potworm *Enchytraeus albidus*. After 21 days of exposure, Lock and Janssen (2001) discovered that survival in the control was on average, at least 90%. Moreover, survival was not affected at any of the metal concentrations tested. Reproduction of *E. albidus* in the positive controls that were exposed to one toxic unit (EC_{50}) of a single metal varied from 46 to 64% of the control reproduction. When *E. albidus* was exposed to one toxic unit of the equitoxic mixture, reproduction was significantly higher ($P<0.05$) than reproduction in the positive controls in which the organisms were exposed to metal concentrations equal to the EC_{50} values of the four metals separately.

Lock and Janssen (2001) argued that the effect of the mixtures of cadmium and zinc on the growth of *F. candida*, as discussed by Van Gestel and Hensbergen (1997) was antagonistic, while the effect on reproduction was additive. The experiment with four metals in the study by Lock and Janssen (2001) indicated that not all terms of a

complex equation can be estimated correctly due to the high background variation of the response to ecotoxicological effects. These authors concluded that as the ecotoxicity of metals can vary over several orders of magnitude depending on the soil characteristics, it would be interesting to study the effect of the soil composition on the toxicity of metal mixtures.

Accounting comprehensively for mixture toxicity via direct observation is possible only for a few selected cases as mixture occurrence in the environment is too variable and divergent to be comprehensively investigated (Altenburger et al., 2013). In their report on the state of the ecotoxicological combined effects from chemical mixtures written on behalf of the Federal Environment Agency (Germany), Altenberger et al. (2013) emphasised that in experimental studies on mixture toxicity, models that allow calculation of expected combined effects on the basis of knowledge about the components biological activities have become an established means for assessment.

Altenberger et al. (2013) acknowledged a situation of a limited database for mixture toxicity studies. These shortcomings in the field of mixture toxicity are: several specific aspects relevant to an adequate exposure assessment of biologically active compounds; technical problems to be encountered when trying to perform mixture assessments with the data available in current documentations for product authorization; and notwithstanding that a lack of reliable empirical indicators for mixture synergism based on the individual components effects is to be acknowledged. Altenberger et al. (2013) concluded that provisions to account for a lack of data or conceivable interactive effects may well be taken when dealing with resulting uncertainties.

1.3) Biological importance of soil

On the basis of organic matter content, soils are characterized as mineral or organic. Mineral soils form most of the world's cultivated land and may contain from a trace to 30% organic matter. Organic soils are naturally rich in organic matter, principally for

climatic reasons. Soil organic matter is any material produced originally by living organisms (plant or animal) that is returned to the soil and goes through the decomposition process. At any given time, it consists of a range of materials from the intact original tissues of plants and animals to the substantially decomposed mixture of materials known as humus (Bot and Benites, 2005). Most soil organic matter originates from plant tissue. Plant residues contain 60 to 90% moisture. The remaining dry matter consists of carbon (C), oxygen, hydrogen (H) and small amounts of sulphur (S), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg). Although present in small amounts, these nutrients are very important from the viewpoint of soil fertility management (Bot and Benites, 2005).

When plant residues are returned into soil, various organic compounds undergo decomposition. Decomposition is a biological process that includes the physical breakdown and biochemical transformation of complex organic molecules of dead material into simpler organic and inorganic molecules (Juma, 1998). The continual addition of decaying plant residues to the soil surface contributes to the biological activity and the carbon cycling process in the soil. Breakdown of soil organic matter as well as root growth and decay also contribute to these processes. Carbon cycling is the continuous transformation of organic and inorganic carbon compounds by plants as well as micro-and macro-organisms between the soil, plants and the atmosphere (Bot and Benites, 2005). Decomposition of organic matter is largely a biological process that occurs naturally. Its speed is determined by three major factors: soil organisms, the physical environment and the quality of the organic matter (Brussaard, 1994).

Soil organisms use soil organic matter as food. As they break down the organic matter, any excess nutrients are released into the soil in forms that plants can use. This release process is called mineralization. The waste products produced by micro-organisms are also soil organic matter. This waste material is less decomposable than the original plant and animal material, but it can be used by a large number of organisms. By breaking down carbon structures and rebuilding new ones or storing the carbon into their own biomass, soil biota (among them, woodlice) plays the most important role in nutrient cycling processes and, thus, in the ability of soil to provide crops with sufficient nutrients to harvest a healthy product. Woodlice form a considerable part of the macrofauna in soil ecosystems (A'Bear et al., 2014).

Woodlice, even at low density, consistently reduce mycelial biomass and exert selective pressures strong enough to alter the outcomes of competitive interactions. Extensive mycelial ingestion by a widely distributed woodlouse species, *Oniscus asellus* has been shown to reduce soil extracellular enzyme activities and increase collembola abundance by releasing the more easily ingested micro fungi from competitive suppression (Crowther et al., 2013). Their capacity to maintain high fungal diversity and mycophagous mesofaunal abundance has led to woodlice being suggested as keystone grazers in temperate woodland soil (Crowther et al., 2013). The capacity for field woodlouse populations to regulate decomposer community structure and function determines their potential to moderate climate-induced stimulation of decomposition and CO_2 efflux from temperate soil (Jones and Hopkin, 1998). However, woodlice experience stress when exposed to high levels of metals in the diet, which can reduce feeding rates and may combine with natural stresses to reduce fitness and lower 'performance', thereby increasing the probability of early mortality. Woodlice with very high concentrations of metals can have significantly lower energy reserves, decreased moult frequency and can show reduced locomotion (Jones and Hopkin, 1998). Therefore, metal pollution may result in a reduction in the ability of woodlice to fulfil its ecological function.

1.4) Woodlice as biomonitor organisms

1.4.1) Metals in woodlice

Woodlice (also called sow bugs, pill bugs and slaters) are terrestrial isopods (Class Crustacea, Sub-Order Isopoda) of the Family Oniscidea, which have invaded terrestrial habitats from aquatic environments. Most species can still tolerate submersion in water saturated with O_2 (Maurizio and Hassall, 1999). Most woodlice species are small to medium sized organisms (1.2–30mm), with approximately 5000 species distributed worldwide from deserts to forests, rangelands, agro-ecosystems, up mountains and in subterranean caves. There are several life forms: runners,

which have large eyes, long legs, and sometimes mimetic colours; rollers, capable of rolling into a tight ball when disturbed; clingers, less mobile than the preceding forms and with depressed margins of the body which they press down on flat surfaces; and creepers, which have developed tergal ribs and live in narrow interstices and caves. The body surface is covered by setae, scales, glands and sometimes ornaments in various shapes. Strategies to improve body impermeability have been developed to colonize terrestrial environments (Maurizio and Hassall, 1999).

The diet of woodlice consists of decaying organic matter in soil and occasionally other animal materials, thus representing a species intimately mixing within the surface soil horizons. They have a broad tolerance of certain contaminants and can accumulate metals to relatively high levels (Gal et al., 2008). Single individuals also provide sufficient material for chemical analysis. High levels of metals, especially copper (Hopkin and Martin, 1982), but also zinc, lead and cadmium are accumulated in vesicles such as lysosomes. Considerable concentrations of copper are present in the haemolymph of isopods as this contains haemocyanins with copper at their active sites (Gal et al., 2008). Woodlice have demonstrated a potential as environmental monitors of metal contamination in soil. Most studies of metals in woodlice have involved common metals such as cadmium, lead, copper, zinc and iron, and mostly in single exposures (Gal et al., 2008).

Woodlice are predated by an array of different animals, including vertebrates such as birds, frogs, lizards, mammals (particularly shrews) and invertebrates including spiders such as *Dysdera crocata*, some scorpions, chilopods, ground beetles and other polyphagous insects. Since woodlice are predated by a wide range of invertebrates and vertebrates, the bioaccumulation of toxic metals in their bodies can also have ramifications for trophic levels higher up the food chain (Maurizio and Hassall, 1999).

Soil invertebrates have been used quite extensively to assess metal contamination in soil (Hopkin and Drobne, 1995; Loureiro et al., 2005; Žaltauskaitė and Sodienė, 2010). Generally, the potential hazards of various environmental toxicants to soil invertebrates are assessed by bioassays with the keystone species being earthworms and woodlice (Žaltauskaitė and Sodienė, 2010). Woodlice like many other soil invertebrates, occur in the soil litter component of terrestrial ecosystems.

This zone is regarded as an important sink for metal contaminants (Coughtrey et al., 1979). Terrestrial isopods are one of the animal groups that fulfil most of the criteria required for an animal group to be regarded as good organisms for toxicity testing and biomonitoring (Drobne, 1997). They are frequently used for testing the effects of chemicals (Odendaal and Reinecke, 2007).

The toxic effects of metals refer to the harmful effects of metals to organisms when consumed above the recommended limits. In a study by Odendaal and Reinecke (2003), it was shown that the body mass of terrestrial isopods (*Porcellio leavis*) were negatively impacted after they have been exposed to cadmium and zinc. It has also been found that when exposed to certain levels of cadmium, copper and lead, isopods may face mortality (Ginneken et al., 2015).

Biomonitors have been widely studied in the assessment of terrestrial and aquatic ecosystems, particularly for metal contamination. The biomonitoring capability of woodlice appears to be promising in terms of metal pollution of contaminated sites (Gal et al., 2008). Good biological indicators of metal contamination in the environment include earthworms, fruit flies, ants, slugs, snails and woodlice (Hopkin et al., 1986). Woodlice are probably one of the best candidates because they are able to store a range of metals to very high concentrations, are common in a diverse range of habitats in rural and urban areas and consume a wide variety of dead plant material (Hassall & Rushton, 1984).

Woodlice generally respond quickly to environmental contamination, with increased mortality, loss of biomass and a decrease in the number of species, resulting from different levels of pollution. Woodlice can act as bioaccumulators of metal pollutants because they adopt a tolerance strategy of accumulating and immobilizing metals rather than preventing absorption or increasing efficiency of excretion. As they are also large, conspicuous and very easy to collect, they are well suited to act as biomonitors of metal contamination in saprophagous food chains. Woodlice are useful for monitoring metal pollution in industrialized and urbanized areas (Paoletti et al., 1988).

Hopkin et al. (1986) discovered the presence of considerable concentrations of zinc and copper in the hepatopancreas of *Porcellio scaber*. Woodlice are very efficient in storing metals in the hepatopancreas, resulting in low metal concentrations in the

body fluid (Martina et al., 2004). The hepatopancreas of woodlice contains high-density granules to which metals are bound and this increases its storage capacity. When metal storage capacity of these granule-containing hepatopancreas cells is exceeded or when storage cannot keep up with uptake rate, woodlice will suffer from metal toxicity (Martina et al., 2004).

A study by Hopkin et al. (1986), described a practical example of biological monitoring in which the concentrations of zinc, cadmium, lead and copper have been determined in the hepatopancreas and whole body of *Porcellio scaber*, as well as samples of leaf litter and soil, collected from 89 sites in the counties of Avon and Somerset, south-west England. The specific objective of the study was to determine how closely the concentrations of zinc, cadmium, lead and copper were correlated in soil, leaf litter, whole woodlice and the hepatopancreas. From the findings, Hopkin et al. (1986) deduced that the determination of concentrations of zinc, cadmium, lead and copper in soil and leaf litter does not enable the concentrations of these metals in woodlice to be accurately predicted. One of the most significant findings of this survey was the presence of considerable concentrations of zinc and copper in the hepatopancreas of *Porcellio scaber* from uncontaminated sites.

