
Histological change in the hepatopancreas of terrestrial isopods as potential biomarker of cadmium and zinc exposure

BY

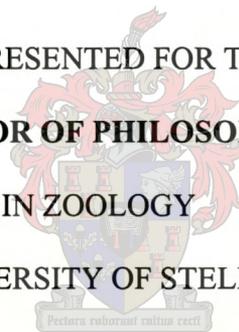
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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Abstract

Metals occur naturally in the environment but since the industrial revolution the amounts of bioavailable metals in the environment have increased significantly. There are various anthropogenic sources of metals in the environment. In recent years there has been an increasing interest in the use of biomarkers in terrestrial invertebrates for the assessment of the potential adverse effects of chemicals in soil ecosystems. Terrestrial isopods are one of the groups that play an important role in the decomposition process as fragmentors of dead plant material in soil ecosystems. They are also known to accumulate contaminants in their bodies, especially in the hepatopancreas. Cadmium has no known biological function and is deposited in the environment through a variety of sources. Zinc on the other hand is one of the essential metals and play an important role in the normal metabolism of animals. Zinc too, is deposited in the environment through a variety of anthropogenic sources. The aim of this study was to undertake an experimental and field evaluation of the role cellular change in the hepatopancreas of terrestrial isopods can play in conjunction with other sublethal responses to contribute to the assessment of the impact of cadmium and zinc in terrestrial ecosystems.

Porcellio laevis was used as test species in the laboratory experiments in this study, to conduct sublethal toxicity tests. Cadmium- and zinc sulphate were used as the contaminants, applied separately and as mixtures. The isopods were weighed before the beginning of the exposures and every week throughout the exposure period of six weeks. At the end of the exposure period they were dissected to remove the hepatopancreas. The zinc and cadmium content of the hepatopancreases and rest of the bodies of the isopods

were determined by atomic absorption spectrophotometry. Hepatopancreas samples were also prepared for histological analysis, and measurements of the Percentage Cellular Area (PCA) were made using image analysis. Specimens of *Porcellionides pruinosus* were collected from contaminated- and uncontaminated field sites. Hepatopancreas- and rest of the body samples of *P. pruinosus* were also analysed for cadmium and zinc. Histological sections of the hepatopancreas were similarly prepared and analysed, as in the laboratory experiments.

Exposure to cadmium- and zinc sulphate affected the mass of *P. laevis* negatively in the single metal exposures. However, these two metals "neutralised" each other's effects in the mixture toxicity exposures, pointing towards an antagonistic interaction. Administered cadmium- and zinc sulphate resulted in the accumulation of cadmium and zinc in the isopods, especially in the hepatopancreas. In the mixture toxicity experiments cadmium and zinc influenced the accumulation of each other. Cadmium- and zinc sulphate, whether administered separately or as mixtures, changed the structure of the hepatopancreas and this was quantitatively shown through PCA measurements. PCA of the hepatopancreas of terrestrial isopods was shown to be a good general biomarker of exposure and effects for cadmium as well as for zinc. It is suggested that PCA could serve as a general biomarker to predict possible impairment of growth or mass change of isopods exposed to cadmium and zinc mixtures. The findings of the field survey also suggest that PCA may be suitable to be used as a general biomarker to measure metal induced stress in woodlice from contaminated field sites.

Uittreksel

Metale kom natuurlik voor in die omgewing, maar sedert die industriële revolusie het die hoeveelheid bio-beskikbare metale in die omgewing betekenisvol toegeneem. Daar is verskeie antropogeniese bronne van metale in die omgewing. Die afgelope paar jaar was daar toenemende belangstelling in die gebruik van biomerkers by terrestriële invertebrate vir die assessering van potensiële negatiewe effekte van chemiese stowwe in grondekosisteme. Terrestriële Isopoda is een van die groepe wat 'n belangrike rol speel in die ontbindingsproses as fragmenteerders van dooie plantmateriaal in grondekosisteme. Hulle akkumuleer ook kontaminante in hul liggame, veral in die hepatopankreas. Kadmium het geen biologiese funksie nie, en word vrygestel in die omgewing deur verskeie bronne. Sink, aan die ander kant, is een van die essensiële metale en speel 'n belangrike rol in die metabolisme van diere. Sink word ook in die omgewing vrygestel deur 'n verskeidenheid antropogeniese bronne. Die doel van die studie was om deur eksperimentele en veldondersoek die rol van sellulêre veranderinge in die hepatopankreas van terrestriële Isopoda in samehang met ander subletale response te evalueer om by te dra tot die assessering van die impak van kadmium en sink op terrestriële ekosisteme.

Porcellio laevis is as toets-spesie in die laboratorium eksperimente gebruik om subletale toksisiteitstoetse mee uit te voer. Kadmium- en sinksulfaat is as kontaminante gebruik, en is apart en gemeng toegedien. Die houtluise is geweeg voor die begin van die blootstellings en elke week deur die loop van die blootstellingsperiode van ses weke. Aan die einde van die blootstellingsperiode is hulle gedissekteer om die hepatopankreas

te verwyder. Die sink en kadmium inhoude van die hepatopankreas en res van die liggame van die houtluise is bepaal met behulp van atoomabsorpsiespektrofotometrie. Monsters van die hepatopankreas is ook voorberei vir histologiese analise, en metings van die Persentasie Sellulêre Oppervlak (PSO) is gedoen met behulp van beeldanalise. Eksemplare van *Porcellionides pruinosus* is versamel van 'n gekontameneerde- en 'n ongekontameneerde gebied. Monster van die hepatopankreas en res van die liggaam van *P. pruinosus* is ook geanaliseer vir kadmium en sink. Histologiese sneë van die hepatopankreas was ook voorberei en geanaliseer, soos in die laboratorium eksperimente.

Blootstelling aan kadmium- en sinksulfaat het die massa van *P. laevis* negatief beïnvloed in die enkelmetaal-eksperimente. Hierdie twee metale het egter mekaar se effekte in die mengseltoksisiteitstoetse "geneutraliseer". Dit dui op 'n antagonistiese interaksie. Toegediende kadmium- en sinksulfaat het gelei tot die akkumulاسie van kadmium en sink in the houtluise, veral in die hepatopankreas. In die mengseltoksisiteitstoetse het kadmium en sink mekaar se akkumulاسie beïnvloed. Kadmium- en sinksulfaat, apart of as mengsels toegedien, het die struktuur van die hepatopankreas verander, en dit is kwantitatief aangetoon deur die PSO metings. PSO van die hepatopankreas van houtluise is 'n bruikbare algemene biomerker van blootstelling en effek vir kadmium en sink. Dit word voorgestel dat PSO kan dien as 'n algemene biomerker om negatiewe effekte op die groei of massaverandering van houtluise te voorspel wat aan kadmium en sink mengsels blootgestel word. Die bevindinge van die veldondersoek dui daarop dat PSO gepas mag wees om gebruik te word as 'n algemene biomerker om metaal-geïnduseerde stres by houtluise vanaf 'n gekontameneerde gebied te meet.

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Figure 13: The mean zinc concentrations (mg.kg^{-1} ; dry mass) ($\pm\text{SD}$) in hepatopancreas samples of *P. laevis* after 6 weeks of exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg^{-1} CdSO_4 with 1000 mg.kg^{-1} ZnSO_4 ; mix2 = 80 mg.kg^{-1} CdSO_4 with 4000 mg.kg^{-1} ZnSO_4 ; mix3 = 160 mg.kg^{-1} CdSO_4 with 8000 mg.kg^{-1} ZnSO_4 . $n=5$. See Table 11 for statistics.

Figure 14: The mean zinc concentrations (mg.kg^{-1} ; dry mass) ($\pm\text{SD}$) in the rest of the body samples (excluding hepatopancreas) of *P. laevis* after 6 weeks of exposure to

various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. n=5. See Table 11 for statistics.

Figure 15: Comparison between cadmium concentrations (mg.kg⁻¹; dry mass) (±SD) in the hepatopancreas of *P. laevis* after exposure to cadmium sulphate (20, 80, 160 mg.kg⁻¹) and exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. Stat. sign. diff. between single metal exposure and joint exposure indicated by (*) (P<0.05). n=5.

Figure 16: Comparison between cadmium concentrations (mg.kg⁻¹; dry mass) (±SD) in the rest of the body samples of *P. laevis* after exposure to cadmium sulphate (20, 80, 160 mg.kg⁻¹) and exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. Stat. sign. diff. between single metal exposure and joint exposure indicated by (*) (P<0.05). n=5.

Figure 17: Comparison between zinc concentrations (mg.kg⁻¹; dry mass) (±SD) in the hepatopancreas of *P. laevis* after exposure to zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) and exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. Stat. sign. diff. between single metal exposure and joint exposure indicated by (*) (P<0.05). n=5.

Figure 18: Comparison between zinc concentrations (mg.kg⁻¹; dry mass) (±SD) in the rest of the body samples of *P. laevis* after exposure to zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) and exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. Stat. sign. diff. between single metal exposure and joint exposure indicated by (*) (P<0.05). n=5

Figure 19: Examples of histological sections, stained with hematoxylin and Eosin, in the Z3 zone of hepatopancreas samples of *P. laevis* after six weeks of exposure to various concentrations of cadmium sulphate. **A:** control; **B:** 20 mg.kg⁻¹ CdSO₄ exposure; **C:** 80 mg.kg⁻¹ CdSO₄ exposure; **D:** 160 mg.kg⁻¹ CdSO₄ exposure. Magnification = X250.

(L = lumen; B = B-cells; S = S-cells.)

Figure 20: Percentage Cellular Area (PCA) measurements (±SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after 6 weeks of exposure to various concentrations of cadmium sulphate, with **A:** control; **B:** 20 mg.kg⁻¹; **C:** 80 mg.kg⁻¹; **D:** 160 mg.kg⁻¹. n=40. See Table 14 for statistics.

Figure 21: PCA measurements (±SD) of hepatopancreas sections, within a particular zone (Z1-Z4), of cadmium sulphate exposed groups (20, 80 and 160 mg.kg⁻¹), with **A:** Z1; **B:** Z2; **C:** Z3; **D:** Z4. n=40. See Table 14 for statistics.

Figure 22: Examples of histological sections, stained with hematoxylin and eosin, in the Z3 zone of hepatopancreas samples of *P. laevis* after six weeks of exposure to various concentrations of zinc sulphate. **A:** control; **B:** 1000 mg.kg⁻¹ ZnSO₄ exposure; **C:** 4000 mg.kg⁻¹ ZnSO₄ exposure; **D:** 8000 mg.kg⁻¹ ZnSO₄ exposure. Magnification = X250.

(L = lumen; B = B-cells; S = S-cells.)

Figure 23: Percentage Cellular Area (PCA) measurements (±SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after 6 weeks of exposure to various concentrations of zinc sulphate, with **A:** control; **B:** 1000 mg.kg⁻¹; **C:** 4000 mg.kg⁻¹; **D:** 8000 mg.kg⁻¹. n=40. See Table 15 for statistics.

Figure 24: PCA measurements (±SD) of hepatopancreas sections, within a particular zone (Z1-Z4), of zinc sulphate exposed groups (1000, 4000 and 8000 mg.kg⁻¹), with **A:** Z1; **B:** Z2; **C:** Z3; **D:** Z4. n=40. See Table 15 for statistics.

Figure 25: Examples of histological sections, stained with hematoxylin and eosin, in the Z3 zone of hepatopancreas samples of *P. laevis* after six weeks of exposure to various mixtures of cadmium- and zinc sulphate. **A:** control; **B:** mixture of 20 mg.kg⁻¹ CdSO₄ and 1000 mg.kg⁻¹ ZnSO₄; **C:** mixture of 80 mg.kg⁻¹ CdSO₄ and 4000 mg.kg⁻¹ ZnSO₄; **D:** mixture of 160 mg.kg⁻¹ CdSO₄ and 8000 mg.kg⁻¹ ZnSO₄. Magnification = X250.

(L = lumen; B = B-cells; S = S-cells.)