In woodlice, metals such as zinc, cadmium, lead and copper are accumulated in the S and B cells of the hepatopancreas. To enter the hepatopancreas, metals have to be soluble in the liquid component of the food (Hopkin, 1989). It was explained by Hopkin (1989) that digestive enzymes are secreted by the hepatopancreas into food as it passes through the foregut and into the anterior chamber. Products of digestion are forced back along the typhlosole channels from the papillate region to the foregut. This liquid that may contain metals released into solution by digestive enzymes passes through the filters into the hepatopancreas where absorption can take place. Hopkin (1989) further argued that because the hindgut is lined with cuticle, the only route of loss of metal via the lumen of the digestive system is likely to be from the hepatopancreas by lysis of cells. A site of storage-excretion other than the hepatopancreas has not been discovered in terrestrial isopods (Hopkin, 1989).

1.4.2) Metal mixtures in woodlice

Little is known about the effects of a mixture of metals on soil fauna (Altenberger et al., 2013). Relative to other types of investigations, only a few studies had been undertaken to investigate the effects of mixed metals on soil fauna (Khalil et al., 1996). To date, most environmental toxicity tests and experiments are based on single metal exposures and thus have little relevance as far as the combined effect of metals is concerned as fauna could react differently to mixtures of metals than singly employed metals (Enserink et al., 1991). Mixture toxicity experiments reflect environmental pollution in a more realistic manner and it is also a proven fact that different mixtures of metals have different effects on fauna (Odendaal and Reinecke, 2003).

In their study, Odendaal and Reinecke (2003) determined the accumulation of cadmium and zinc in *Porcellio laevis* after separate and mixed exposure to these metals. This was an attempt to give an indication whether cadmium and zinc influence each other's bioaccumulation. After six weeks of exposure to mixtures of cadmium and zinc, cadmium accumulation in the hepatopancreas samples of *P. laevis* of all exposure groups were significantly higher than in the control group. In the rest of the body samples (excluding the hepatopancreas) of *P. laevis*, after six weeks of exposure to mixtures of cadmium and zinc, cadmium concentrations in all the exposure groups were statistically significantly higher than that of the control group. However, the concentrations of zinc in the hepatopancreas of *P. laevis* after six weeks of exposure to mixtures of cadmium and zinc did not show any statistical significant differences between the exposure groups and control group. However, the concentrations of zinc in the rest of the body samples of *P. laevis* of all the exposure groups after 6 weeks of exposure to mixtures of cadmium and zinc differed significantly from that of the control group and also differed significantly from the other exposure groups. Odendaal and Reinecke (2003) concluded that the hepatopancreas was the main organ of bioaccumulation for cadmium and zinc in both single and mixture exposures. Cadmium and zinc also exhibited the ability to influence the bioaccumulation of each other in the mixed metal exposures. They also came to the conclusion that the interaction of these two metals was at least partly

dependent on the ratio of exposure concentrations, the actual exposure concentrations, and also the period of exposure.

In a study by Witzel (2000), three patterns of metal accumulation in *P. scaber* were distinguished in relation to cadmium, zinc and lead. During exposure, bioaccumulation of cadmium continued in the animal, even on uncontaminated food. Woodlice exposed to lead showed a decreasing aluminium concentration with gradual decontamination of the food source. They eventually reached the concentration of the control group. Animals exposed to zinc showed an increase of zinc bioaccumulation throughout the experiment. When woodlice were exposed to a mixture of zinc and lead, zinc bioaccumulation in the animal was significantly higher than when the woodlice were exposed to zinc alone. When zinc was combined with cadmium, the zinc concentration in woodlice was significantly higher than when they were exposed to zinc alone. These results, according to Witzel (2000), confirmed the changes in zinc and cadmium when they are combined in high concentrations. While the uptake and loss of cadmium was not influenced by the presence of zinc, the uptake of zinc itself was elevated by the presence of lead and cadmium. Witzel (1998) found that once cadmium is assimilated and stored by woodlice, it cannot be excreted, and is stored at 80% in the hepatopancreas. In contrast to this, Witzel (2000) revealed significant excretion of cadmium when combined with zinc in high concentrations. Witzel (2000) argued that the presence of zinc interferes with the storage of cadmium in S cells at high concentrations which therefore made the uptake and storage of cadmium not permanent. The conclusion was that when combined with zinc, at high concentrations, cadmium can be excreted separately or simultaneously with zinc.

1.5) Statement of the research problem

Human activities results in the deposition of a large variety of contaminants into the environment on a continual basis, which effectively ensures human and wildlife exposure to complex mixtures of contaminants. Soil ecosystems in urban, rural and agricultural environments receive chemical input from diverse sources of

contamination, such as wastewater, industrial discharge, agricultural and urban runoff, vehicle leakages, landfill seepage and animal waste overspill. Agricultural activities, transportation and industrial activities are suspected to be the highest sources of metal contamination in Cape Town. Although scientists generally have a good understanding of the toxicity of individual chemical pollutants, there is a great need to bridge the gap between our understanding of the toxic effects of exposure to individual contaminants and those effects from exposure to mixtures of these chemicals (Olmstead and Leblanc, 2004).

Woodlice and other soil detritivores have a particularly important ecosystem function in mineralising organic matter. They transform litter into faecal pellets, which decompose rapidly. For example, woodlice utilise more than 10% of the annual litter, increasing fourfold the surface available to micro-organisms (Souty-Grosset et al., 2005). Woodlice experience stress when exposed to toxic levels of metals in the diet, which can reduce feeding rates and may combine with natural stresses to reduce fitness and lower 'performance', thereby increasing the probability of early mortality. Woodlice with very high concentrations of metals can have significantly lower energy reserves, decreased moult frequency and can show reduced locomotion (Jones and Hopkin, 1997). These factors can contribute in the inability of the woodlice to completely fulfil its ecological function of mineralising organic matter.

Aluminium and manganese were chosen for this study because of their apparent prevalence in the soil environment in and around Cape Town, as shown by previous unpublished studies.

1.6) Objectives of the research

The aim of this study was to investigate the effect of mixtures of aluminium and manganese on the bioaccumulation of these metals in experimentally exposed *Porcellio scaber*. The objectives derived from this aim were:

- To compare how aluminium and manganese are bioaccumulated in *Porcellio scaber* in terms of the contribution of the hepatopancreas in metal storage compared to the rest of the body.
- To determine whether mixtures of aluminium and manganese affect each other's bioaccumulation and distribution in *Porcellio scaber*.

CHAPTER TWO: MATERIALS AND METHODS

2.1) Field sampling

2.1.1) Site of collection for woodlice and oak leaves

The Kirstenbosch Botanical Garden in Cape Town was chosen as a site for woodlice and oak leaf collection. Kirstenbosch was chosen for this purpose because there is no apparent source of contamination in its immediate environment. Woodlice of the species *Porcellio scaber* (Crustacea, Isopoda) were used in this study. Oak leaves were used as a means of metal exposure and a food source for woodlice in the experimental exposures.

2.1.2) Sample collection method

Decaying oak leaves were hand collected and stored in plastic bags. Leaves were collected away from paths and picnic areas. Woodlice were also hand collected and kept in a plastic container with a sieve lid to allow air to move into the container. Because woodlice feed on decaying plant material, oak leaves were added into the container to allow the individuals to feed normally and thereby minimize stress on the animals.

2.2) Preparations for the experimental exposures

2.2.1) Leaves

After collection, decaying leaves were stored in a plastic bag. Leaves were shredded in a blender into smaller pieces to make it easier to contaminate them with the metal in a more homogeneous fashion. After shredding, leaves were spread on a flat

surface to allow them to dry before contamination with the metals. The experimental set up and metal exposure procedure followed in the present study was based on Odendaal (1997).

2.2.2) Metal contamination of leaves and experimental exposure groups

Aluminium and manganese were administered as $AlSO_4$ and $MnSO_4$ solutions to pre-weighed dried oak leaves. The leaves were homogenously sprayed with the $AlSO_4$ and $MnSO_4$ solutions. This was only done once at the beginning of the experimental exposures. These leaves were offered to the woodlice as their food source. Woodlice were exposed to environmentally relevant low and relatively high concentrations of $AlSO_4$ and $MnSO_4$ in single metal and mixed metal experiments. The calculated low concentration of $AlSO_4$ was 450mg/kg, while the relatively high concentration was 2000mg/kg (dry mass). The low concentration of $MnSO_4$ was 150mg/kg and the relatively high concentration was 300mg/kg (dry mass). These concentrations are realistic in terms of aluminium and manganese concentrations previously found in unpublished studies in Cape Town.

Plastic containers holding 10 woodlice each were prepared with the following exposure groups (5 replicates per exposure group): one group where woodlice were provided with uncontaminated oak leaves was used as the control; one group with a low concentration of aluminium (AlL); one group with a high concentration of aluminium (AlH); one group with a low concentration of manganese (MnL); one group with a high concentration of manganese (MnH); one group with a mixture of low concentrations of aluminium and manganese (AlL/MnL), one group with a mixture of high concentrations of aluminium and manganese (AlH/MnH); one group with a mixture of a low concentration of aluminium and high concentration of manganese (AlL/MnH); and one group with a high concentration of aluminium and a low concentration of manganese (AlH/MnL).

Exposures were carried out for a period of five weeks in round plastic containers with a plaster of Paris bottom and covered with a plastic sieve to allow air to circulate into

the container. The containers were filled with 50g of shredded oak leaves, contaminated with $AlSO_4$ and $MnSO_4$.

At week 0 and after five weeks of exposure, one woodlouse out of five replicate containers from each exposure group was digested according to the acid digestion process described below and analysed for aluminium and manganese. The woodlice were first dissected to remove the hepatopancreas so that this organ and the rest of the body could be digested separately. Prior to dissection for acid digestion, woodlice were kept in moist petri dishes for 24 hours to clear their gut (Hames and Hopkin, 1989).

2.3) Acid digestion of samples

2.3.1) Leaf digestion

Leaf samples were dried for 48 hours in a Memmert oven at 60^{o}C and a subsample of 0.3 g was weighed on a Precisa XB 220A balance. This subsample was then placed into a labelled, metal free glass test tube for digestion. The test tubes with the samples, as well as a blank (test tube with only 10ml nitric acid, to check for possible contamination) were placed in a Grant heating block in a fume cabinet and digested with 10 ml 65% nitric acid according to the method used by Odendaal and Reinecke (2003). After cooling, samples were filtered through Whatman no 6 (90 mm) filter paper and diluted to 20 ml with distilled water using labelled 20 ml volumetric flasks. The samples were finally filtered through Whatman 0.45 µm cellulose nitrate membrane filters using a syringe and Millipore filter holders. 1ml of the filtrate were introduced into plastic volumetric tubes and diluted with 9 ml distilled water to obtain a total volume of 10 ml. The prepared samples were stored in a fridge until it was taken to the ICP-MS laboratory at the University of Stellenbosch to determine the metal concentrations in the samples. Metal concentrations in leaf samples were within 15% of the calculated concentrations.

2.3.2) Rest of the body digestion

Rest of the body samples were dried for 48 hours in a Memmert oven at 60^{o}C and weighed on a Precisa XB 220A balance. The same acid digestion and metal analysis

process that were used for the leaf samples were used for the rest of the body samples.

2.3.3) Hepatopancreas digestion

Glass tubes were washed, dried then weighed to obtain the empty weight. Dissected hepatopancreas samples were then placed in separate, pre-weighed and labelled glass tubes. The glass tubes with the hepatopancreas samples were then placed into a Memmert oven to dry for 48 hours at 60°C. Tubes containing the dried hepatopancreas were then weighed again using a Precisa XB 220A balance. The dry weight of the hepatopancreas was obtained by subtracting the weight of the empty glass tubes from the weight of the glass tubes containing the hepatopancreas. The same acid digestion and metal analysis process that were used for the leaf samples were used for the hepatopancreas samples.

2.4) Metal analysis

The metal concentrations in the samples were determined by means of Inductively Coupled Plasma Mass Spectrophotemetry (ICP-MS) and calculated using the following formula:

$$\frac{(ICP\ reading - Blank)\ x\ [dilution\ factor]}{Dry\ mass\ of\ sample\ (g)}$$

Metal concentrations were expressed as mg/kg.

2.5) Statistical analysis of data

The data in this study was analysed by using the Sigmaplot 12.3 computer software package. The probability level used for statistical significance were $P<0.05$. ANOVA on Ranks with a post hoc test (Student Newman Keuls) were used to statistically compare the aluminium and manganese concentrations found in hepatopancreas and rest of the body samples.

CHAPTER THREE: RESULTS AND DISCUSSION: ALUMINIUM

3.1) Results

3.1.1) Comparisons of aluminium concentrations in the hepatopancreas at week 0 and week 5

The aluminium concentrations in the hepatopancreas before exposure (week 0) were compared to the aluminium concentrations in the hepatopancreas after 5 weeks of exposure. The hepatopancreas of woodlice at week 0 is termed H0 and the hepatopancreas of woodlice after 5 weeks of exposure is termed H5. The control is termed ctrl (Table 1).

There were no statistically significant differences found in the hepatopancreas when the aluminium concentrations at H0 were compared to H5 for the control group (P = 0.548); the groups with AlL (P = 0.548); the groups with AlH (P = 0.690); the groups with AlL/MnL: (P = 0.905); the groups with AlH/MnL (P = 0.841); the groups with AlL/MnH (P = 0.222) and the groups with AlH/MnH (P = 0.730) (Table 1).

Table 1: The mean aluminium concentrations (mg/kg) ± SD in the hepatopancreas of week 0 woodlice (N=5) and in the hepatopancreas of week 5 woodlice (N=5) for all the exposure groups.

		DIFFERENT EXPOSURE GROUPS						
		ctrl	AlL	AlH	**AlL**/MnL	**AlL**/MnH	**AlH**/MnL	**AlH**/MnH
Calculated Concentrations			**450**	**2000**	**450**/150	**450**/300	**2000**/150	**2000**/300
1) H0	**Mean**	**^{a}103.35**	**^{a}103.35**	**^{a}103.35**	**^{a}103.35**	**^{a}103.35**	**^{a}103.35**	**^{a}103.35**
	SD	105.35	105.35	105.35	105.35	105.35	105.35	105.35
2)H5	**Mean**	**^{a}39.3**	**^{a}46.84**	**^{a}41.76**	**^{a}52.67**	**^{a}612.04**	**^{a}112.28**	**^{a}51.56**
	SD	27.56	24.05	17.68	46.48	721.75	56.31	38.20

Statistical significant differences between H0 and H5 are indicated with different superscripted letters.

3.1.2) Comparisons of aluminium concentrations in the rest of the body at week 0 and week 5

The rest of the body represents the body of the woodlice without the hepatopancreas.

The aluminium concentrations in the rest of the body of woodlice before exposure (week 0) were compared to the aluminium concentrations in the rest of the body of woodlice after 5 weeks of exposure (week 5). The rest of the body of week 0 woodlice is termed R0 and the rest of the body of week 5 woodlice is termed R5 (Table 2).

The aluminium concentrations showed no statistically significant differences between R0 and R5 in the control group (P = 0.690); the groups with AlL (P = 0.841) and the groups with AlH (P = 0.151).

Statistical comparisons between R0 and R5 showed statistically significant differences in the aluminium concentrations for the exposure groups: AlL/MnL (P = 0.032), AlL/MnH (P = 0.008), AlH/MnL (P = 0.008) and AlH/MnH (P = 0.016) (Table 2).

Table 2: The mean aluminium concentrations (mg/kg) ±SD in the rest of the body of week 0 woodlice (N=5) and in the rest in the body of week 5 woodlice (N=5) for all exposure groups.

		DIFFERENT EXPOSURE GROUPS						
		Ctrl	AlL	AlH	**AlL**/MnL	**AlL**/MnH	**AlH**/MnL	**AlH**/MnH
Calculated Concentrations			**450**	**2000**	**450**/150	**450**/300	**2000**/150	**2000**/300
1) R0	**Mean**	**^{a}13.94**	**^{a}13.94**	**^{a}13.94**	**^{a}13.94**	**^{a}13.94**	**^{a}13.94**	**^{a}13.94**
	SD	7.90	7.90	7.90	7.90	7.90	7.90	7.90
2)R5	**Mean**	**^{a}13.98**	**^{a}64.91**	**^{a}195.74**	**^{b}574.95**	**^{b}406.78**	**^{b}432.67**	**^{b}381.98**
	SD	16.69	99.06	214.17	377.84	36.35	246.10	28.26

Statistical significant differences between R0 and R5 are indicated with different superscripted letters.

3.1.3) Comparison of the aluminium concentrations in the hepatopancreas and the rest of the body after five weeks of exposure

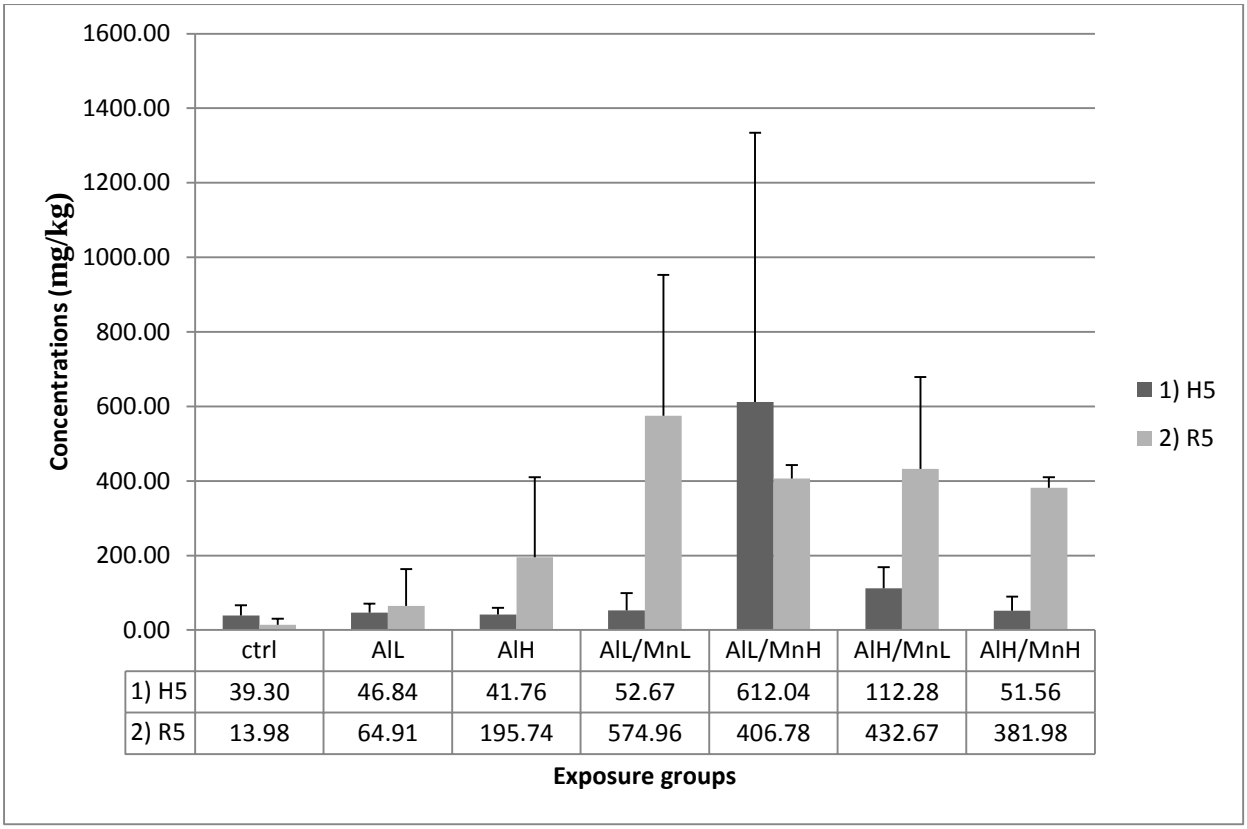
The aluminium concentrations in the rest of the body was compared to the aluminium concentrations in the hepatopancreas after 5 weeks of exposure for all the exposure groups (Table 3; Figure 1).

No statistically significant differences were found between the aluminium concentrations of H5 and R5 for the following exposure groups: control (P = 0.117); AlL (P = 0.548); AlH (P = 0.151); and AlL/MnH (P = 0.543). Statistical comparisons showed significant differences between the aluminium concentrations of H5 and R5 for the groups exposed to AlL/MnL (P = 0.016), AlH/MnL (P = 0.032); and AlH/MnH (P < 0.001) with the concentrations in R5 always higher than in H5.

Table 3: The mean aluminium concentrations (mg/kg) ±SD in the hepatopancreas and the rest of the body samples of woodlice after 5 weeks of exposure to various concentrations (N=35).

		DIFFERENT EXPOSURE GROUPS						
		Ctrl	AlL	AlH	**AlL**/MnL	**AlL**/MnH	**AlH**/MnL	**AlH**/MnH
Calculated Concentrations.			**450**	**2000**	**450**/150	**450**/300	**2000**/150	**2000**/300
1) H5	**Mean**	**[a]39.3**	**[a]46.84**	**[a]41.76**	**[a]52.67**	**[a]612.04**	**[a]112.28**	**[a]51.56**
	SD	27.56	24.05	17.68	46.48	721.75	56.31	38.20
2) R5	**Mean**	**[a]13.98**	**[a]64.91**	**[a]195.74**	**[b]574.96**	**[a]406.78**	**[b]432.67**	**[b]381.98**
	SD	16.69	99.06	214.17	377.84	36.35	246.10	28.26

Statistical significant differences between H5 and R5 are indicated with different superscripted letters.



	ctrl	AlL	AlH	AlL/MnL	AlL/MnH	AlH/MnL	AlH/MnH
1) H5	39.30	46.84	41.76	52.67	612.04	112.28	51.56
2) R5	13.98	64.91	195.74	574.96	406.78	432.67	381.98

Figure 1: The mean aluminium concentrations (mg/kg) ±SD in the hepatopancreas and the rest of the body samples at week 5 for all exposure groups (N=35).

3.1.4) Comparisons of single metal exposure groups (AlL and AlH)

Aluminium concentrations in the hepatopancreas and in the rest of the body exposed to a single contamination of aluminium (AlL and AlH) were compared after 5 weeks of exposure (Table 4).

No statistically significant differences were found between the Ctrl, AlL and AlH exposure groups in terms of aluminium concentrations in the hepatopancreas ($P = 0.05$).

Statistical comparisons also showed no significant differences in the aluminium concentrations in the rest of the body between the Ctrl, AlL and AlH ($P = 0.05$).