Figure 26: Percentage Cellular Area (PCA) measurements (±SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after 6 weeks of exposure to various mixtures cadmium- and of zinc sulphate, with **A:** control; **B:** mix1; **C:** mix2; **D:** mix3. n=40. See Table 16 for statistics. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

Figure 27: PCA measurements (±SD) of hepatopancreas sections, within a particular zone (Z1-Z4), of groups exposed to mixtures of cadmium- and zinc sulphate (mix1, mix2 and mix3), with **A:** Z1; **B:** Z2; **C:** Z3; **D:** Z4. n=40. See Table 16 for statistics. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

Figure 28: Comparison of Percentage Cellular Area (PCA) measurements (±SD) in the different zones (**A:** Z1, **B:** Z2, **C:** Z3, **D:** Z4) of the hepatopancreas of *P. laevis* after six weeks of exposure to cadmium sulphate (20, 80, 160 mg.kg⁻¹) and combined exposure to various mixtures of cadmium- and zinc sulphate (mix1, mix2, mix3). n=40. Stat. sign. diff. between single metal exposure and combined exposure indicated by (*). mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

Figure 29: Comparison of Percentage Cellular Area (PCA) measurements (±SD) in the different zones (**A:** Z1, **B:** Z2, **C:** Z3, **D:** Z4) of the hepatopancreas of *P. laevis* after six weeks of exposure to zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) and combined exposure to various mixtures of cadmium- and zinc sulphate (mix1, mix2, mix3).

n=40. Stat. sign. diff. between single metal exposure and combined exposure indicated by (*). mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

Figure 30: Relationship ($r = -0.93$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to cadmium sulphate (20, 80, 160 mg.kg⁻¹), and cadmium exposure concentrations.

Figure 31: Relationship ($r = -0.73$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to zinc sulphate (1000, 4000, 8000 mg.kg⁻¹), and zinc exposure concentrations.

Figure 32: Relationship ($r = -0.58$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to various mixtures of cadmium- and zinc sulphate, and cadmium and zinc exposure concentrations. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

Figure 33: Relationship ($r = -0.88$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to cadmium sulphate (20, 80, 160 mg.kg⁻¹), and cadmium concentrations in the hepatopancreas.

Figure 34: Relationship ($r = -0.40$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to zinc sulphate (1000, 4000, 8000 mg.kg⁻¹), and zinc concentrations in the hepatopancreas.

Figure 35: Relationship ($r = -0.96$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to mixtures of cadmium- and zinc sulphate, and cadmium concentrations in the hepatopancreas. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

Figure 36: Relationship ($r = 0.23$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to mixtures of cadmium- and zinc sulphate, and zinc concentrations in the hepatopancreas. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

Figure 37: Relationship ($r = 0.99$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to various concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹), and percentage mass change.

Figure 38: Relationship ($r = 0.80$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹), and percentage mass change.

Figure 39: Relationship ($r = -0.08$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to various mixtures of cadmium- and zinc sulphate, and percentage mass change. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

Figure 40: Examples of histological sections, stained with hematoxylin and eosin, in the Z3 zone of hepatopancreas samples of *P. pruinosis* collected from an A: uncontaminated site and B: contaminated site. Magnification = X250.

(L = lumen; B = B-cells; S = S-cells)

Figure 41: Percentage Cellular Area's (PCA) (\pm SD) in the Z3 zone of the hepatopancreas of *P. pruinosis* collected from the uncontaminated- and contaminated sites. n=40. P=<0.001.

1.

Introduction

1.1. Background

The industrial activities of modern society involve chemical and physical transformations of matter of great diversity and scale. As a result impure materials may be produced which may disperse into the environment. Many such materials may not have played a significant part in the chemical environment of life on any large geographical scale until the last two centuries, or would not have been found at such high environmental concentrations until that time. Processes such as the combustion of fossil fuels with special additives such as antiknock compounds in vehicle fuel, along with the extraction and smelting of metals and a variety of chemical industries, all result in the emission of small particles to the atmosphere. Many of these particles contain various metals in one chemical form or another and may have atmospheric residence times of up to a month. As a result, such particles may be deposited not only close to the site of emission but also in ecosystems over a larger region. There is evidence that shows there to be much greater deposition of lead, copper, zinc, nickel and cadmium on ecosystems remote from industry and roads in the present era than was the case either two hundred years ago or in the more distant past (Hughes et al. 1980).

1.2. Sources of metal pollution

Metals occur naturally in the environment but since the industrial revolution the amounts of metals in the environment increased significantly (Hopkin 1989). There are various anthropogenic sources of metals in the environment. The major sources

of metal pollutants in terrestrial ecosystems are briefly discussed below.

1. *Mining:* Exposure of ore bodies by mining activity far exceeds exposure due to natural weathering. Serious pollution of the soil is usually restricted to the immediate area on and around spoil tips where metals are too low to be extracted economically but are at a high enough level to exert toxic effects on plants and animals. Metals may be transported away from mining areas via wind-blown particles or dissolved in acidic groundwater (Khan & Frankland 1983; Hopkin 1989; Gummow et al. 1991). The South African mining industry is quite large and various metals that could potentially pollute the environment such as copper, zinc and lead are extracted from ores.

2. *Smelting:* Once ores have been mined the metals they contain must be purified by smelting. An example is the smelting unit of the copper mine in Phalaborwa, South Africa, which causes unnaturally high levels of copper in grass in the surrounding area (Gummow et al. 1991). Various other smelters occur in South Africa where primary or secondary smelting of metals take place. Metals may also be recovered from scrap by secondary smelting. In modern smelting works efforts have been made to help reduce the amount of metals released into the atmosphere. Waste gasses are passed through fine filters and treated with electrostatic precipitators to remove most metal-containing particles from gasses before they are released into the atmosphere. Unfortunately even the most modern equipment working at removal efficiencies approaching 100% is unable to completely remove metals from these gasses (Hopkin 1989). At the Avonmouth smelting plant in England huge amounts of zinc, lead and cadmium are produced yearly, while some 50 tonnes of zinc, 30 tonnes of lead and 3 tonnes of cadmium are released into the atmosphere each year (Harrison & Williams 1983; Coy 1984). Contamination of soil as far as 25 km away from this

plant had been reported (Martin & Coughtrey 1982; Hopkin et al. 1986).

3. *Combustion of fossil fuels*: The burning of coal, oil and natural gasses for the domestic production of heat, or the generation of electricity in power stations release metals into the environment, however, this is not one of the major sources of pollution. The main contributor of contamination due to the combustion of fossil fuels is the addition of lead to petrol to serve as an anti-knock agent. Lead is added to petrol in the tetraethyl form to increase the octane rating and to prevent the premature ignition of fuel in the engine cylinders (Hopkin 1989). About 75% of this lead is emitted from the exhaust as fine particles.

4. *Agriculture*: The use of pesticides in agriculture is one of the major sources of metal pollution. Before the Second World War, pesticides containing arsenic, tin and mercury were used extensively for the control of agricultural pests. Their long residence times in the environment made them apparently ideal for preventing investment by fungal and invertebrate pests, like molluscs (Godan 1983). In South Africa fungicides such as copper oxychloride, and manganese and zinc containing mancozeb are widely used in vineyards and orchards of the Western Cape (London & Myers 1995). However, the very factors that make metals effective for long term pest control, namely their toxicity and long residence times in the environment, also resulted in serious pollution problems (Hopkin 1989). The consequences of the use of metal-containing pesticides can be devastating, as illustrated by the incident in Iraq in the early 1970's when seeds sprayed with mercury fungicide were accidentally made into bread. Several hundred people died before the source of the mercury poisoning was traced (Bakir et al. 1973). Large quantities of metals are washed into drains from roads, industry and domestic households. Most of these metals are discharged into the sea in untreated sewage. However, concern over pollution of rivers and estuaries

has led to the increase in the proportion of this waste which is treated in sewage works. The residue of the breakdown of organic material in sewage, is a sludge rich in nitrogen, which can be used as a fertilizer. Unfortunately the sludge may contain considerable amounts of metals (Berrow & Webber 1972; Lake et al. 1984). Repeated application of sludge to soil may lead to the accumulation of metals in the upper soil layers (Soon 1981).

5. *Other:* Metals are used in plastic formulations, where they may play roles such as stabilizers, plasticizers, antioxidants, colourants and fire retardants. Upon pyrolysis metals contained in plastics may be released and become. An example of this can be seen in the fire in Thetford, which caused a major plastic run-off. Elevated concentrations of metals in the area surrounding the site were recorded (Sorensen et al. 1997).

1.3. Biomarkers

In recent years there has been an increasing interest in the use of so-called biomarkers in terrestrial invertebrates for the assessment of the potential adverse effects of chemicals in soil ecosystems (Kammenga et al. 2000). There are, however, a considerable amount of confusion and differences in the definition and understanding of the term biomarker (Van Gestel & Van Brummelen 1996). Different definitions of biomarker have been put forward; some of which overlap with other terms (eg. Peakall 1994; Peakall & Shugart 1992; Depledge & Fossi 1994; Van Gestel & Van Brummelen 1996). The definition that will be accepted for the purpose of this study is that of Van Gestel & Van Brummelen (1996). A biomarker is any biological response to an environmental chemical at the below individual level, measured inside an organism or its products (urine, faeces, hair, feathers, etc) indicating a departure

from the normal status, that cannot be detected from the intact organism. According to Van Gestel & Van Brummelen (1996) the advantage of this definition is that a clear distinction is made between responses at different levels of biological organisation. Overlap with other definitions are also avoided. Morgan et al. (1999) stated that suborganismal functional endpoints of a physiological, cellular, biochemical or molecular nature can be referred to as biomarkers.

Biomarkers also have potentially useful features. Two of these features are predominant: 1) Their responsiveness and sensitivity may give an early alarm of toxicant impacts on organisms, well before ecological disturbances can be discerned; 2) they may give a more direct and accurate insight into the relationship between toxicant exposure (cause) and the biological response (effect) (Weeks 1998; Morgan et al. 1999). However, there are certain challenges to be faced if potential biomarkers is to be put forward for use in environmental monitoring. Pollutants hardly ever occur singly, which means that their toxic impact depends on outcome of interactions between components of the mixture. Secondly, biomarkers may impact individual organisms but the effects on the ecological level are of special concern for the environmental policy makers. Thirdly, fast reliable and robust techniques are needed to measure the biological effect of pollutants (Morgan et al. (1999). Jenkins & Sanders (1992) stated that from the point of view of environmental protection it is the ecological-level changes that are paramount, but that these effects are evoked through stress-induced disturbances of cellular and molecular functions in individual organisms. Thus, it should theoretically be possible to use biomarkers not only as early warning stress indicators but also to anticipate or predict population and community responses.

One of the major reasons for the current interest in biomarkers is the limitation

of the classical approach to environmental toxicology that involves measuring the amount of the chemical in an animal and then relating this to adverse effects on mortality, reproduction and growth. First of all, in the classical approach, the bioavailability and toxicity of a compound may differ in laboratory tests compared to those observed in the field. Secondly, under field conditions, multiple toxicants may be present simultaneously, producing an even more complex situation. Finally, only a few conventional endpoints from the classical approach can be assessed as in situ experiments or surveys. The strength of the biomarkers approach lies in its potential to circumvent the serious limitations of the classical approach. Biomarkers deal with the question of bioavailability of chemical by only reacting to the biologically available fraction of the pollutant. Biomarkers also have the advantage that they can exhibit the effect caused by many toxic compounds present at the same time, and they are applicable under laboratory and field conditions (Scott-Fordsmand & Weeks 1998).

There are a number of reasons why soil invertebrates must be taken into consideration when it comes to the ecotoxicological assessment of the effects of environmental contaminants and consequently, biomarkers. They play a major role in the functioning of soil ecosystems by enhancing soil structure and the decomposition of organic material. Invertebrates also represent a major component of all animal species in soils and often are present in high population densities. Thus, samples can be taken for analysis without significantly affecting population dynamics. Ethical and legal considerations also favour their use in contrast to vertebrates. Soil invertebrates have the advantage over most vertebrates that they are in direct contact with soil pore water or food exposure (Kammenga et al. 2000).

A variety of different types of biomarkers had been investigated. Stress

proteins, metallothioneins and other metal-binding proteins, histology and ultrastructure, isozymes, lysosome membrane integrity and other novel biomarkers are examples of biomarkers that had thus far been studied. Some of these examples of biomarkers had been studied to a larger extent than others as is evident in the literature (Kammenga et al. 2000).

One of the most important advantages of biomarkers is that they can give information on the effect of pollutants rather than mere quantification of pollutant levels (Peakall & Walker 1994). Biomarkers must be designed to assess, at an early stage, damage / change at the below-individual level (Van Gestel & Van Brummelen 1996) before sublethal effects become apparent (Kammenga et al. 2000). Cellular and histological alterations reflect the 'health' state of a cell, which may be a measure for the presence of toxicants. Histological changes may serve as biomarkers and are effective tools to evaluate the toxicity of environmentally relevant chemicals in a predictive as well in a retrospective fashion, together with other types of biomarkers. However, research on histological and cellular changes in terrestrial invertebrates due to pollutants is still scarce (Kammenga et al. 2000). The epithelium of the intestinal tract and digestive gland (hepatopancreas) is the first barrier against the poisoning of the whole organism (Pawert et al. 1996). Changes or damage due to pollutants should thus be detected first in these parts of the organism. The use of the hepatopancreas of *P. laevis* in the present study for potential biomarker research is thus relevant. Very little research on the effect of pollutants on the hepatopancreas structure of terrestrial isopods had been done previously (Drobne & Strus 1996; Köhler et al. 1996).

1.4. Choice of test organism

The question must be asked; which animals could be used in ecotoxicological testing? The animals chosen must be suited to be used as bioindicator- or biomonitor species. A set of criteria was laid down by Moore (1966). They should be easily available, i.e. widely distributed, relatively abundant and easy to collect. It must be possible to make chemical analysis of single animals or of bulk samples. They should be sufficiently large. Ideally, it should be possible to ascertain the age of the animals by inspection. Species that, in a preliminary survey, show relatively high contamination are most suitable. The range of the species must be known. They must be sedentary in their habits or have extensive ranges, depending on the type of study to be conducted.

In addition, there must be sufficient background knowledge on the biology of the species, its sublethal responses to chemicals, and information that enables differentiation of measured effects from natural background variability. Terrestrial isopods are one of the animal groups that fulfill most of the criteria (Drobne 1997).

Other than earthworms and springtails, terrestrial isopods are perhaps one of the most frequently used terrestrial invertebrate groups for testing the effects of chemicals (Beyer et al. 1984; Beyer & Anderson 1985; Van Capelleveen 1985; Bercovitz & Alikhan 1989; Donker & Bogert 1991; Van Wensem et al. 1992; Crommentuijn et al. 1994; Drobne & Hopkin 1994; Alikhan 1995; Odendaal & Reinecke 1999). These are only a few examples of studies conducted on terrestrial isopods. Terrestrial isopods are distributed throughout the world and are abundant in many different terrestrial environments (Hopkin et al. 1993). They inhabit the upper layer of the soil and surface leaf litter in a variety of urban and natural habitats. (Drobne 1997). The soil and leaf litter are the parts of the terrestrial ecosystems

where metals accumulate mostly (Tyler 1972, 1984). Coughtrey et al. (1979) stated that, the soil-litter component of terrestrial ecosystems is an important sink for metal contaminants. Terrestrial isopods, like many other soil invertebrates, occur in this zone .

The normal functioning of terrestrial ecosystems depend on litter breakdown and decomposition for its supply of several important nutrients (Tyler 1984). Soil invertebrates are directly responsible for only about 5 - 10% of the chemical decomposition of leaf litter (Petersen & Luxton 1982). However, they act as "catalysts" by stimulating the activities of bacteria and fungi which conduct the majority of chemical decomposition (Anderson & Ineson 1984; Anderson 1988). They do this by fragmenting leaf litter into small particles, increasing the total litter surface available for further attack by micro-organisms (Eijsackers 1994). The fragmented leaf litter is also voided as faecal pellets, which are favourable substrate for microbial breakdown (Eisenbeis & Wichard 1987). Terrestrial isopods are one of the groups that play an important role in the decomposition process as fragmentors of dead plant material (Hassall et al. 1987; Van Wensem 1989; Drobne 1997). Any changes in the feeding rates of terrestrial isopods affect the decomposition processes and subsequently affects flow of matter and energy through ecosystems (Drobne 1997). A reduction in the rate of decomposition of dead plant material may also occur in the presence of metal contaminants (Rühling & Tyler 1973; Williams et al. 1977; Kratz & Weigmann 1987; Hopkin 1994). Thus, if the rate of decomposition is reduced in the presence of metal contaminants, it may be partly due to the effect of the metals on the isopods.

One of the reasons why one would choose terrestrial isopods for toxicity testing is that the toxicity tests presently available for routine use only cover the

insects and annelids, which mainly dwell in the soil. Isopods could be used to assess the relative toxicity of the litter itself (Drobne 1997). The litter layer is the compartment of terrestrial ecosystems that accumulate metals in high concentrations in relation to other compartments (Tyler 1972, 1984). Carefoot (1993) also suggested them to be suitable for experimental research studies due to their hardiness in laboratory cultures.

1.5. The hepatopancreas of terrestrial isopods

The hepatopancreas of terrestrial isopods was the focus of a wide range of studies over the past years (Holdich 1973; Holdich & Mayes 1975; Prosi et al. 1983; Hames & Hopkin 1989). The hepatopancreas consists of a pair of bilobed structures, each connected to the digestive tract at the junction of the foregut and hindgut by a common hepatopancreatic duct (Fig. 1) (Bettica et al. 1984). A study by Hames & Hopkin (1989) determined that the hepatopancreas is a very important organ in the digestive system of isopods. Digestive enzymes are secreted from the hepatopancreas to help digest food. Products of digestion are transported into the hepatopancreas, where absorption can take place (Hames & Hopkin 1989). The hepatopancreas consists of two cell types, namely the larger B-cells and the smaller S-cells (Vernon et al. 1974). The B-cells secrete digestive enzymes and are responsible for the absorption of food (Storch 1984). The S-cells, on the other hand function as storage cells, especially for contaminants such as metals (Prosi et al. 1983). A series of studies were also conducted where accumulation of metals in the hepatopancreas of terrestrial isopods was investigated (Wieser 1968; Wieser & Klima 1969; Alikhan 1972; Hopkin & Martin 1982a, 1984; Dallinger & Prosi 1988; Prosi & Dallinger 1988; Hopkin 1990; Morgan et al. 1990). The hepatopancreas of other organisms,

such as marine molluscs, showed a variety of changes in its structure and also other related alterations when exposed to a polluted environment (Lowe 1988; Lowe & Clarke 1989).

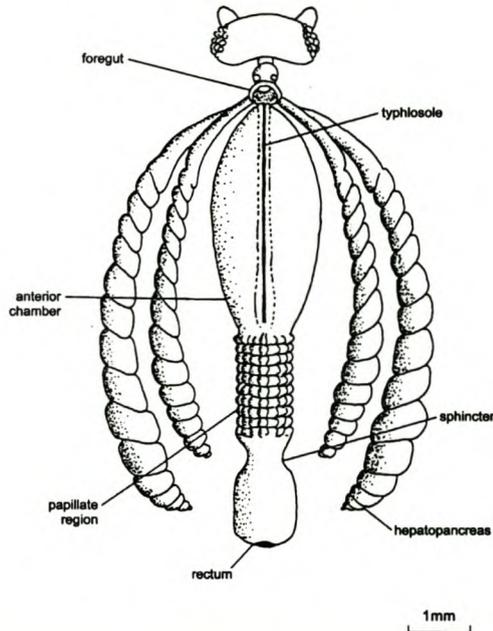


Figure 1: The digestive system of a terrestrial isopod, showing the hepatopancreas. (Hopkin & Martin 1984).

1.6. Cadmium and Zinc

Among the various metals that contaminate terrestrial ecosystems, cadmium and zinc were chosen to be used in this study. Cadmium does not occur naturally in organisms and may be potentially toxic if found at metabolically active sites. It is also labelled as a non-essential metal (Depledge et al. 1994). Zinc on the other hand is one of the essential metals and play an important role in the normal metabolism of animals (Miller 1983; Moore & Ramamoorthy 1984).

Cadmium has no known biological function and may be extremely toxic to life

(Hiatt & Huff 1975). One of the major uses of cadmium is in electroplating, where the object is to provide a bright appearance and resistance to corrosion. The end-products include parts in the automobile and aircraft industries, industrial and builders hardware, marine hardware, radio and television parts, and household appliances. Cadmium is also used in the packing industries (Moore & Ramamoorthy 1984). It is used as plastic stabilizers (Richardson & Gangolli 1993) in the production of PVC (Moore & Ramamoorthy 1984). Cadmium sulfides give yellow to orange colours and cadmium sulfoselenides give pink to red and maroon. These pigments are used in the plastic industry, ceramics, paints and coatings. Cadmium pigments are also used in traffic paints (Moore & Ramamoorthy 1984). Cadmium is extensively employed in batteries due to its perfectly reversible electrochemical reactions at a wide range of temperatures, low rate of self discharge, and easy recovery from dead batteries. Such batteries are used in consumer items such as battery-operated toothbrushes, shavers, drills and handsaws, medical appliances, communication devices and emergency lighting supplies, airplanes, satellites and missiles, and even ground equipment for polar regions (Moore & Ramamoorthy 1984). Cadmium may also be used in nickel-cadmium batteries (Richardson & Gangolli 1993). Cadmium is also used in a variety of other products such as fluorescent lamps, tubes in television sets, x-ray screens, phosphorescent tapes, and a variety of alloys (Moore & Ramamoorthy 1984). Small amounts are used in fungicide production (Hiatt & Huff 1975). Phosphate fertilisers contain significant amounts of cadmium due to the biogeochemical processes which occur during the formation of phosphorite rock. Up to $170 \mu\text{g}\cdot\text{g}^{-1}$ cadmium has been found in some samples of superphosphate as a result of cadmium in both the phosphorite and sulphuric acid (Samarawickrama 1983). In the U.K. waste disposal by means of incineration is a large source of airborne cadmium (Barratt 1988).

Cadmium is commonly found associated with zinc in carbonate and sulphide ores. Cadmium is also obtained as a by-product in the refining of other metals (Moore & Ramamoorthy 1984; Wren et al. 1995). The production of copper, lead and zinc, thus resulted in the unknowing pollution of the environment with cadmium (Moore & Ramamoorthy 1984). Cadmium may also enter the environment through the combustion of fuel (Wren et al. 1995). Cigarettes are also a source of cadmium. Smoking of 20 cigarettes results in the inhalation of about 2-4 μg cadmium (Elinder 1991). The compound of cadmium used in this study, cadmium sulphate, is used in electrodeposition of cadmium, copper and nickel and as a nematocide. Experimental evidence indicates that the biological half-life of cadmium in the body is very long, which means that its effects may last longer (Richardson & Gangolli 1993). In mammals cadmium tends to accumulate in and damage the kidneys, liver, pancreas and spleen. Severe chromosome anomalies may also occur in workers exposed to high concentrations of cadmium (Richardson & Gangolli 1993).

In contrast to cadmium, zinc is essential for normal life in humans and other organisms. It has unique and extensive roles in the biochemistry of enzymes and other biological molecules (Jackson 1989). Zinc is a cofactor for more than 200 enzymes. In fact, zinc is the only essential metal that occurs in all six categories of enzymes (Harris 1991; Miller 1983). Zinc plays a key role in numerous essential processes including protein synthesis, DNA and RNA metabolism, carbohydrate and lipid metabolism, energy metabolism and many others (Miller 1983). Because of the ease of extraction from ores, zinc has a long history of use by humans. It ranks fourth among the metals next to steel, aluminium and copper in annual global consumption. Global production of zinc has increased steadily during the last century (Moore & Ramamoorthy 1984). The largest use of zinc is in galvanising iron and steel products.

This provides a corrosion-resistant coating. Such products are used in construction, automobile industries, building industries for roofing, appliance casings, office equipment, heating and ventilation ducts. Alloys such as zinc-aluminium have been developed as protective coatings. Zinc diecast products are used in a variety of components of automobiles. The development of nickel-zinc batteries for use in electric vehicles will provide a significant new market for zinc in coming years. Another major consumption category for zinc is the production of brass. Brass is used in a variety of applications from decorative hardware to plumbing and heat exchange units. Rolled zinc is used in dry battery production, photo engraving, lithographic printing plates, roofing, and rain water gutters and pipes. Zinc oxide is used as a catalyst in the vulcanisation of rubber, and is also required for paints, and other end-products such as photocopy paper, agricultural products, and cosmetic products (Moore & Ramamoorthy 1984). Wood combustion and waste incineration is other important sources of zinc in the environment (Moore & Ramamoorthy 1984). Zinc contamination can give rise to a number of conditions and effects in humans and other animals. Excessive zinc can impair the intestinal absorptions of iron, and also affect the pancreas (Richardson & Gangolli 1994). Inhalation of zinc oxide fumes can lead to so called "Zn fume fever" or "metal fume fever". Zinc contamination can also lead to a severe depression of growth in rodents (Harris 1991). High zinc concentrations near to a zinc smelter was found to have inhibited root elongation in seedling trees (Beyer & Storm 1995). Despite the high production of zinc and apparent negative effects on humans and other animals, relatively few studies on the toxicity of zinc to terrestrial invertebrates had been conducted (Hopkin & Spurgeon 2001). In the past there has been an emphasis on the effect of zinc deficiency, while the effects of excess zinc in terrestrial ecosystems, with a few exceptions, had largely

been overlooked (Hopkin & Spurgeon 2001).