Table 4: The mean aluminium concentrations (mg/kg) ±SD in the hepatopancreas and rest of the body of woodlice exposed to aluminium only (AlL and AlH) (N = 15).

		DIFFERENT EXPOSURE GROUPS		
		ctrl	AlL	AlH
Calculated Concentrations			**450**	**2000**
1) H5	**Mean**	**[a]39.3**	**[a]46.84**	**[a]41.76**
	SD	27.56	24.05	17.68
2) R5	**Mean**	**13.98 [1]**	**64.91 [1]**	**195.74 [1]**
	SD	16.69	99.06	214.17

Statistical significant differences between the different exposure groups are indicated by different superscripted letters (for the hepatopancreas) and numbers (for the rest of the body).

3.1.5) Effect of the presence of manganese on the aluminium bioaccumulation in hepatopancreas samples in the AlL exposure groups

The hepatopancreas aluminium concentrations of the exposure group with a single low aluminium concentration (AlL) was compared to the aluminium concentrations of the exposure groups with a mixture of aluminium and manganese, containing a low concentrations of aluminium (AlL/MnL, AlL/MnH) (Table 5).

No statistically significant differences were found between the AlL group and the ctrl ($P = 0.657$), the AlL/MnL group ($P = 0.813$), or the AlL/MnH group ($P = 0.151$) in terms of aluminium concentrations of the hepatopancreas.

3.1.6) Effect of the presence of manganese on aluminium bioaccumulation in the rest of the body in the AlL exposure groups

The rest of the body aluminium concentrations of the exposure group with a single low aluminium concentration (All) was compared to the aluminium concentrations of the exposure groups with a mixture of aluminium and manganese, containing a low concentration of aluminium (AlL/MnL, AlL/MnH) (Table 5).

Statistical comparisons showed no significant differences in terms of the aluminium concentrations in the rest of the body between AlL and the control (P = 0.421) and between AlL/MnL and AlL/MnH (P = 0.690).

Statistical significant differences in terms of aluminium concentrations in the rest of the body were found between the control and AlL/MnL (P = 0.008), the control and AlL/MnH (P = 0.008), AlL and AlL/MnL (P = 0.0032) and AlL and AlL/MnH (P = 0.008).

Table 5: The mean aluminium concentrations (mg/kg) ±SD in the hepatopancreas and rest of the body of woodlice exposed to AlL, AlL/MnL and AlL/MnH (N = 20).

		DIFFERENT EXPOSURE GROUPS			
		ctrl	AlL	AlL/MnL	AlL/MnH
Calculated Concentrations			**450**	**450**/150	**450**/300
1) H5	**Mean**	**39.3**	**46.84**	**52.67**	**612.04**
	SD	27.56	24.05	46.48	721.75
2) R5	**Mean**	**13.98** ^{cd}	**64.91** ^{cd}	**574.95** ^{ab}	**406.78** ^{ab}
	SD	16.69	99.06	377.84	36.35

Statistically significant differences from ctrl = a, AlL = b, AlL/MnL = c, AlL/MnH = d.

3.1.7) Effect of the presence of manganese on aluminium bioaccumulation in hepatopancreas samples in the AlH exposure groups

The hepatopancreas aluminium concentrations of the exposure group with a single high aluminium concentration (AlH) were compared to the aluminium concentrations of the exposure groups with a mixture of aluminium and manganese, containing a high concentration of aluminium (AlH/MnL, AlH/MnH) (Table 6).

No statistically significant differences were found in the aluminium concentration in the hepatopancreas between the control group and the AlH group (P = 0.455).

Mean aluminium concentrations in the hepatopancreas showed a statistically significant difference between the AlH group and the AlH/MnL group (P = 0.008).

No statistically significant differences were found in the mean aluminium concentrations in the hepatopancreas between the AlH group and the AlH/MnH group (P = 0.514).

3.1.8) Effect of the presence of manganese on the aluminium bioaccumulation in the rest of the body in the AlH exposure groups

The aluminium concentrations in the rest of the body of the group exposed to a single high aluminium concentration (AlH) was compared to the aluminium concentrations in the rest of the body of the groups exposed to a mixture of aluminium and manganese, containing a high concentration of aluminium (AlH/MnL, AlH/MnH) (Table 6).

Statistical comparisons showed no statistically significant differences in the aluminium concentrations in the rest of the body between the AlH group and the following groups: control (P = 0.095); AlH/MnL (P = 0.095); and AlH/MnH (P = 0.053).

Table 6: The mean aluminium concentrations (mg/kg) ±SD in the hepatopancreas and rest of the body exposed to AlH, AlH/MnL and AlH/MnH (N = 20).

		DIFFERENT EXPOSURE GROUPS			
		ctrl	AlH	AlH/MnL	AlH/MnH
L calculated Concentrations			**2000**	**2000**/150	**2000**/300
1) H5	**Mean**	**39.3**	**[c]41.76**	**[d]112.28**	**51.56**
	SD	27.56	17.68	56.31	38.20
2) R5	**Mean**	**13.98**	**195.74**	**432.67**	**381.98**
	SD	16.69	214.17	246.10	28.26

Statistically significant differences from ctrl = a, AlH = b, AlH/MnL = c, AlH/MnH = d.

3.2) Discussion

3.2.1) Bioaccumulation of aluminium in the hepatopancreas

Aluminium concentrations in the hepatopancreas of *Porcelio scaber* after 5 weeks of exposure did not differ significantly when compared to the aluminium concentrations in the hepatopancreas of the woodlice before exposure (week 0). No statistically significant differences were found when these two groups were compared in all the exposure groups. Therefore, no bioaccumulation of aluminium took place relative to uncontaminated hepatopancreas samples. Two different scenarios are suspected to be the cause of this finding. Woodlice avoided contaminated leaves during the exposure period, or they consumed leaves that were contaminated, but regulated the aluminium that was taken up. The first option is quite unlikely as the leaves that woodlice fed on during exposure were contaminated in such a way that no uncontaminated spot were left. In a similar experiment on crayfish, it was found that little aluminium accumulated in the hepatopancreas or other organs of crayfish following aqueous exposure (Alexopoulos et al., 2003). Woodburn et al. (2011) showed both accumulation and subsequent removal of ingested aluminium in crayfish fed with aluminium contaminated food. Therefore, there may have been uptake of aluminium by woodlice, followed by regulation, which resulted in no statistically significant differences being found when the aluminium concentrations in the hepatopancreas at week 0 were compared to the aluminium concentrations at week 5.

Protasowicky et al. (2013) suggested that essential metals such as aluminium are involved in vital functions in the body of crayfish as they may impair the immune system, reproduction, heart rhythm, breathing processes, regeneration and moulting processes. Aluminium may also cause changes in pigmentation, increase glucose concentration, change pH of digestive juices and induce histopathological changes in the hepatopancreas of crayfish (Protasowicky et al., 2013). In terms of the proportions of aluminium storage between the rest of the body and the hepatopancreas, it is not yet established what the normal dynamics should be. In their study, Protasowicky et al. (2013) mentioned that there are only a few publications on iron, manganese, vanadium, lithium, and aluminium in crustaceans.

From the findings in the studies by Dallinger and Rainbow (1991) and Woodburn et al. (2011), it is understandable why in the present study, the aluminium concentrations in the hepatopancreas of *P. scaber* after exposure were not statistically different from the aluminium concentration in the hepatopancreas of woodlice before exposure. From the study by Woodburn at al. (2011) it was revealed that loss of aluminium from the hepatopancreas of crayfish is enhanced at high tissue burdens as proportionally more aluminium was removed from this organ. It is possible that in the present study, aluminium accumulated in the hepatopancreas of *P. scaber* was removed from this organ like in the case of cadmium and lead in *P. scaber* (Dallinger and Rainbow, 1991) and aluminium in the case of *Pacifastacus leniusculus* (Woodburn et al., 2011).

Histopathological analysis of the hepatopancreas of crayfish (*Pacifastacus leniusculus*) fed with aluminium contaminated food by Woodburn et al. (2011) revealed a significant reduction in the height of the tubular epithelial cells in the hepatopancreas as compared to the control group. In addition, all the hepatopancreas samples of crayfish fed with aluminium contaminated food showed increased vacuolisation of the tubular epithelial cells, while the control group showed a similar degree of the vacuolisation in one out of six groups. The diameter of the tubule lumen, expressed as the percentage of the tubule diameter was significantly greater in crayfish fed with aluminium contaminated food compared to control crayfish. Furthermore, the mean percentage lumen diameter for each crayfish was positively but non-linearly correlated with the amount of aluminium ingested. Woodburn et al. (2011) concluded that crayfish fed with aluminium contaminated food accumulated significant concentrations of aluminium in the hepatopancreas and excreted a proportion of this, most likely via the antennal glands. Following the ten-day clearance period, a reduction in the concentration of aluminium in the hepatopancreas of crayfish fed with aluminium contaminated food occurred but complete removal was not observed. Findings from these studies correlates further to the suggestion that most of the aluminium ingested by woodlice in this study was later excreted out of the hepatopancreas, hence the observed statistical result between aluminium concentrations in the hepatopancreas of woodlice at week 0 and at week 5.

When the aluminium concentrations in the hepatopancreas of *P. scaber* of the group exposed to a single low aluminium concentration (AlH) were compared to the groups with a mixture of low aluminium with low and high manganese concentrations (AlL/MnL, AlL/MnH), no statistically significant differences were found. However, there was a tendency of a slight increase of aluminium concentrations in the hepatopancreas in the groups with a mixture of aluminium and manganese. Aluminium concentrations in the hepatopancreas of the group exposed to a single high concentration of aluminium (AlH) were partly different from the aluminium concentrations in the hepatopancreas samples of the groups exposed to a mixture of high aluminium and manganese (AlH/MnL, AlH/MnH). Only the AlH/MnL group showed a statistical difference from the AlH group.

In most of the exposure groups, manganese did not affect aluminium bioaccumulation. However, manganese affected the bioaccumulation of Al in the AlH/MnL exposure group. Therefore, it seems like relatively low manganese concentrations can affect aluminium bioaccumulation in the hepatopancreas. Beyer et al. (1982) suggests that the ratio of the metals in a mixture and the actual concentrations can affect how metals interact. Both these factors could have played a role in the way manganese affected the bioaccumulation of aluminium in woodlice hepatopancreas.

3.2.2) Bioaccumulation of aluminium in the rest of the body

Aluminium was found to concentrate significantly in the rest of the body of *P. scaber* in this study. This finding is similar to that of Dallinger and Rainbow (1991) when they realised that in both uncontaminated and metal-loaded *P. scaber*, cadmium was shown to accumulate in the posterior part of the alimentary tract and it has been suggested that this fraction of the intestine may be involved in metal uptake. In the groups exposed to aluminium alone (AlL and AlH), no statistically significant differences were found in aluminium concentration in the rest of the body after five weeks of exposure when compared to week 0. However, the aluminium concentrations in the rest of the body of the groups exposed to various mixtures of aluminium and manganese after five weeks of exposure were statistically higher than

the aluminium concentrations in the rest of the body at week 0. This suggests interactions between aluminium and manganese that caused aluminium to accumulate more in the rest of the body over time (week 0 vs week 5). In a study by Yang (2009), where the mixed toxicity of aluminium and manganese was investigated in soybean (*Glycine max)*, aluminium concentrations in roots increased with increasing aluminium concentrations in nutrient solution under independent aluminium treatment. Addition of a dose of manganese increased aluminium accumulation in roots. This does not explain entirely why aluminium accumulation in the rest of the body of woodlice in this study increased in the presence of manganese, but in the absence of literature on the mixture toxicity of aluminium and manganese in *P. scaber*, it can be concluded that the addition of manganese stimulated the bioaccumulation of aluminium in the rest of the body of woodlice. It is also important to highlight the finding by Van Straalen and Donker (1994) that showed that metals are also stored in parts of the rest of the body (exoskeleton and head). This may partly explain the finding in this study where aluminium accumulated more in the rest of the body.