1.7. Mixture toxicity

Cadmium is commonly found associated with zinc in carbonate and sulfide ores. The refining and smelting of zinc over the years thus sometimes unknowingly resulted in the pollution of the environment, not only with zinc, but also with cadmium (Moore & Ramamoorthy 1984). It is thus likely that a particular area known to be polluted with zinc will also be polluted with cadmium. However, little is known about the effects of a mixture of cadmium and zinc on soil fauna. 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. 1996a; Van Gestel & Hensbergen 1997; Korthals et al. 2000; Witzel 2000). Toxicity tests used to evaluate the environmental risk of metals are also largely based on the use of single metals. Environmental criteria are based on these single metal tests but however, have little real environmental relevance 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 (Kraak et al. 1994). It is also a proven fact that a particular mixture of metals have different effects on different fauna (Berger et al. 1993; Witzel 2000). Therefore, much work is needed in the field of mixture toxicity.

1.8. Aims of the study

The aim of this study was to undertake an experimental and field evaluation of the role cellular change in the hepatopancreas of terrestrial isopods can play in conjunction with other sublethal responses to contribute to the assessment of the impact of cadmium and zinc in terrestrial ecosystems. In order to achieve this aim the

following studies were undertaken.

- To determine the sublethal effects of cadmium sulphate and zinc sulphate and mixtures of these metal salts on the mass of terrestrial isopods.
 - Change in mass is a commonly accepted sensitive endpoint in ecotoxicity testing. It is a subtle effect that could have long term effects on populations. Differing metal mixtures could have different effects on mass than when metals are administered separately.
- To determine the accumulation of cadmium and zinc in the isopods' hepatopancreas after singular and mixed exposure to these metals.
 - This gives an indication of the degree of accumulation of these metals in the isopods because those animal groups that accumulate pollutants in their bodies are most suitable for biomonitoring. Exposure to mixed metals could change the accumulation patterns of metals.
- To determine the effects of cadmium and zinc administered separately and mixed on the histological structure of the hepatopancreas of the isopods to assess it's possible role as biomarker of metal exposure.
 - The hepatopancreas is a target organ of metal contamination in invertebrates and is therefore important in the search for usable and relevant biomarkers.
- To quantify the possible changes to the hepatopancreas and to relate these to the mass change and accumulation of metals.
 - For suggested biomarkers to be relevant the biomarker response should ideally be connected and correlated with other toxicological parameters.

- To determine the extent of the change or damage and whether it is uniformly spread over the length of the hepatopancreas or restricted to certain areas of the organ.
 - This is important as possible zoning of damage in the hepatopancreas could result in the unknowing misinterpretation of results.
- To evaluate the biomarker responses of the hepatopancreas under field conditions at a polluted field site.
 - For a suggested biomarker to be useful and practical it must be employable in a field situation.

2. *Materials and Methods*

2.1. Laboratory experiments

2.1.1. *Study animal*

2.1.1.1. *Classification*

The isopods used in this study were identified with the help of descriptions of Hopkin (1991). The original description by Latreille (1804) was not available. The classification, based on Hopkin (1991) is the following:

Phylum: Arthropoda

Class: Crustacea

Order: Isopoda

Suborder: Oniscidea

Family: Porcellionidae

Genus: *Porcellio*

Species: *P. laevis* (Latreille, 1804)

2.1.1.2 *General*

Porcellio laevis is a member of the class Crustacea, which also includes crabs, shrimps and lobsters. Most of the crustaceans are aquatic and the order Isopoda is one of only two orders that contain species which are able to live their whole life cycle away from water. Terrestrial isopods occur from the supralittoral zone of the seashore to the temperate, tropical desert areas of the world. Contrary to previous

thoughts, terrestrial isopods are very well adapted to land as they evolved several sophisticated physiological and behavioural mechanisms to control water loss on land. These include the modification of the pleopods to form 'lungs' and a capillary conducting system, which allows nitrogenous waste to be excreted as ammonia gas, without significant loss of water (Hopkin 1991).

P. laevis is mostly found in synanthropic sites such as compost heaps and gardens and also around buildings. Where found, they are usually abundant (Hopkin 1991). Specimens of *P. laevis* used in this study were collected from the compost heaps of the Botanical Gardens of the University of Stellenbosch.

2.1.1.3. Morphology

Specimens of *P. laevis* reach a maximum length of about 18 mm and exhibit a smooth glossy surface with a brownish-grey colour (Hopkin 1991). Occasionally the surface is very feebly tubercular on the posterior margins of pleon segments (Barnard 1931-1934). Each of the eyes has numerous black ocelli (Hopkin 1991). The frontal lobe of the head is not so prominent as the lateral lobes. The telson is broader than long with the apex acute and dorsally grooved (Barnard 1931-1934). The outline of the edges of the pereon and pleon is a smooth line. Two pairs of lungs (pseudotracheae) occur on the first two pairs of pleopods (Hopkin 1991). The uropods are longer than in other *Porcellio* species, with their widest part nearest to the body. The flagellum of antenna 2 has two joints with the first joint slightly longer than the second. Male woodlice can be distinguished from females by the externally visible elongation of the endopodite of the first pleopod present in males (Katakura 1984).

2.1.1.4. Rearing of woodlice

It is quite easy to keep and rear woodlice under laboratory conditions. Woodlice were kept in plastic containers with a perforated lid, which allows air to pass through. Decaying oak leaf litter with a moisture content of about 65-70 % was used as a food source. A light spray of water was also applied to preserve the moisture in the leaf litter. The woodlice containers were kept in a climate room at a relative humidity of ± 60 % and a temperature of ± 18 °C.

2.1.2. Sublethal Toxicity Tests

Sublethal toxicity tests were conducted using specimens of *P. laevis* with a length of between 7 and 9 mm. Containers used in these tests were specially made for this purpose. PVC cylinders of 110 mm in diameter were cut to a length of 130 mm each. An artificial bottom was created in each of the cylinders by a plaster of Paris layer of about 20 mm thick (Joosse et al. 1981). The plaster of Paris bottom was functional in that it could be kept moist to provide a constant moist environment in a container. The tops of these containers were covered by a metal sieve with openings 1 mm in diameter. This made the free flow of air through the containers possible.

Decaying oak leaves (*Quercus robur*) were collected from the Botanical Gardens of the University of Stellenbosch and used as substrate in the experiments, as it was suggested by Depledge et al. (1994) that food is the major route of metal uptake in soil arthropods. Collected leaves were kept moist, before the commencement of the tests, in closed plastic bags to ensure that the microorganisms on them kept flourishing and did not disappear. The leaves were shredded to small pieces in a food processor after which they were spread out on a flat surface to dry at room

temperature. In each of the containers used, 50 g dry mass leaves were added. The metals used in this study were cadmium and zinc, which were administered, respectively as cadmium sulphate and zinc sulphate solutions to the dried leaves. Concentrations used were 20, 80 and 160 mg/kg (dry mass) cadmium sulphate and 1000, 4000 and 8000 mg/kg (dry mass) zinc sulphate. A calculated mass of the metal salts were weighed and dissolved in distilled water. The solutions of metal salts were sprayed on the leaves (50 g dry mass) to give the desired concentration. A range of experiments were done, where the cadmium and zinc were administered separately. In another range of experiments cadmium and zinc salts were mixed before administration to the leaves. The cadmium and zinc mixtures used were 20 mg/kg CdSO_4 with 1000 mg/kg ZnSO_4 , 80 mg/kg CdSO_4 with 4000 mg/kg ZnSO_4 and 160 mg/kg CdSO_4 with 8000 mg/kg ZnSO_4 . The leaves in the experimental containers were covered with a plastic sheet to prevent the moisture from evaporating. For each of the concentrations, 5 replicates were prepared. 20 isopods were weighed on a Sartorius microbalance and put in each of the containers. The masses of the isopods were monitored every week for 6 weeks. A control in which the leaves were sprayed only with distilled water was also prepared. The pH of the leaves were measured using a Crison micropH 2001 (KCl-electrode) pH meter and the moisture content of the leaves was measured with a Sartorius infrared moisture detector. The pH levels were found to be between 5.96 and 6.79, while the moisture content of the leaves remained between 65% and 70 %.

After the 6 week period 10 woodlice were randomly picked out of each of the containers and dissected to remove the hepatopancreas. They were starved beforehand for 24 hours (Hames & Hopkin 1989) to rid the gut of any food. After the 24 hour period the woodlice were killed with chloroform. The dissections were done

in the following manner. An isopod was put with its ventral side up. A dissection needle was inserted right behind the head and then pushed forward to remove the head. In the forward pushing movement the yellow coloured hepatopancreatic lobes came out of the body, still attached to the head. Whenever this procedure was unsuccessful causing the hepatopancreas to break loose from the head while still in the body, dissection tweezers were used to remove it from the body.

2.1.3. Acid digestion and metal analysis

The hepatopancreases of ten isopods out of each of the experimental containers were pooled together for acid digestion and metal analysis. Rest of the body samples of ten isopods out of each of the experimental containers were also pooled for acid digestion and metal analysis. The samples were weighed (wet mass) and were temporarily stored in small polytops in a freezer.

Hepatopancreas and the rest of the body samples were thawed before acid digestion. The digestion procedures for the hepatopancreas and the rest of the body samples were slightly different, and will be dealt with separately.

The digestion of the hepatopancreas samples was as follows: Each of the samples were placed in 2ml 55% nitric acid and left overnight. The samples were then heated at 40 °C for 1 hour, after which the temperature was raised to 120 °C for 30 minutes. During the digestion process the samples were shaken until a brown-yellowish vapour came off. The now totally liquid samples were left to cool. A blank digestion was also performed with each of the sets of digestions. The blanks were treated the same as all the digestions but contained no tissue. They were analysed for lead and/or cadmium. This helped to keep a check on possible contamination.

The procedure for the digestion of the rest of the body samples was slightly different. After thawing of the samples, 8 ml 55% nitric acid were added to each and left overnight. They were then heated to 40°C for 1 hour after which the temperature was raised to 120°C for 90 minutes. As in the digestion procedure for the hepatopancreas samples, the samples were shaken during the process until a brown-yellowish vapour came off. They were then left to cool down. A blank digestion was also performed with each of the sets of digestions like in the case of the hepatopancreas samples.

The samples were filtered through a 0.45 µm Millipore filter, made up to 10 ml and stored in plastic containers (Ebdon 1982) in a refrigerator until the analyses for zinc and cadmium with a Varian AA-1275 flame atomic absorption spectrophotometer. Concentrations were divided by ten to get a mean concentration for one hepatopancreas, and one sample of the rest of the body, as samples were pooled for the digestions. The cadmium and lead concentrations in the hepatopancreas samples and rest of the body samples were expressed on a wet mass basis. Twelve hepatopancreas and rest of the body samples were oven dried at 60°C for 24 hours. The percentage mass loss was calculated to be 91.58% (S.D. ±3.84) for the hepatopancreas samples and 74.66% (S.D. ±3.59) for the rest of the body samples. These conversion factors were used to express the cadmium and zinc concentrations on a dry mass basis (Odendaal & Reinecke 1999).

After the experiments samples of the leaves were also digested in acid to determine the metal content. Samples from each of the experimental containers were dried in a drying oven at 60 °C for 48 hours. The samples were then ground to a powder and digested in the following way. 10 ml 55% nitric acid were added to each sample and left overnight. They were heated to a temperature of 40 °C and then to

120 °C for 3 hours. The samples were shaken as in the previously described digestion procedures and left to cool after digestion. The samples had a whitish coloured sludge, which had to be removed before filtration. Each sample was made up to 20 ml and filtered through Whatman No.1 filter paper. They were subsequently filtered using 0.45 µm Millipore filters. A blank digestion was also performed together with each of the sets of digestions. This helped to keep a check on possible contamination.

The samples were temporarily stored in a refrigerator in plastic containers (Ebdon 1982) until they were analysed for the metals with a Varian AA-1275 atomic absorption spectrophotometer. The lower detection limit of the AAS was 0.01 ppm. To determine percentage recovery uncontaminated woodlice were spiked with cadmium- and zinc sulphate and analysed for cadmium and zinc using the same method as described in this chapter. Percentage recovery was found to be in the region of 90%. Concentrations of cadmium and zinc in the leaves used in the sublethal toxicity tests were within 10% of the desired concentrations. Cadmium and zinc concentrations in control leaves were 0 and 67.6 (± 11.0) mg.kg⁻¹, respectively.

2.1.4. Histological technique

2.1.4.1. Preparation of hepatopancreas samples

After the exposure period woodlice were dissected to remove the hepatopancreas. The various hepatopancreas samples were fixated in Bouin's fluid (Preece 1972) for about 18 hours. The samples were then placed in tissue processing cassettes and rinsed in 50% ethanol for 40 minutes, after which the hepatopancreas samples were taken through various steps of dehydration in a range of ethanols. There after the samples were taken through a clearing process in xylene, and finally impregnation

with Paraplast wax at 58 °C (Table 1). Finally, the hepatopancreas samples were embedded in Paraplast in metal base moulds, and left to cool overnight.

Table 1: The various steps of rinsing, dehydration, clearing and impregnation during histological preparation of hepatopancreas samples of *Porcellio laevis*.

Chemicals / Steps	Time	Function
50% ethanol	40 mins.	Rinsing
70% ethanol	40 mins.	Dehydration
70% ethanol	20 mins.	
90% ethanol	20 mins.	
96% ethanol	20 mins.	
100% ethanol	20 mins.	
100% ethanol	20 mins.	
Xylene 1	20 mins.	Clearing
Xylene 2	10 mins.	
Wax 1 (1:1 Paraplast + Xylene)	60 mins.	Impregnation
Wax 2 (Paraplast)	60 mins.	
Wax 3 (Paraplast)	30 mins. (vacuum) *	Remove bubbles *

* In vacuum pump chamber at 300mmHg

2.1.4.2. Sectioning

Cross sections of hepatopancreas samples were made with a Leica microtome, at a thickness of 6µm. Sections were carefully placed on microscope slides. A few drops of Mayer's egg albumin and glycerol mixture (Preece 1972) was added on microscope slides to spread the wax and also to act as a adherent agent to help the sections to stick to the slides. The slides were left to dry on a hotplate at ±35 °C.

2.1.4.3. Staining and mounting

Slides were stained with the routine Hematoxylin and Eosin method. The Hematoxylin used was prepared according the recipe of Gill, while the Eosin was

ethanol soluble (Presnell & Schreiber 1997). The slides were taken through a range of steps: rinsing, dehydration, staining, differentiation and clearing (Table 2). After the whole process a few drops of Entellan mounting fluid were immediately put on the slides. Finally, a coverslip was carefully placed on each microscope slide. The slides were then left to dry overnight.

Table 2: The various steps of rinsing, dehydration, staining, differentiation and clearing in the staining process of sections of the hepatopancreas of *P. laevis*.

Chemicals / Steps	Time	Function
Xylene	3 mins.	Removes wax
Xylene	3 mins.	
100% ethanol	Dip	Removes xylene
100% ethanol	3 mins.	Dehydration
96% ethanol	3 mins.	
96% ethanol	2 mins.	
70% ethanol	2 mins.	
50% ethanol	2 mins.	
Distilled water	2 mins.	Removes ethanol
Gill's Hematoxylin	±15 mins.*	Staining of nuclei
Flowing tap water	2 mins.	Provides blue colour to nuclei
Scott's solution	3 mins.	Provides alkaline medium
Flowing tap water	2 mins.	Rinsing
1% Alc. sol. Eosin	40 secs.	Staining of cytoplasm
70% ethanol	Dip	Differentiation
96% ethanol	Dip	Dehydration
100% ethanol	3 mins.	
100% ethanol	3 mins.	
Xylene	4 mins.	Clearing
Xylene	5 mins.	

* depending on strength of prepared staining solution.

2.1.5. Image analyses and determination of PCA

Slides of four individuals of each exposure group were studied under a Leica compound microscope at 10X magnification. The Leica QWin image analysis software package was used for area measurements of the hepatopancreas sections. The area of the lumen was subtracted from the area of the total section to calculate the area covered by the cells in a particular section. The cellular area in a section was expressed as a percentage of the total area of the section, and was termed the Percentage Cellular Area (PCA). All the hepatopancreas samples used were divided in four equal zones from the front to the back of the lobe (i.e. Z1, Z2, Z3 and Z4). An example: if a hepatopancreas lobe were sectioned and filled 40 microscope slides, slide 1-10 would be Z1, 11-20 would be Z2, 21-30 would be Z3, and 31-40 would be Z4. A total of 40 PCA measurements were made in each zone for all the exposure groups, in order to evaluate consistency of measurements and to standardize methods.

2.2. Field survey

2.2.1. Study animal

2.2.1.1. Classification

The isopods used in the field survey were identified with the help of descriptions of Barnard (1931-1934) and Hopkin (1991). The original description by Brandt (1833) was not available. The classification, based on Hopkin (1991) is the following:

Phylum: Arthropoda

Class: Crustacea

Order: Isopoda

Suborder: Oniscidea

Family: Porcellionidae

Genus: *Porcellionides*

Species: *P. pruinus* (Brandt 1833)

2.2.1.2. Morphology

Specimens of *P. pruinus* reach a maximum length of about 11 - 12 mm. They are slaty-grey, and faintly mottled, with the margins of the pereon and pleon segments sometimes pale (Barnard 1931-1934). Sometimes specimens exhibit a orange colour. A plum-like 'bloom', that can easily be rubbed off, usually covers the surface of life animals (Hopkin 1991). In *P. pruinus* the outline of the edges of the pereon and pleon is a stepped line. This is in contrast to the situation in *Porcellio laevis* where the outline of the edges of the pereon and pleon is a smooth line. Each of the eyes in *Porcellionides pruinus* consists of numerous black ocelli. Two pairs of lungs

(pseudotracheae) occur on the first two pairs of pleopods (Hopkin 1991). The flagellum of antenna 2 has two joints with the first joint slightly longer than the second (Barnard 1931-1934).

2.2.2. Field sites

Two field sites were chosen for this part of the study. The uncontaminated site (33°56'15"S; 18°52'5"E) was the botanical gardens of the University of Stellenbosch. The pH, as determined with a Crison micropH 2001, of the soil was found to be between 6.91 and 6.99 and the leaf litter ± 6.51 . The contaminated site (33°56'30"S; 18°41'30"E) was nearby a smelting works in Kuilsriver. The pH of the soil was between 6.36 and 6.42 and the leaf litter between 6.63 and 6.70. Specimens of *P. pruinus* were collected by hand from the leaf litter present at both sites. Soil and leaf litter samples were also collected from the same spots where the woodlice were collected.

2.2.3. Acid digestion and metal analysis

Woodlice (*P. pruinus*) were sampled from the uncontaminated and contaminated sites. The same procedures used in the laboratory based experiments were utilised to acid digest hepatopancreas and rest of the body samples of the woodlice. Soil and leaf samples of both sites were also digested, using the same procedure described earlier in this chapter. Cadmium and zinc concentrations were measured with a Varian AA-1275 flame atomic absorption spectrophotometer.

2.2.4. Histological technique

The same procedures, as described earlier in this chapter, were used for the preparation, sectioning, staining and mounting of hepatopancreas samples.

2.2.5. Image analyses and determination of PCA

Hepatopancreas sections of woodlice from both the uncontaminated and contaminated sites were used for Percentage Cellular Area (PCA) measurements. Measurements were only made in the 3rd zone (Z3) of the hepatopancreas.

2.3. Statistical Analyses

The data of the sublethal toxicity tests were analysed by means of the Kruskal-Wallis One Way Anova on Ranks. Multiple pairwise comparisons of data of the above mentioned tests were done using Dunn's method. Linear regression analysis was used to determine the relationships between Percentage Cellular Area (PCA) and the measured parameters (exposure concentrations, metal concentrations in the hepatopancreas, and mass change). Data from the field survey was analysed by means of the t-test (Jandel Scientific Sigmastat 2.0).

3.

Results

3.1. Laboratory experiments

3.1.1. Influence of cadmium- and zinc sulphate exposure on mass change of *P. laevis*

3.1.1.1. Cadmium sulphate

The changes in body mass of *P. laevis* after 6 weeks exposure to various concentrations of cadmium sulphate are illustrated in Table 3 and Fig. 2. Pairwise multiple comparison of the exposure groups revealed the following pattern. After one week of exposure the percentage mass change of the 20 mg.kg⁻¹ exposure group did not differ significantly from that of the the control group, while the percentage mass changes of the 80 and 160 mg.kg⁻¹ groups were statistically significantly lower than that from the control group (P<0.05). The 20 vs 80 mg.kg⁻¹ and 20 vs 160 mg.kg⁻¹ comparisons showed statistically significant differences, when percentage mass changes were compared with each other (P<0.05), with the percentage mass changes of woodlice from the 80 mg.kg⁻¹ group lower than that of the 20 mg.kg⁻¹ group and the mass of the woodlice from the 160 mg.kg⁻¹ group decreasing. After two weeks of exposure percentage mass changes of the 80 and 160 mg.kg⁻¹ exposure groups differed significantly from that of the control group (P<0.05). The 20 vs 80 mg.kg⁻¹ comparison also differed significantly from each other, in terms of mass change (P<0.05). After three weeks of exposure only the 160 mg.kg⁻¹ exposure group differed statistically significantly from the control group, concerning mass change (P<0.05). After four weeks none of the comparisons differed significantly from each

other ($P>0.05$). After five and six weeks of exposure the percentage mass changes of the 80 and 160 mg.kg^{-1} exposure groups differed statistically significantly from that of the control group ($P<0.05$).

Table 3: The mean percentage mass change (\pm SD) of *P. laevis* over 6 weeks of exposure to various nominal concentrations of cadmium sulphate (20, 80, 160 mg.kg^{-1}). Numbers in [brackets] indicate the number of woodlice per sample. Stat. sign. diff. from: control=a; 20=b; 80=c; 160=d.