In the rest of the body, the aluminium concentrations in the group exposed to a single low concentration of aluminium (AlL) was statistically lower than the aluminium concentrations of the groups with a mixture of low aluminium and manganese (AlL/MnL and AlL/MnH). The aluminium concentrations were not statistically different between the group exposed to a single high aluminium concentration (AlH) and the groups exposed to a mixture of high aluminium and manganese. It must be noted that, although not statistically, aluminium concentrations were generally higher in the mixed metal exposure groups than in the single high aluminium (AlH) group after 5 weeks of exposure. As indicated earlier in this discussion, different factors such as the ratio of the metals in the mixture and the actual concentrations of the metals in the mixture may affect the degree of the metal interaction (Beyer et al., 1982). In the present study, the effect of manganese on the bioaccumulation of aluminium varied from one mixture exposure group to the next. This may be due to factors as explained by Beyer et al. (1982) above.

3.2.3) Compartmentalisation of aluminium (Hepatopancreas vs rest of the body)

Dallinger and Rainbow (1991) reported that metals accumulated by earthworms, isopods, terrestrial gastropods and spiders are not evenly distributed in the body, which means that some organs and tissues are involved in metal accumulation, while others are not. In most cases, digestive tissues such as gut epithelia or digestive glands, which play a crucial role in the nutrition physiology of these organisms, are the predominant sites of metal accumulation. Differences in accumulation patterns of metals by terrestrial invertebrates are probably caused by differences in metal kinetics in the organism and the exposure route (Heikens et al., 2000). A metal dependent increase in body concentration was observed by Heikens et al. (2000). In their study, Heikens et al. (2000) found that isopods presented a higher metal concentration than other soil invertebrates. It was also found in the same study that the body concentrations increased with increasing soil concentrations for lead, cadmium and copper in most terrestrial invertebrates. Aluminium appears to be regulated and kept at low internal concentrations compared to environmental concentrations such as in the earthworm *Dendrobaena octaedra* (Holmstrup et al., 2010). The same tendency can be seen in the woodlice of the present study.

The aluminium concentrations in the hepatopancreas samples were compared to the aluminium concentrations in the rest of the body in all the exposure groups after five weeks of exposure. In the groups exposed to aluminium alone (AlL and AlH), no statistically significant differences were found between the hepatopancreas and the rest of the body. Most of the groups exposed to mixtures of aluminium and manganese showed statistically significant differences between aluminium concentrations in the hepatopancreas and the rest of the body, with the rest of the body exhibiting higher concentrations. To explain this, it is important to understand the dynamics of metals and the functioning of the digestive system in isopods. The hepatopancreas of isopods is the main organ where metals normally accumulate and although it accounts for only 5% of body weight it can contain up to 75–95% of the metals accumulated (Mazzei et al., 2014). The digestive system of *P. scaber* is divided into five regions: the foregut, the anterior chamber, the papillate region, the rectum and the hepatopancreas (Van Straalen and Donker, 1994). Except for the

hepatopancreas, all components of the digestive system have an ectodermal lining. The hepatopancreas opens in the foregut and consists of four blind-ending tubules. Food passes via the oesophagus to the foregut where it is mixed with digestive enzymes from the hepatopancreas and passes caudate to the hindgut. Digestion takes place in the anterior chamber and digested fluids are forced back in the rostral direction via typhlosole channels in the dorsal wall of the hindgut. These fluids are filtered in the foregut and then pass into the lumen of the hepatopancreas where absorption of nutrients takes place (Hopkin, 1989).

When an animal is given contaminated food, the internal concentration will rise until equilibrium is reached between uptake and elimination (Van Straalen, 1994). Metals are assimilated from the food at rates lower than the efficiency with which the food as a whole is assimilated (Van Straalen and Donker, 1994). After metals have been taken up, they are distributed though the body in a species-specific way. For *P. scaber*, the internal distribution of metals has been investigated because their size allows dissection of the different organs. It appears from the study by Van Straalen and Donker (1994) that the hepatopancreas is by far the most important organ for the storage or metals. Although it represents only 5% of the dry weight of the animal, it may contain something like 75% of the zinc, 95% of the calcium, 80% of the lead, and 85% of the copper in the whole body of an isopod (Hopkin and Martin, 1982). Van Staalen and Donker (1994) advise that these figures by Hopkin and Martin (1982) are not constant as there are differences between species and there are also differences between metal-adapted and non-adapted populations of *P. scaber*. Other parts of the body contributing to metal storage are the exoskeleton and the head (Van Straalen and Donker, 1994). These studies by Van Staalen and Donker (1994) and Hopkin and Martin (1982) did not investigate the toxicity of aluminium in *P. scaber*, neither did they take into account mixture toxicity of the metals. From the study by Van Staalen and Donker (1994), we understand that detoxification only happens when the metal uptake have reached a certain level of bioaccumulation in the animal. The fact that there were no statistically significant differences in the bioaccumulation of aluminium between the hepatopancreas and the rest of the body of *P. scaber* in the present study when they were exposed to single concentrations of aluminium could be an indication of the regulation process used by woodlice when the level of contaminant reaches a certain threshold.

For the groups exposed to mixtures of aluminium and manganese, the aluminium concentrations in the hepatopancreas samples were mostly statistically lower than the aluminium concentrations in the rest of the body. When a low concentration of manganese was mixed with a low concentration of aluminium (AlL/MnL), aluminium accumulated in the rest of the body more than the hepatopancreas. However, when a high concentration of manganese was mixed with a low concentration of aluminium (AlL/MnH), there were no statistical differences between the aluminium concentrations in the hepatopancreas and the aluminium concentrations in the rest of the body. The presence of manganese clearly stimulated the bioaccumulation of aluminium in woodlice particularly in the rest of the body. Manganese did not have this stimulating effect on aluminium bioaccumulation in the hepatopancreas. The reasons for this phenomenon seems to be unclear, since there is a scarcity of literature on aluminium and manganese in woodlice and their effects on each other's bioaccumulation.

The individuals with low levels of aluminium bioaccumulation in the hepatopancreas might have eliminated most of the metal ingested through a detoxification mechanism at the time they were sampled at week 5. As much as woodlice are good accumulators of metals, they can also efficiently regulate metal concentrations in the hepatopancreas (Woodburn et al., 2011).

Elangovan et al. (2000) found that aluminium entering the digestive gland lumen of the freshwater snail, *Lymnaea stagnalis*, follows the same pathway as food and other ingested particles. Elongovan et al. (2000) also reported that up to 40% of the aluminium accumulated by *L. stagnalis* is incorporated into the digestive gland. In ecotoxicological studies, there are different classes of metals. 'Class A' metals such as aluminium is known for having a preference for binding with ligands with oxygen as the donor atom (Nieboer & Richardson, 1980). Metal detoxification in 'class A' metals happens by incorporating into concentrically layered type A granules (Hopkin, 1986) with a high inorganic content and localized in the calcium cells. Although aluminium is a 'class A' metal, large amounts can also be stored in type B granules as was previously found in molluscs (Brooks & White, 1995). Aluminium was also previously found in excretory granules of a land snail (Brooks & White, 1995). Aluminium in the hepatopancreas of woodlice in the present study could have been partly regulated by means of similar excretory granules. This polyvalence of

aluminium in the digestive system of invertebrates gives an indication on its behaviour in woodlice. In this study it is unclear why aluminium unlike many other metals accumulated more in the rest of the body than in the hepatopancreas. From the literature and the result of this study, it is evident that aluminium bioaccumulation does not follow a specific rule. The interpretation of aluminium bioaccumulation results in woodlice is complicated by the lack of previous studies on aluminium bioaccumulation as well as aluminium and manganese interactions in woodlice. Mixture toxicity of metals in terrestrial invertebrates has previously been investigated (Lock and Janssen, 2001; Van Gestel and Hensbergen, 1997; Odendaal and Reinecke, 2003). However, when it comes to mixture toxicity of aluminium and manganese, very few investigations were found. Those found focused on plants (Blair and Taylor, 1996; Yang, 2009).

CHAPTER FOUR: RESULTS AND DISCUSSION: MANGANESE

4.1) Results

4.1.1) Comparison of manganese concentrations in the hepatopancreas at week 0 and week 5

The manganese concentrations in the hepatopancreas of week 0 woodlice were compared to the manganese concentrations in the hepatopancreas of week 5 woodlice (Table 7).

There were no statistically significant differences found in the hepatopancreas when the manganese concentrations of H0 were compared to the manganese concentrations of H5 for the control group (P = 0.151); the group with MnL (P = 0.151), the group with MnH (P = 0.222), the group with AlL/MnL (P = 0.190), the group with AlH/MnL (P = 0.222), the group with AlL/MnH (P = 0.421) and the group with AlH/MnH (P = 0.190) (Table 7).

Table 7: The mean manganese concentrations (mg/kg) ±SD in the hepatopancreas of week 0 woodlice (N = 5) and in the hepatopancreas of week 5 woodlice (N = 5) for all the exposure groups.

		DIFFERENT EXPOSURE GROUPS						
		ctrl	MnL	MnH	AlL/MnL	AlL/MnH	AlH/MnL	AlH/MnH
Calculated Concentrations			**150**	**300**	450/**150**	450/**300**	2000/**150**	2000/**300**
1) H0	**Mean**	**^{a}76.87**	**^{a}76.87**	**^{a}76.87**	**^{a}76.87**	**^{a}76.87**	**^{a}76.87**	**^{a}76.87**
	SD	99.28	99.28	99.28	99.28	99.28	99.28	99.28
2)H5	**Mean**	**^{a}2.62**	**^{a}3.14**	**^{a}3.93**	**^{a}1.89**	**^{a}13.4**	**^{a}6.65**	**^{a}1.01**
	SD	2.34	2.93	3.52	1.71	12.52	9.52	0.53

Statistical significant differences between H0 and H5 are indicated with different superscripted letters.

4.1.2) Comparisons of manganese concentration in the rest of the body at week 0 and week 5

The manganese concentrations in the rest of the body of week 0 woodlice were compared to the manganese concentrations in the rest of the body of week 5 woodlice (Table 8).

Statistical comparisons between R0 and R5 showed statistically significant differences in the manganese concentrations for all the exposure groups: ctrl (P = 0.008), MnL (P = 0.008), MnH (P = 0.008), AlL/MnL (P = 0.008), AlL/MnH (P = 0.008), AlH/MnL (P = 0.008), AlH/MnH (P = 0.016).

Table 8: The mean manganese concentrations (mg/kg) ±SD in the rest of the body of week 0 woodlice (N = 5) and for the rest of the body of week 5 woodlice (N = 5) for all exposure groups.