Exposure time (weeks)	Exposure concentrations (mg.kg^{-1})			
	Control	20	80	160
1	2.9 (\pm 1.5) ^{cd} [99]	3.4 (\pm 1.1) ^{cd} [100]	0.3 (\pm 1.4) ^{ab} [100]	0.2 (\pm 1.9) ^{ab} [98]
2	4.0 (\pm 1.2) ^{cd} [98]	2.6 (\pm 0.9) ^c [100]	1.1 (\pm 0.8) ^{ab} [100]	1.3 (\pm 1.5) ^a [98]
3	3.6 (\pm 1.7) ^d [96]	2.3 (\pm 2.5) [97]	1.7 (\pm 1.3) [99]	0.2 (\pm 2.4) ^a [94]
4	5.0 (\pm 3.3) [91]	1.9 (\pm 4.7) [95]	0.9 (\pm 1.9) [97]	0.5 (\pm 2.4) [91]
5	5.4 (\pm 2.7) ^{cd} [81]	0.4 (\pm 4.3) [88]	-0.1 (\pm 3.6) ^a [90]	0.7 (\pm 2.8) ^a [89]
6	6.2 (\pm 3.3) ^{cd} [80]	1.3 (\pm 4.5) [78]	-2.2 (\pm 3.3) ^a [86]	-3.5 (\pm 3.5) ^a [82]

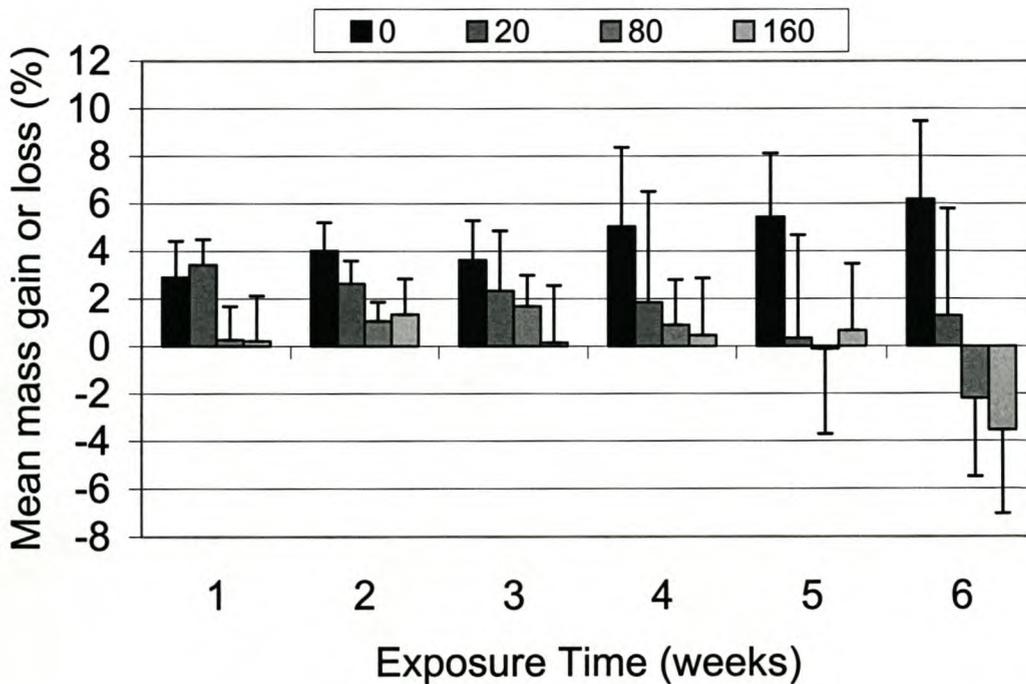


Figure 2: The mean percentage mass change (\pm SD) of *P. laevis* over 6 weeks of exposure to different nominal concentrations of cadmium sulphate (20, 80, 160 mg.kg^{-1}). See Table 3 for samples sizes and statistical comparisons.

3.1.1.2. Zinc sulphate

Changes in body mass of *P. laevis* after exposure to various concentrations of zinc sulphate, over a period of 6 weeks, are presented in Table 4 and illustrated in Fig. 3.

There was a statistically significant difference between the woodlice of all exposure groups over the exposure period in terms of changes in body mass ($P < 0.001$). Pairwise multiple comparisons showed the following pattern. After one week of exposure to zinc sulphate only the woodlice of the 8000 mg.kg⁻¹ exposure showed significant differences from the control group's woodlice in terms of mass change ($P < 0.05$). The 1000 vs 8000 mg.kg⁻¹ and 4000 vs 8000 mg.kg⁻¹ comparisons were also significantly different, concerning percentage mass change of *P. laevis* ($P < 0.05$). After two weeks of exposure the mass change of the 1000 and 8000 mg.kg⁻¹ exposure groups differed significantly from that of the control group ($P < 0.05$). The 1000 vs 8000 mg.kg⁻¹ and 4000 vs 8000 mg.kg⁻¹ comparisons differed significantly after two weeks, concerning mass change ($P < 0.05$). After three weeks of exposure the mass change of the 4000 and 8000 mg.kg⁻¹ exposure groups differed statistically significantly from that of the control group ($P < 0.05$). The comparisons 1000 vs 4000 mg.kg⁻¹, 1000 vs 8000 mg.kg⁻¹ and 4000 vs 8000 mg.kg⁻¹ all differed significantly, in terms of percentage mass change ($P < 0.05$). After four weeks the 4000 and 8000 mg.kg⁻¹ exposure groups differed significantly from the control group, in terms of percentage mass change ($P < 0.05$). The 1000 vs 8000 mg.kg⁻¹ and 4000 vs 8000 mg.kg⁻¹ comparisons showed significant differences after four weeks ($P < 0.05$). The mass changes of the 4000 and 8000 mg.kg⁻¹ exposure groups differed significantly from that of the control group after five and six weeks of exposure ($P < 0.05$). The 1000 vs 4000 mg.kg⁻¹ and 1000 vs 8000 mg.kg⁻¹ comparisons also differed significantly after five and six weeks, in terms of percentage mass change ($P < 0.05$).

Table 4: The mean percentage mass change (\pm SD) of *P. laevis* over 6 weeks of exposure to various nominal concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹). numbers in [brackets] indicate the number of woodlice per sample. Stat. sign. diff. from: control=a; 1000=b; 4000=c; 8000=d.

Exposure time (weeks)	Exposure concentrations (mg.kg ⁻¹)			
	Control	1000	4000	8000
1	2.9 (\pm 1.5) ^d [99]	1.8 (\pm 1.6) ^d [96]	1.7 (\pm 1.8) ^d [94]	-1.7 (\pm 1.4) ^{abc} [100]
2	4.0 (\pm 1.2) ^{bd} [98]	2.3 (\pm 0.7) ^{ad} [91]	2.0 (\pm 2.7) ^d [93]	-3.3 (\pm 1.2) ^{abc} [99]
3	3.6 (\pm 1.7) ^{cd} [96]	3.5 (\pm 1.1) ^{cd} [88]	-0.3 (\pm 1.3) ^{abd} [86]	-3.5 (\pm 1.6) ^{abc} [96]
4	5.0 (\pm 3.3) ^{cd} [91]	1.5 (\pm 3.7) ^d [76]	-1.9 (\pm 1.5) ^{ad} [84]	-4.4 (\pm 1.1) ^{abc} [94]
5	5.4 (\pm 2.7) ^{cd} [81]	2.4 (\pm 3.6) ^{cd} [70]	-2.3 (\pm 1.7) ^{ab} [76]	-4.3 (\pm 1.4) ^{ab} [91]
6	6.2 (\pm 3.3) ^{cd} [80]	2.9 (\pm 4.2) ^{cd} [64]	-3.4 (\pm 2.0) ^{ab} [72]	-5.2 (\pm 1.9) ^{ab} [88]

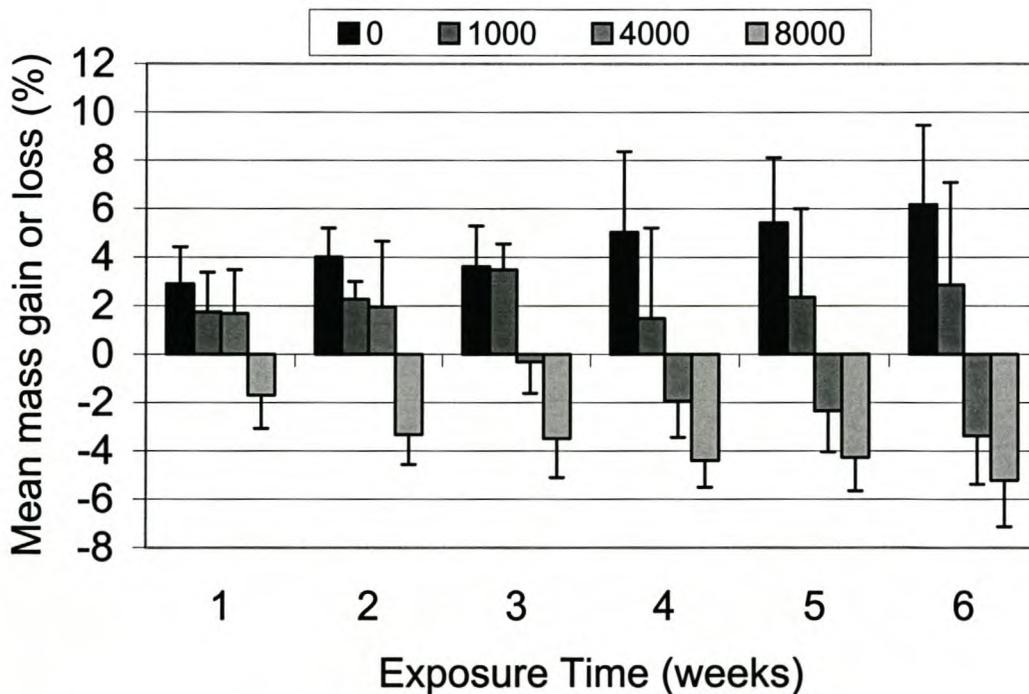


Figure 3: The mean percentage mass change (\pm SD) of *P. laevis* over 6 weeks of exposure to different nominal concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹). See Table 4 for sample sizes and statistical comparisons.

3.1.1.3. Combined effect of cadmium- and zinc sulphate

The changes in body mass of *P. laevis* during 6 weeks of combined exposure to mixtures of cadmium- and zinc sulphate are showed in Table 5 and Fig. 4. Pairwise multiple comparisons of the mass change data revealed the following differences. After one and two weeks of exposure none of the exposure groups differed significantly from each other, in terms of percentage mass change ($P>0.05$). After three, four, five and six weeks of exposure only the 20/1000 mg.kg⁻¹ (Cd/Zn) exposure group differed statistically significantly from the control group, in terms of mass change ($P<0.05$). After three weeks only the 20/1000 vs 80/4000 mg.kg⁻¹ comparison showed a statistical significant difference, in terms of mass change ($P<0.05$). After four, five and six weeks of exposure the 20/1000 vs 80/4000 mg.kg⁻¹ and 20/1000 vs 160/8000 mg.kg⁻¹ comparisons showed statistical significant differences, concerning mass change ($P<0.05$).

Table 5: The mean percentage mass change (\pm SD) of *P. laevis* after combined exposure to mixtures (Cd/Zn) of cadmium- and zinc sulphate. Numbers in [brackets] indicate number of woodlice per sample. Stat. sign. diff. from: control=a; 20/1000=b; 80/4000=c; 160/8000=d.

Exposure time (weeks)	Exposure concentrations (mg.kg ⁻¹)			
	Control	20/1000	80/4000	160/8000
1	2.9 (\pm 1.5) [99]	4.6 (\pm 1.6) [100]	2.3 (\pm 1.7) [99]	2.7 (\pm 1.7) [99]
2	4.0 (\pm 1.2) [98]	5.6 (\pm 1.4) [100]	3.7 (\pm 1.0) [95]	4.5 (\pm 1.9) [97]
3	3.6 (\pm 1.7) ^b [96]	7.2 (\pm 0.9) ^{ac} [99]	5.2 (\pm 1.1) ^b [92]	5.0 (\pm 2.5) [97]
4	5.0 (\pm 3.3) ^b [91]	9.8 (\pm 1.9) ^{acd} [98]	5.9 (\pm 2.9) ^b [89]	5.7 (\pm 3.0) ^b [92]
5	5.4 (\pm 2.7) ^b [81]	12.4 (\pm 2.8) ^{acd} [93]	5.6 (\pm 1.5) ^b [85]	3.9 (\pm 2.5) ^b [89]
6	6.2 (\pm 3.3) ^b [80]	13.8 (\pm 2.5) ^{acd} [89]	4.6 (\pm 2.1) ^b [82]	4.3 (\pm 1.5) ^b [83]

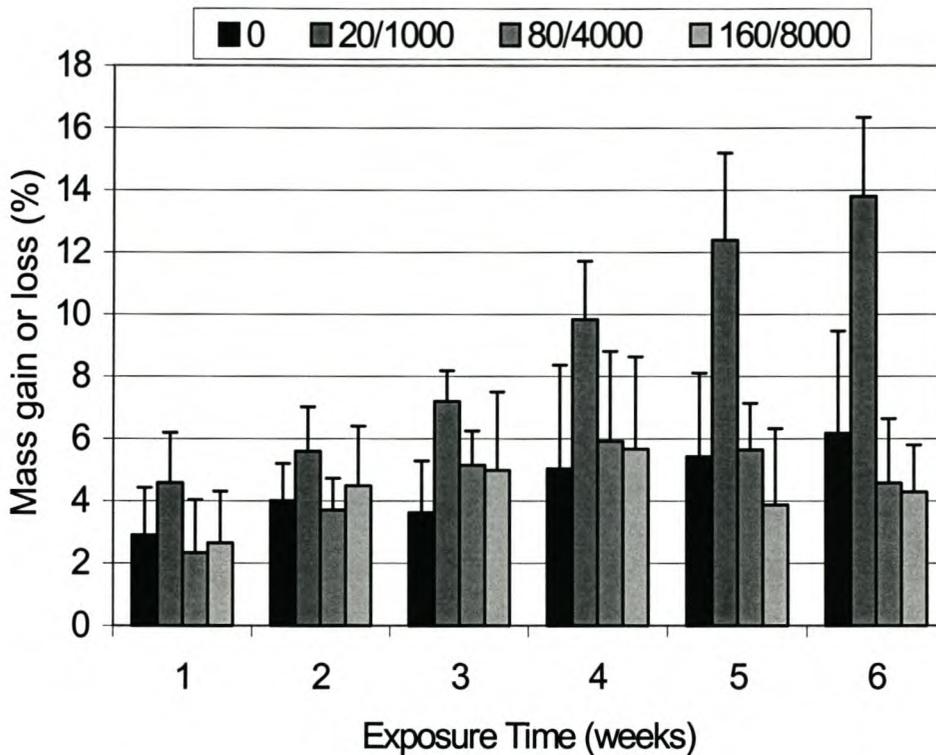


Figure 4: The mean percentage mass change (\pm SD) of *P. laevis* after combined exposure to different mixtures (Cd/Zn) of cadmium- and zinc sulphate. See Table 5 for samples size and statistical comparisons.

3.1.1.4. Comparison between singular concentrations & binary combinations of cadmium- and zinc sulphate

The comparisons of mass changes between exposure groups where cadmium sulphate was administered separately and combined with zinc sulphate are shown in Table 6 and Fig. 5. The following comparisons were found to be statistically different from each other after pairwise multiple comparison analysis. The 20 vs 20/1000 mg.kg⁻¹ (Fig. 5A) and 80 vs 80/4000 mg.kg⁻¹ (Fig. 5B) comparisons differed statistically significantly after two, three, four, five and six weeks of exposure, concerning percentage mass change ($P < 0.05$). The 160 vs 160/8000 mg.kg⁻¹ comparison (Fig. 5C) showed statistically significant differences after two, three, four and six weeks of exposure, concerning mass change ($P < 0.05$).

Table 6: Comparison between percentages mass change (\pm SD) of *P. laevis* during exposure for 6 weeks to cadmium sulphate (20, 80, 160 mg.kg⁻¹) and combined exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. Numbers in [brackets] indicate number of woodlice per sample. See Fig. 5 for statistics.

Exposure time (weeks)	Exposure concentrations (mg.kg ⁻¹)						
	Control	20	mix1	80	mix2	160	mix3
1	2.9 (\pm 1.5) [99]	3.4 (\pm 1.1) [100]	4.6 (\pm 1.6) [100]	0.3 (\pm 1.4) [100]	2.3 (\pm 1.7) [99]	0.2 (\pm 1.9) [98]	2.7 (\pm 1.7) [99]
2	4.0 (\pm 1.2) [98]	2.6 (\pm 0.9) [100]	5.6 (\pm 1.4) [100]	1.1 (\pm 0.8) [100]	3.7 (\pm 1.0) [95]	1.3 (\pm 1.5) [98]	4.5 (\pm 1.9) [97]
3	3.6 (\pm 1.7) [96]	2.3 (\pm 2.5) [97]	7.2 (\pm 0.9) [99]	1.7 (\pm 1.3) [99]	5.2 (\pm 1.1) [92]	0.2 (\pm 2.4) [94]	5.0 (\pm 2.5) [97]
4	5.0 (\pm 3.3) [91]	1.9 (\pm 4.7) [95]	9.8 (\pm 1.9) [98]	0.9 (\pm 1.9) [97]	5.9 (\pm 2.9) [89]	0.5 (\pm 2.4) [91]	5.7 (\pm 3.0) [92]
5	5.4 (\pm 2.7) [81]	0.4 (\pm 4.3) [88]	12.4 (\pm 2.8) [93]	-0.1 (\pm 3.6) [90]	5.6 (\pm 1.5) [85]	0.7 (\pm 2.8) [89]	3.9 (\pm 2.5) [89]
6	6.2 (\pm 3.3) [80]	1.3 (\pm 4.5) [78]	13.8 (\pm 2.5) [89]	-2.2 (\pm 3.3) [86]	4.6 (\pm 2.1) [82]	-3.5 (\pm 3.5) [82]	4.3 (\pm 1.5) [83]

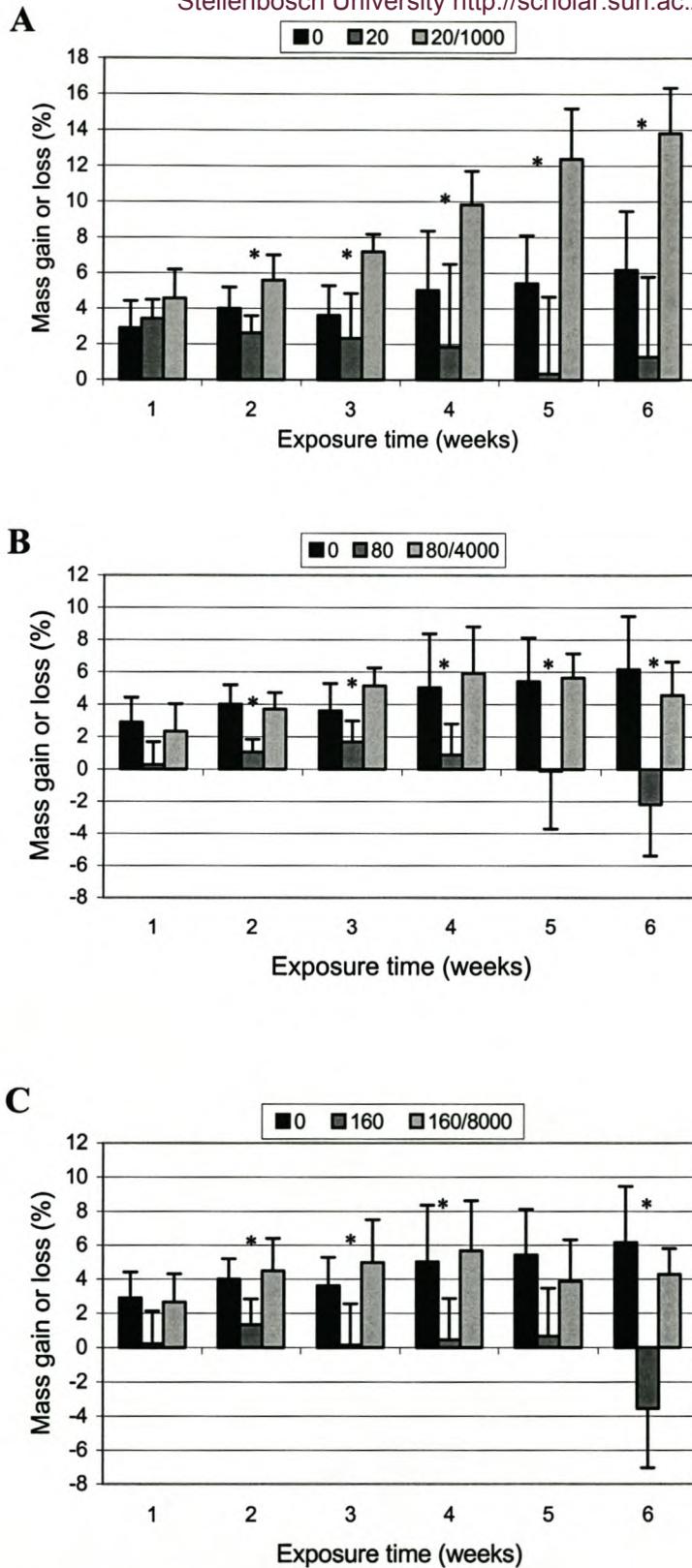


Figure 5: Comparison between mass change percentages (\pm SD) of *P. laevis* during 6 weeks exposure to nominal concentrations of cadmium sulphate (A:20, B:80, C:160 mg.kg^{-1}) and combined exposure to various mixtures of cadmium- and zinc sulphate (A:20/1000, B:80/4000, C:160/8000 mg.kg^{-1}). Stat. sign. diff. between single metal exposure and combined exposure indicated by (*). See Table 6 for sample sizes.

The comparisons of mass changes between exposure groups where zinc sulphate was administered separately and combined with cadmium sulphate are shown in Table 7 and Fig. 6. The 1000 vs 20/1000 mg.kg⁻¹ comparison (Fig. 6A) showed statistical significant differences throughout the exposure period, in terms of mass change ($P < 0.05$). There were statistical significant differences, in terms of mass change, between woodlice exposed to 4000 mg.kg⁻¹ zinc sulphate and woodlice exposed to the 80/4000 mg.kg⁻¹ cadmium- and zinc sulphate mixture (Fig. 6B) after three, four, five and six weeks of exposure ($P < 0.05$). The 8000 vs 160/8000 mg.kg⁻¹ comparison (Fig. 6C) showed statistical significant differences throughout the exposure period of six weeks, in terms of mass change ($P < 0.05$).

Table 7: Comparison between percentages mass change (\pm SD) of *P. laevis* during exposure for 6 weeks to zinc sulphate (1000, 4000, 8000 mg.kg^{-1}) and combined exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg^{-1} CdSO_4 with 1000 mg.kg^{-1} ZnSO_4 ; mix2 = 80 mg.kg^{-1} CdSO_4 with 4000 mg.kg^{-1} ZnSO_4 ; mix3 = 160 mg.kg^{-1} CdSO_4 with 8000 mg.kg^{-1} ZnSO_4 . Numbers in [brackets] indicate number of woodlice per sample. See Fig. 6 for statistics.

Exposure time (weeks)	Exposure concentrations (mg.kg^{-1})						
	Control	1000	mix1	4000	mix2	8000	mix3
1	2.9 (\pm 1.5) [99]	1.8 (\pm 1.6) [96]	4.6 (\pm 1.6) [100]	1.7 (\pm 1.8) [94]	2.3 (\pm 1.7) [99]	-1.7 (\pm 1.4) [100]	2.7 (\pm 1.7) [99]
2	4.0 (\pm 1.2) [98]	2.3 (\pm 0.7) [91]	5.6 (\pm 1.4) [100]	2.0 (\pm 2.7) [93]	3.7 (\pm 1.0) [95]	-3.3 (\pm 1.2) [99]	4.5 (\pm 1.9) [97]
3	3.6 (\pm 1.7) [96]	3.5 (\pm 1.1) [88]	7.2 (\pm 0.9) [99]	-0.3 (\pm 1.3) [86]	5.2 (\pm 1.1) [92]	-3.5 (\pm 1.6) [96]	5.0 (\pm 2.5) [97]
4	5.0 (\pm 3.3) [91]	1.5 (\pm 3.7) [76]	9.8 (\pm 1.9) [98]	-1.9 (\pm 1.5) [84]	5.9 (\pm 2.9) [89]	-4.4 (\pm 1.1) [94]	5.7 (\pm 3.0) [92]
5	5.4 (\pm 2.7) [81]	2.4 (\pm 3.6) [70]	12.4 (\pm 2.8) [93]	-2.3 (\pm 1.7) [76]	5.6 (\pm 1.5) [85]	-4.3 (\pm 1.4) [91]	3.9 (\pm 2.5) [89]
6	6.2 (\pm 3.3) [80]	2.9 (\pm 4.2) [64]	13.8 (\pm 2.5) [89]	-3.4 (\pm 2.0) [72]	4.6 (\pm 2.1) [82]	-5.2 (\pm 1.9) [88]	4.3 (\pm 1.5) [83]