		DIFFERENT EXPOSURE GROUPS						
		ctrl	MnL	MnH	AlL/MnL	AlL/MnH	AlH/MnL	AlH/MnH
Calculated Concentrations			**150**	**300**	450/**150**	450/**300**	2000/**150**	2000/**300**
1) R0	**Mean**	**^{a}1.04**	**^{a}1.04**	**^{a}1.04**	**^{a}1.04**	**^{a}1.04**	**^{a}1.04**	**^{a}1.04**
	SD	1.18	1.18	1.18	1.18	1.18	1.18	1.18
2)R5	**Mean**	**^{b}26.03**	**^{b}22.91**	**^{b}56.26**	**^{b}41.82**	**^{b}52.00**	**^{b}32.49**	**^{b}48.56**
	SD	14.24	5.89	29.72	19.64	11.8	16.32	6.4

Statistical significant differences between R0 and R5 are indicated with different superscripted letters.

4.1.3) Comparison of manganese concentrations in the hepatopancreas and the rest of the body after five weeks of exposure

Manganese concentrations in the hepatopancreas after exposure were compared to the manganese concentrations in the rest of the body for all the exposure groups (Table 9, Figure 2).

Statistically significant differences were found between the mean manganese concentrations in the hepatopancreas and the rest of the body after 5 weeks of exposure for all the exposure groups: Ctrl ($P = 0.007$); MnH ($P = 0.016$); MnL ($P = 0.001$); AlL/MnL ($P = 0.016$); AlH/MnL ($P = 0.016$); AlL/MnH ($P = 0.001$); AlH/MnH ($P = 0.029$). The manganese concentrations were always higher in the rest of the body than in the hepatopancreas.

Table 9: The mean manganese concentrations (mg/kg) ±SD in the hepatopancreas and the rest of the body samples of woodlice after 5 weeks of exposure to various concentrations (N = 35).

		DIFFERENT EXPOSURE GROUPS						
		ctrl	MnL	MnH	AlL/MnL	AlL/MnH	AlH/MnL	AlH/MnH
Calculated Concentrations			**150**	**300**	450/**150**	450/**300**	2000/**150**	2000/**300**
1) H5	**Mean**	**[a]2.62**	**[a]3.14**	**[a]3.93**	**[a]1.89**	**[a]13.4**	**[a]6.65**	**[a]1.01**
	SD	2.34	2.93	3.52	1.71	12.52	9.52	0.53
2) R5	**Mean**	**[b]26.03**	**[b]22.91**	**[b]56.26**	**[b]41.82**	**[b]52.00**	**[b]32.49**	**[b]48.56**
	SD	14.24	5.89	29.72	19.64	11.80	16.32	6.40

Statistical significant differences between H5 and R5 are indicated with different superscripted letters.

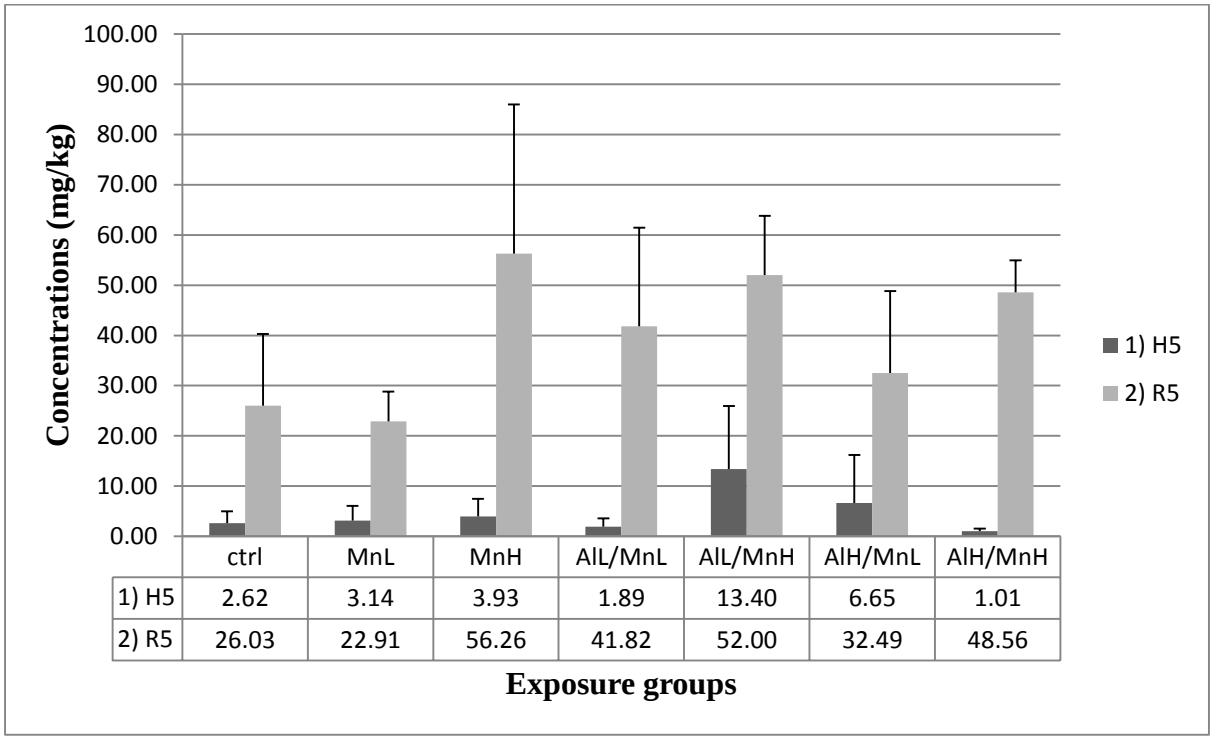


Figure 2: The mean manganese concentrations (mg/kg) ±SD in the hepatopancreas and rest of the body samples at week 5 for all exposure groups (N=35).

4.1.4) Comparisons of single metal exposure groups (MnL and MnH)

Manganese concentrations in the hepatopancreas and in the rest of the body of the woodlice exposed to manganese alone (MnL and MnH) were compared after 5 weeks of exposure (Table 10).

Statistical comparisons of the manganese concentrations in the hepatopancreas showed no statistically significant differences between the control group and MnL (P = 0.548), between the control group and MnH (P = 0.310) or between MnL and MnH (P = 0.548).

In the rest of the body, the manganese concentrations also showed no statistical differences between the control group and MnL (P = 0.590), between the control group and MnH (P = 0.095) or between MnL and MnH (P = 0.151).

Table 10: The mean aluminium concentrations (mg/kg) ±SD in the hepatopancreas and the rest of the body of woodlice exposed to manganese only (MnL and MnH) (N=15).

		EXPOSURE GROUPS		
		ctrl	MnL	MnH
Calculated Concentrations			**150**	**300**
1) H5	**Mean**	**2.62[a]**	**3.14[a]**	**3.93[a]**
	SD	2.34	2.93	3.52
2) R5	**Mean**	**26.03[1]**	**22.91[1]**	**56.26[1]**
	SD	14.24	5.89	29.72

Statistical significant differences between the different exposure groups are indicated by different superscripted letters (for hepatopancreas) and numbers (for rest of the body).

4.1.5) Effect of the presence of aluminium on manganese bioaccumulation in hepatopancreas samples in the MnL exposure groups

The hepatopancreas manganese concentrations of the exposure groups with a single low manganese concentration (MnL) were compared to the manganese

concentrations of the exposure groups with a mixture of aluminium and manganese, containing a low concentration of manganese (AlL/MnL, AlH/MnL) (Table 11).

Statistical comparisons showed no significant differences in the manganese concentrations in the hepatopancreas between MnL and the following groups: Ctrl (P = 0.548), AlL/MnL (P = 0.413) and AlH/MnL (P = 0.841).

4.1.6) Effect of the presence of aluminium on manganese bioaccumulation in the rest of the body in the MnL exposure groups

The manganese concentrations in the rest of the body of the groups exposed to a single low manganese concentration (MnL) was compared to the manganese concentrations in the rest of the body of the groups exposed to a mixture of aluminium and manganese, containing a low concentration of manganese (AlL/MnL, AlH/MnL) (Table 11).

No statistically significant differences were found when the manganese concentrations in the rest of the body in the MnL group were compared to the control group (P = 0.690), to the AlL/MnL group (P = 0.151) or to the AlH/MnL group (P = 0.310).

Table 11: The mean manganese concentrations (mg/kg) ±SD in the hepatopancreas and rest of the body of woodlice exposed to MnL, AlL/MnL and AlH/MnL (N = 20).

		DIFFERENT EXPOSURE GROUPS			
		ctrl	MnL	AlL/MnL	AlH/MnL
Calculated Concentrations			**150**	450/**150**	2000/**150**
1) H5	**Mean**	**2.62**	**3.14**	**1.89**	**6.65**
	SD	2.34	2.93	1.71	9.52
2) R5	**Mean**	**26.03**	**22.91**	**41.82**	**32.49**
	SD	14.24	5.89	19.64	16.32

Statistically significant differences from ctrl = a, MnL = b, AlL/MnL = c, AlH/MnL = d.

4.1.7) Effect of the presence of aluminium on manganese bioaccumulation in hepatopancreas samples in the MnH exposure groups

The hepatopancreas manganese concentrations of the group exposed to a single high manganese concentration (MnH) were compared to the manganese concentrations of the groups with a mixture of aluminium and manganese, containing a high concentration of manganese (AlL/MnH, AlH/MnH) (Table 12).

Mean manganese concentrations in the hepatopancreas showed a statistically significant difference between MnH and AlH/MnH ($P = 0.016$). Manganese concentrations in the hepatopancreas showed no statistically significant difference between MnH and AlL/MnH ($P = 0.095$). No statistically significant differences were also found between the control group and MnH ($P = 0.310$).

4.1.8) Effect of the presence of aluminium on manganese bioaccumulation in the rest of the body in the MnH exposure groups

The manganese concentrations in the rest of the body of the group exposed to a single high manganese concentrations (MnH) were compared to the manganese concentrations in the rest of the body of the groups exposed to a mixture of aluminium and manganese, containing a high concentration of manganese (AlL/MnH, AlH/MnH) (Table 12).

No statistically significant differences were found between MnH and the following groups: control ($P = 0.095$), AlL/MnH ($P = 0.310$) and AlH/MnH ($P = 0.190$).

Table 12: The mean manganese concentrations (mg/kg) ±SD in the hepatopancreas and rest of the body of woodlice exposed to MnH, AlL/MnH and AlH/MnH (N = 20).

		DIFFERENT EXPOSURE GROUPS			
		ctrl	MnH	AlL/MnH	AlH/MnH
Calculated Concentrations			**300**	450/**300**	2000/**300**
1) H5	**Mean**	**2.62**	**3.93[d]**	**13.4**	**1.01[b]**
	SD	2.34	3.52	12.52	0.53
2) R5	**Mean**	**26.03**	**56.26**	**52.00**	**48.56**
	SD	14.24	29.72	11.8	6.4

Statistically significant differences from ctrl = a, MnH = b, AlL/MnH = c, AlH/MnH = d.

4.2) Discussion

4.2.1) Bioaccumulation of manganese in the hepatopancreas

In this study, the manganese concentrations in the hepatopancreas of *P. scaber* exposed for 5 weeks did not show statistically significant differences when compared to the manganese concentrations in the hepatopancreas of week 0 individuals. For both the hepatopancreas of week 0 and week 5 woodlice, the manganese concentrations measured were relatively low. On average, the lowest manganese concentrations in the hepatopancreas were recorded in week 5 woodlice. However, the concentrations in the hepatopancreas of week 0 woodlice were very variable, resulting in no statistical difference between H0 and H5. Hopkin (1989) reported that the hepatopanceas samples of *P. scaber* collected from the spoil tips of disused mining areas contained more than 1.5% copper on a dry weight basis, while for *Oniscus asellus* the concentrations of zinc, cadmium, lead and copper in the hepatopancreas of individuals of this species from sites contaminated with metals may exceed 1.2%, 0.4%, 2.5% and 3.4% of the dry weight, respectively, with no apparent ill effect. We understand (as stated in chapter 3) that the hepatopancreas is the primary organ of accumulation of metals in woodlice. While zinc, cadmium, lead and copper have been widely studied in woodlice and proportions of accumulation in the hepatopancreas is known, it is unknown what proportions and concentrations of manganese can be stored by the hepatopancreas. It may be incorrect at this stage to state that the concentrations of manganese observed in the hepatopancreas of woodlice in this study are normal. However, we can notice that as the concentrations of manganese increased in the food source, there were a tendency for manganese concentrations in the hepatopancreas to decrease. In a study by Raessler et al. (2005) the skin of *P. scaber* collected after moult contained 142 mg/kg manganese, while the animal itself contained only 77.6 mg/kg of manganese. This led to the conclusion that moulting is a means of manganese decontamination in *P. scaber*. From this experience, we can say that the tendency of manganese to decrease in the hepatopancreas when concentrations increased in the food source in this study can be seen as part of a detoxification mechanism developed by woodlice to eliminate surplus manganese that is not needed for their metabolism when excessive concentrations are taken up. The presence of calcium and magnesium in

the exoskeleton of woodlice might account for the deposition of metals in this tissue. Woodlice use their exoskeleton as a storage-excretion device with calcium being moved out of the carapace at each moult for retention and reutilisation (Beeby, 1991). The moulting cycle was not taken into account in this study. This phenomenon may also explain the higher manganese concentrations in the rest of the body, since it included the exoskeleton.

Manganese belongs to the group of metals considered as essential for life. They have to be supplied to an organism but excess concentrations may be harmful (Protasowicki et al., 2013). It is also known that essential metals such as manganese are regulated at the individual level, while for non-essential metals such as mercury (Hg), cadmium (Cd) and silver (Ag) there is only weak evidence of controls on accumulation (Gibson et al., 2006). Under constant ambient conditions, the net balance between inward and outward fluxes of metals provides the underlying control on tissue burdens and, in general, metals that exchange rapidly (metals that are regulated) tend to be accumulated less efficiently than metals that exchange slowly (Gibson et al., 2006). Manganese is a micronutrient that may be toxic at high concentrations. It is therefore, regulated efficiently in woodlice, explaining the rather low concentrations in the hepatopancreas.

Manganese concentrations in the hepatopancreas of the group exposed to a low concentration of manganese (MnL) did not show a statistically significant difference when compared to the groups exposed to mixtures of low manganese concentrations and aluminium (AlL/MnL, AlH/MnL). In a study by Musibono (1998) it was reported that when an organism is chronically exposed to a low concentration of a pollutant, the organism will inevitably take up some of the pollutant through ordinary metabolic processes such as feeding, filtering through the gills or digestion. But if the pollutant is not biodegradable (as in the case of manganese), its concentration in the body will increase up to the first signal of danger. The low manganese exposure concentration in this study may not have been high enough to present any danger to the woodlice, hence the low concentrations observed in the hepatopancreas of woodlice exposed to this concentration. Also, since manganese is an essential micronutrient with a regulatory mechanism, the manganese may have simply been regulated to the low concentration measured in the hepatopancreas. Once the first signal of danger is present, the organism may develop a protective

response (such as stopping to feed). It cannot maintain this state for long (it needs to feed), therefore, the pollutant will again enter the body. The concentration of the pollutant in the animal body will increase and will either lead to death or to survival if they have long-term protective mechanisms like excretion or storage in a non-toxic form. In a study by Musibono (1998), it was noticed that when the freshwater amphipod, *Paramelita nigroculus* was exposed to low concentrations of aluminium and manganese mixed in acidic waters (90 mg/l Al and 93.8 mg/l Mn) the mixture was not acutely toxic as 61% of *P. nigroculus* survived after 96 hours. The same study showed that when *P. nigroculus* were exposed to a mixture of aluminium, copper and manganese, manganese and copper predominated as Mn^{2+} and Cu^{2+} while aluminium present as Al^{3+} was removed from the solution as diaspore and Cu^{2+} and Mn^{2+} were absorbed into the surface of the diaspore surface, reducing the bioavailable concentration of the metal species and therefore the toxicity. Musibono (1998) further explained that Mn (II) allows the the precipitation of Al (III), which explains why the combination of aluminium, manganese and copper is least toxic. The mixture of low aluminium and low manganese in this study may have not been toxic enough to create any toxic hazard to woodlice. That is why the concentrations of manganese in the hepatopancreas of the group exposed to a low concentration of manganese (MnL) was not statistically different from the group exposed to a mixture of low manganese and low aluminium (AlL/MnL).

Beeby (1991) reported that isopods need copper for haemocyanin production but will store far more in the hepatopancreas than their respiratory needs. The antagonistic nature of the reaction between copper and manganese (Sunda et al., 1983) suggest a competition between these metals for a critical intracellular site within the hepatopancreas. This led to the finding by Alikhan (1989) that manganese assimilation could be affected by copper. It therefore helps to explain the low accumulation of manganese in the hepatopancreas of *P. scaber* in this study, providing an understanding that manganese accumulation in the hepatopancreas can be subject to competition with copper, which is more important for woodlice because of its role in the production of heamocyanin.

Manganese concentrations in the hepatopancreas of the group exposed to a high manganese concentration (MnH) were compared to the groups exposed to a mixture of aluminium and high manganese (AlL/MnH and AlH/MnH). It appeared that in the

hepatopancreas, manganese concentrations increased slightly (but not statistically significantly) between the group with the high manganese concentration exposure (MnH) and the group with a mixture of high manganese and low aluminium (AlL/MnH). Manganese concentrations decreased significantly between the MnH group and the AlH/MnH group, with the lowest concentrations recorded in the hepatopancreas of the AlH/MnH group. Musibuno (1998) found that combined toxicity of aluminium, manganese and copper in a freshwater amphipod with regards to mortality followed the following order: copper + manganese > aluminium + copper > aluminium + manganese > aluminium + copper + manganese. Musibuno (1998) further discussed that the interactions for aluminium + manganese and aluminium + copper + manganese were antagonistic. Chemical speciation showed that Mn (II) allowed the precipitation of Al (III) as the mineral diaspore (aluminium monohydrate), which adsorbed free metal ions Cu (II) and Mn (II), thus reducing the bioavailability of all three ions and therefore decreasing toxicity. Vijver et al. (2010) after reviewing the toxicity of metal mixtures, concluded that the predominant modes of action of metal mixtures were antagonism and synergism, irrespective of the organism and specific environment tested. Son et al. (2016) added that in many cases, the presence of one chemical can influence the toxicokinetics of another chemical. The result of this study and knowing that copper is present as part of the heamocyanin in the hepatopancreas of woodlice as shown earlier, clearly showed that the presence of aluminium in the mixture influenced the bioaccumulation of manganese in the hepatopancreas, with the higher aluminium exposure concentrations inducing lower bioaccumulation of manganese in the hepatopancreas.

4.2.2) Bioaccumulation of manganese in the rest of the body

Manganese concentrations in the rest of the body of week 0 woodlice were generally significantly lower compared to the manganese concentrations in the rest of the body of week 5 woodlice in all the exposure groups. It is therefore evident that manganese accumulated in the rest of the body over the exposure period. Niemiec and Wiśniowska-Kielian (2015) studied the bioaccumulation of manganese in the larvae of *Diptera* (family *Chironomidae*). They reported that manganese is most easily

absorbed by the skin, gills and intestinal epithelium. The same study makes mention of manganese concentrations in muscles of sea fish from the Black Sea and a very high concentration of manganese in *Danio rerio* fish carcasses, depending on the concentration of manganese in the feed. From these findings, we can understand that the increase of the manganese concentrations between week 0 woodlice and week 5 woodlice indicates that manganese accumulates preferentially in the body of woodlice as the concentration increases in the food source. It was found by Musibono (1998) that copper and manganese were present predominantly as Cu^{2+} and Mn^{2+} in freshwater amphipods. Furthermore, Musibono (1998) found that the combination of manganese and copper was less toxic than the combination of copper and aluminium, aluminium and manganese, or all three metals combined. Musibono (1998) demonstrated that manganese was less toxic to the amphipod than aluminium or copper. Amphipods are physiologically close to isopods as they are both crustaceans, therefore it can be assumed that manganese would be less toxic to woodlice as well. This said, it can therefore be concluded that in the present study, manganese toxicity probably did not pose a major threat to woodlice hence its bioaccumulation in the rest of the body samples. This finding can also be explained by the fact that manganese accumulates in the exoskeleton (as shown earlier) as it will easily be eliminated when the woodlice moult.

In a study by Stanek et al. (2014) where metal bioaccumulation and distribution in the Spiny-Cheek Crayfish was studied, it was discovered that manganese accumulated in the internal body and the exoskeleton of the crayfish more than many other metal. The following order of metal accumulation in the internal body and the exoskeleton of the crayfish was established: in the internal body Zn > Mn > Cu > Ni > Pb > Cr >Co > Cd > Hg and in the exoskeleton Mn > Zn > Ni >Pb > Co > Cu > Cd > Cr > Hg. Stanek et al. (2014) also found that mean concentrations of manganese was 5.93 times higher (and statistically significant) in the exoskeleton than the internal body in crayfish from Lake Goplo in Poland and 6.66 times higher in the exoskeleton than in the internal body in the crayfish, *Astacus leptodactylus* from the Aras Dam in Iran. Stanek et al. (2014) argued that a high concentration of manganese in the exoskeleton may be related to its limited ability to complement or substitute Mg^{2+}; this could also probably stem from its chemical similarity to Ca^{2+} where manganese is a metal that is able to substitute the calcium in $CaCO_3$,

which leads to an accumulation during the calcification of the exoskeleton. Furthermore, Alikhan (1989) suggested that since trace metals like zinc, cadmium, copper, magnesium and manganese pass across cell membranes more than a million times faster than would sodium, potassium and calcium, non-essential elements as well as essential metals, which are surplus to requirements, must be rapidly excreted, or stored in an insoluble form to prevent them from diffusing throughout the body to interfere with biochemical reactions within the tissues. The presence of manganese within the exoskeleton and the hepatopancreas S cells as suggested by Alikhan (1989) indicates that isopods strictly regulate manganese concentrations in the haemolymph by controlling the amount of metal that precipitates between the two tissues. While these findings clearly shows that manganese accumulates preferably in the exoskeleton of crayfish, they could also explain why in the present study, manganese was found to have accumulated significantly in the rest of the body of woodlice after exposure. It may be that most of the manganese were actually accumulated in the exoskeleton of the rest of the body samples. In a review by Baden and Eriksson (2006) it has been revealed that manganese is found in high concentrations in calcified parts of crustaceans, mostly in the exoskeleton. These authors also reported that manganese incorporated into the exoskeleton has very little impact on the organism and that this metal's concentration in the exoskeleton change during the moult cycle, thus storage of manganese in the exoskeleton is an effective means of detoxification.

In the rest of the body, manganese concentrations did not show statistical differences between the group exposed to a single high manganese concentration (MnH) and the groups exposed to a mixture of high manganese and aluminium (AlL/MnH; AlH/MnH). Musibono (1998) found after exposing the amphipod *P. nigroculus* to various concentrations of aluminium, manganese and copper, that at high concentrations, bioaccumulation of aluminium and copper was not different but the bioconcentration factor for manganese was about 1/7 of that of aluminium and copper, suggesting that *P. nigroculus* may be a good accumulator of aluminium, a weak accumulator of copper and a poor accumulator of manganese under the mixed metal exposure conditions. Furthermore, Musibono (1998) argued that the interactions of aluminium and manganese were antagonistic. In a study where woodlice were exposed to a mixture of manganese and magnesium, Alikhan (1989)

found that male woodlice (*Porcellio spinicornis*) accumulated 3.33% of manganese in the exoskeleton at 150 ppm dietary manganese against 3.8% in the control and females accumulated 4.85% at the same dietary concentration against 4.02% in the control group. These concentrations were significantly lower than those recorded for manganese in the same exposure conditions (Joosse and Van Vliet,1984). Alikhan (1989) argued that this may imply that most of the ingested manganese unlike magnesium, does not exist as a free ion, but is tightly bound either to a metallothionein or a methalothionein-like protein. Alikhan (1989) additionally argued that manganese as suggested by Stauber and Florence (1985), may have been affected by the presence of the free magnesium ion. In the group exposed to a mixture of high aluminium and manganese in the present study, manganese may have been affected by the free aluminium and this may explain why there were no statistically significant differences between the group exposed to high manganese and the groups exposed to mixtures of high manganese and aluminium.

As stated earlier in the introduction chapter, Altenberger et al. (2013) acknowledged a situation of a limited database for mixture toxicity studies. These shortcomings in the field of mixture toxicity are: several specific aspects relevant to an adequate exposure assessment of biologically active compounds; technical problems to be encountered when trying to perform mixture assessments with the data available in current documentations for product authorization; and notwithstanding that a lack of reliable empirical indicators for mixture synergism based on the individual components' effects is to be acknowledged. Altenberger et al. (2013) concluded that provisions to account for a lack of data or conceivable interactive effects may well be taken when dealing with resulting uncertainties.

4.2.3) Compartmentalisation of manganese (Hepatopancreas vs rest of the body)

Manganese concentrations in the hepatopancreas of *P. scaber* were compared to manganese concentration in the rest of the body after 5 weeks of exposure. In all the exposure groups, manganese concentrations in the hepatopancreas were statistically lower than the manganese concentrations in the rest of the body. It was earlier determined that woodlice are very efficient in storing metals in the

hepatopancreas, resulting in low metal concentrations in the body fluid (Martina et al., 2004). The hepatopancreas of woodlice contains high-density granules to which metals are bound and this increases its storage capacity. When the metal storage capacity of these granule-containing hepatopancreas cells is exceeded or when storage cannot keep up with uptake rate, woodlice will suffer from metal toxicity (Martina et al., 2004). Furthermore, it was found by Hopkin et al. (1986) that *P. scaber* from an uncontaminated site accumulated considerable concentrations of zinc and copper in their hepatopancreas. It is also known that manganese was proven not to pose a significant threat to a freshwater amphipod and accumulated more in the exoskeleton (Musibono, 1998). From these findings, we can deduce that the reaction of woodlice towards metals is that those that pose a toxicological threat to them are stored in the hepatopancreas preferentially, and those that do not pose a major toxicological threat to them can be stored in the rest of the body more specifically in the exoskeleton. Musibuno (1998) established that the combination of aluminium and manganese in the freshwater amphipod resulted in an antagonistic reaction between those metals. This antagonistic reaction was even enhanced in the presence of copper. It was established earlier in this chapter that copper is present in the hepatopancreas of woodlice as part of heamocyanin. Stauber and Florence (1985) suggested that the assimilation of manganese by woodlice may be affected by the presence of copper in the hepatopancreas. The combination of these factors may explain why manganese concentration in the hepatopancreas was significantly lower than in the rest of the body in all the exposure groups.

The concentrations of manganese in this study within the hepatopancreas and the rest of the body did not always vary proportionally to the manganese concentration in the food source. In certain cases, the manganese concentrations in the rest of the body and the hepatopancreas varied independently and decreased with increasing concentrations in the food source. In other cases, it varied the opposite way. It has been clearly shown in a study by Hopkin et al. (1986) that the determination of concentrations of zinc, cadmium, lead and copper in soil and leaf litter does not enable the concentrations of these metals in woodlice to be accurately predicted. In the same study, Hopkin et al. (1986) also found a presence of considerable concentrations of zinc and copper in the hepatopancreas of *Porcellio scaber* from uncontaminated sites. This suggests that non-essential metals are preferably

accumulated in the hepatopancreas as this ensures toxicological safety to woodlice. Musibono (1998) also reported that to reduce metal uptake, some species have developed various short-term adaptations such as avoiding contact with external sources or with contaminated food sources and so temporarily preventing uptake of metals. These short-term strategies cannot, however, explain the entire regulation process. The present study showed that manganese, being an essential element for woodlice, and presenting no major no toxicological hazard (Alikhan, 1989; Musibono, 1998), accumulated preferably in the rest of the body, as compared to the hepatopancreas.

Bordean et al. (2014) demonstrated that the transfer of manganese from soils to terrestrial gastropods occurs independently of food ingestion. However, the ingestion of food is expected to serve as the main route of manganese exposure, but Bordean et al. (2014) emphasises that the direct transfer from soils to snails should not be neglected when precisely assessing the impact of anthropogenic manganese releases on soil ecosystems. It has also been demonstrated that accumulated metal (whole body concentrations) may be poorly, or even negatively, correlated with toxicity (Winner, 1984), and that organisms that tend to bioaccumulate metals to high levels, do so because they are able to store the metals in non-toxic forms (i.e., in granules, or bound to metallothioneins) (Rainbow, 2002). This study provided results that were not always expected. In some cases, the rest of the body or hepatopancreas concentrations varied unproportionally with the metal concentrations in the food source. This translates to the ability of the woodlice to store the metals in a non-toxic form or to eliminate the metal almost completely. Ribeiro et al. (2001) discovered that although ingestion is the major exposure route in *Porcellio dilatatus*, direct surface contact may also contribute to exposure. Dallinger and Rainbow (1991) stated that uptake of metals by terrestrial invertebrates usually takes place from food via the gut. Cutaneous uptake which in aquatic invertebrates is of equal or greater importance of uptake than uptake via the gut may occur only occasionally in some groups of terrestrial invertebrates such as soil-dwelling organisms or andoparasitoids. During experiments in this study, woodlice were usually hidden under the top layer of leaves. They spent most of the time hidden amongst the moist leaves. In this manner, metal uptake via the water pores of the woodlice body was a significant possibility during exposure. It may be that cutaneous uptake also

contributed to the bioaccumulation of manganese in the rest of the body of *P. scaber* in the present study.

CHAPTER FIVE: CONCLUSION

Objective 1

- **To compare how aluminium and manganese are bioaccumulated in *Porcellio scaber* in terms of the contribution of the hepatopancreas in metal storage compared to the rest of the body.**

The results obtained in this study showed that no bioaccumulation of aluminium took place in the hepatopancreas. Statistical analysis of aluminium concentrations in the hepatopancreas samples showed no significant differences between week 0 woodlice and week 5 woodlice. Based on previous findings by Alexopoulos et al. (2003) and Woodburn et al. (2011), it was concluded that aluminium taken up by woodlice was efficiently regulated.

In the rest of the body, bioaccumulation of aluminium was not significantly different from that observed in the hepatopancreas although aluminium concentrations were generally higher in the rest of the body. For the groups exposed to single aluminium in low and high concentrations (AlL and AlH), there was no statistical differences between week 0 and week 5 woodlice. Results also showed that there was no difference in the aluminium concentrations between the hepatopancreas and the rest of the body for the groups exposed to single concentrations of aluminium. Findings in a study by Van Straalen and Donker (1994) led to the understanding that in isopods, detoxification only starts when the metal uptake have reached a certain level of bioaccumulation. It was therefore understood in the case of the present study that the fact that there was no statistical difference in the aluminium bioaccumulation between week 0 and week 5 woodlice and between the hepatopancreas and the rest of the body when woodlice were exposed to single concentrations of aluminium may have been due to the fact that aluminium bioaccumulation was regulated by the animals when high concentrations were reached.

It can therefore be concluded that single aluminium exposes do not necessarily pose a major toxicological problem to woodlice. This study showed that woodlice can effectively manage aluminium when they are exposed to it alone.

There was no bioaccumulation observed in the hepatopancreas of woodlice after the 5 week exposure period when compared to week 0 woodlice. In the rest of the body, manganese bioaccumulation was significantly higher in 5 week exposed woodlice as compared to week 0 woodlice. Manganese concentrations were statistically higher in the rest of the body than the hepatopancreas in all the exposure groups. While the moulting cycle was not taken into account in the present study, based on the observed result, it can be concluded that woodlice were able to store manganese in their exoskeleton and get rid of it when moulting occurred. This may be an avenue of further study.

Objective 2:

- **To determine whether mixtures of aluminium and manganese affect each other's bioaccumulation and distribution in *Porcellio scaber*.**

Within the hepatopancreas of woodlice in this study, no statistical differences were found between the groups exposed to single aluminium and the groups exposed to mixtures of aluminium and manganese, except for the AlH/MnL group that showed a statistical difference from AlH. In the manganese exposure groups, there were no statistical differences between the groups exposed to single manganese and the groups exposed to mixtures of aluminium and manganese in the hepatopancreas. As suggested by Beyer et al. (1982), the ratio of the metals in a mixture and the actual concentrations can affect how metals interact. This in the present study led to the conclusion that relatively low manganese concentrations can affect aluminium bioaccumulation in the hepatopancreas.

In the rest of the body, there was a statistical difference when the aluminium concentration of the group exposed to low aluminium concentrations was compared to the groups with mixtures of low aluminium and manganese concentrations, while there was no statistical differences between the group exposed to high aluminium concentration and the groups exposed to high aluminium concentration and the groups with mixtures of high aluminium and manganese concentrations. These variations of the effect of manganese on the aluminium bioaccumulation from one mixture exposure group to the next in this study was caused by different factors such

as the rates of the metals in the mixture and the actual concentrations of the metals in the mixture as suggested by Beyer et al.(1982).

Aluminium bioacculumulation was statistically higher in the rest of the body than the hepatopancreas in the groups exposed to mixtures of aluminium and manganese. The effect of aluminium on manganese bioaccumulation were not so different from the effect of manganese on aluminium bioaccumulation and manganese concentrations in the rest of the body were statistically higher than manganese concentrations in the hepatopancreas in all exposure groups. It was then concluded based on literature that while detoxification only happens when metal uptake have reached a certain level of bioaccumulation in woodlice. It was also shown in previous studies that woodlice can accumulate manganese in the exoskeleton and can be consequently eliminated with the moult.

This study has shed more light on the effects of mixtures of metals in the environment. The findings in this study are however not enough to state that every combination of metals in the environment will always present a higher bioaccumulation hazard. Most outcomes of this study cannot be accurately related to existing published studies. Very few previous studies could be found on the effect of aluminium and manganese on each other's bioaccumulation. Protasowicki et al. (2013) indicated that there are fewer publications on iron, manganese, vanadium, lithium, and aluminium in crustaceans. There is thus an opportunity for future research projects on the influence of metals, such as, aluminium and manganese, on each other's bioaccumulation and toxicity, since in reality the environment is contaminated by cocktails of pollutants. The effects of metal interactions on bioaccumulation over time could also be investigated by future studies.

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