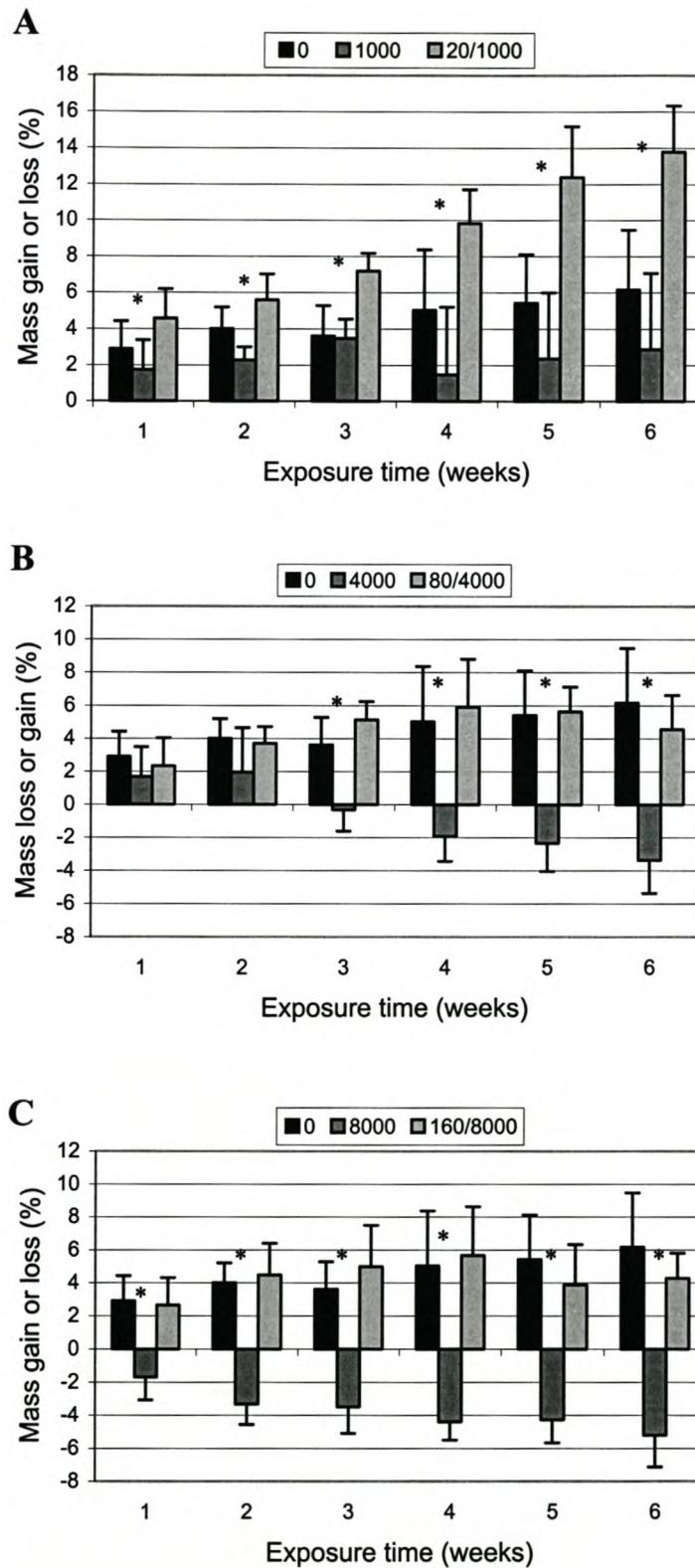


Figure 6: Comparison between mass change percentages (\pm SD) of *P. laevis* during 6 weeks exposure to nominal concentrations of zinc sulphate (A:1000, B:4000, C:8000 mg.kg⁻¹) and combined exposure to various mixtures of cadmium- and zinc sulphate (A:20/1000, B:80/4000, C:160/8000 mg.kg⁻¹). Stat. sign. diff. between single metal exposure and combined exposure indicated by (*). See Table 7 for sample sizes.

3.1.2. Bioaccumulation and compartmentalisation of cadmium and zinc in *P. laevis*

3.1.2.1. Cadmium accumulation in *P. laevis* after exposure to cadmium sulphate

The accumulation of cadmium in the hepatopancreas samples of *P. laevis* after exposure for six weeks to various concentrations of cadmium sulphate is presented in Table 8 and illustrated in Fig. 7. The concentrations of Cd in the hepatopancreas of woodlice of all the exposure groups (20, 80, 160 mg.kg⁻¹) differed statistically significantly from that of the control, after pairwise multiple comparisons ($P < 0.05$). There was also statistically significant differences, in terms of Cd concentrations in the hepatopancreas of woodlice, in the following comparisons after pairwise multiple comparison analysis: 20 vs 80 mg.kg⁻¹, 20 vs 160 mg.kg⁻¹ and 80 vs 160 mg.kg⁻¹ ($P < 0.05$).

The concentrations of cadmium in the rest of the body samples of *P. laevis* after six weeks of exposure to cadmium sulphate (20, 80, 160 mg.kg⁻¹) are shown in Table 8 and Fig. 8. The Cd concentrations in the rest of the body of *P. laevis* exposed to 20, 80 and 160 mg.kg⁻¹ cadmium sulphate differed statistically significantly from that of the control ($P < 0.05$). There was also statistical significant differences in the 20 vs 80 mg.kg⁻¹, 20 vs 160 mg.kg⁻¹ and 80 vs 160 mg.kg⁻¹ comparisons, in terms of Cd concentrations in the rest of the body of the woodlice, after pairwise multiple comparison ($P < 0.05$).

Table 8: The mean cadmium concentrations (mg.kg^{-1} ; dry mass) ($\pm\text{SD}$) in the hepatopancreas and rest of the body samples of *P. laevis* after 6 weeks of exposure to various concentrations of cadmium sulphate (20, 80, 160 mg.kg^{-1}). $n=5$. Stat. sign. diff. from: control=a; 20=b; 80=c; 160=d ($P<0.05$). ND = not detectable.

	Exposure concentrations (mg.kg^{-1})			
	Control	20	80	160
hepatopancreas	ND ^{bcd}	75.2 (± 7.6) ^{acd}	370.9 (± 103.7) ^{abd}	1004.6 (± 239.3) ^{abc}
% of total body load	-----	98.9	98.9	98.9
rest of the body	ND ^{bcd}	0.8 (± 0.06) ^{acd}	4.3 (± 0.8) ^{abd}	11.2 (± 0.9) ^{abc}
% of total body load	-----	1.1	1.1	1.1

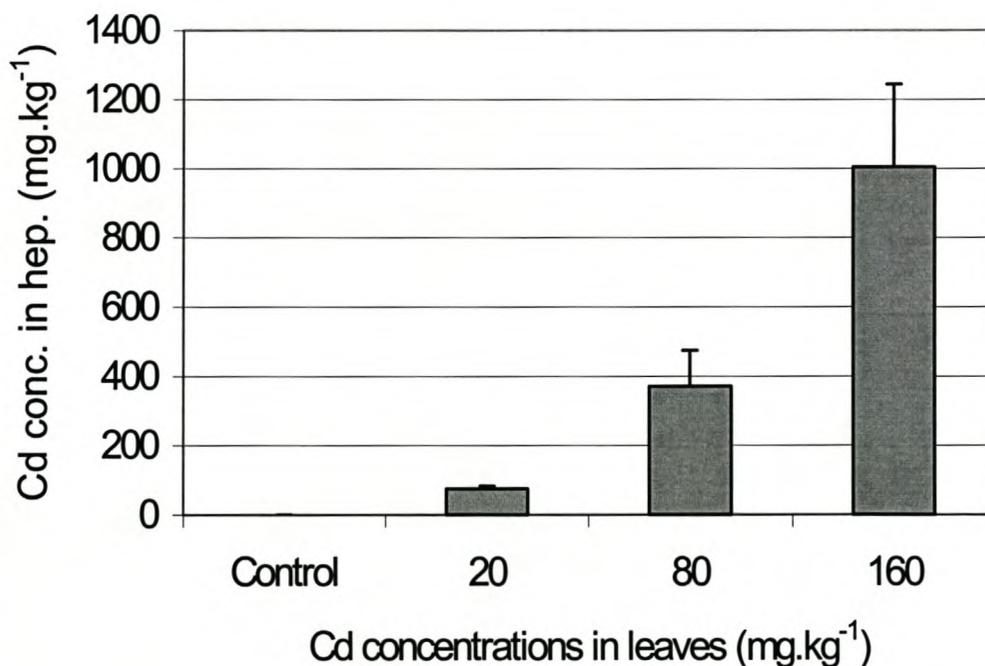


Figure 7: The mean cadmium concentrations (mg.kg^{-1} ; dry mass) ($\pm\text{SD}$) in the hepatopancreas of *P. laevis* after 6 weeks of exposure to various concentrations of cadmium sulphate (20, 80, 160 mg.kg^{-1}). $n=5$. See Table 8 for statistics.

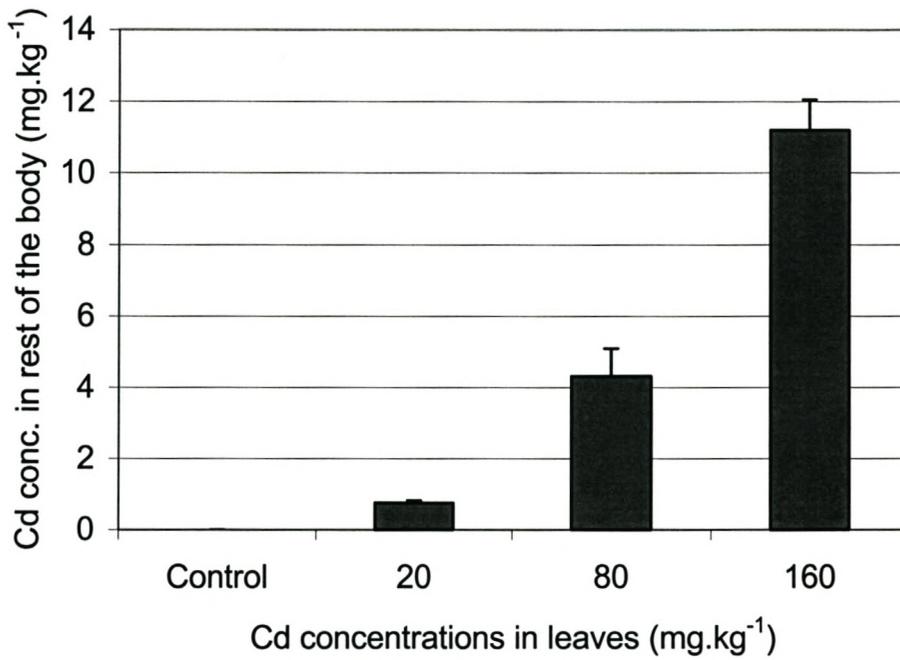


Figure 8: The mean cadmium concentrations (mg.kg⁻¹; dry mass) (\pm SD) in the rest of the body samples (excluding hepatopancreas) of *P. laevis* after 6 weeks of exposure to various nominal concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹). n=5. See Table 8 for statistics.

3.1.2.2. Zinc accumulation in *P. laevis* after exposure to zinc sulphate

The accumulation of zinc in the hepatopancreas of *P. laevis* after exposure to different concentrations of zinc sulphate is shown in Table 9 and Fig. 9. Only the woodlice of the 8000 mg.kg⁻¹ exposure group showed a statistically significantly higher concentration of Zn in the hepatopancreas in comparison to that of the control group after the 6 week exposure period (P<0.05). The concentrations of Zn in the hepatopancreas of woodlice exposed to 8000 mg.kg⁻¹ are statistically significantly higher than concentrations of Zn in the hepatopancreas of woodlice exposed to 1000 and 4000 mg.kg⁻¹ (P<0.05).

The concentrations of zinc in the body rests of *P. laevis* after exposure to zinc sulphate are shown in Table 9 and Fig. 10. Woodlice exposed to 4000 and 8000 mg.kg⁻¹ zinc sulphate had statistically significantly higher Zn concentrations in rest of the body samples than woodlice of the control group after 6 weeks of exposure (P<0.05). There was no statistically significant difference between woodlice exposed to 1000, 4000 and 8000 mg.kg⁻¹ zinc sulphate after the 6 week exposure period, in terms of Zn concentrations in rest of the body samples (P>0.05).

Table 9: The mean zinc concentrations (mg.kg⁻¹; dry mass) (±SD) in the hepatopancreas and rest of the body samples of *P. laevis* after 6 weeks of exposure to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹). n=5. Stat. sign. diff. from: control=a; 1000=b; 4000=c; 8000=d (P<0.05).

	Exposure concentrations (mg.kg ⁻¹)			
	Control	1000	4000	8000
hepatopancreas	2221.9 (±530.9) ^d	1675.7 (±544.1) ^d	2529.6 (±1052.9) ^d	3516.4 (±675.8) ^{abc}
% of total body load	99.7	99.5	99.6	99.7
rest of the body	6.7 (±0.6) ^{cd}	8.8 (±2.8)	9.3 (±4.2) ^a	10.6 (±1.3) ^a
% of total body load	0.3	0.5	0.4	0.3

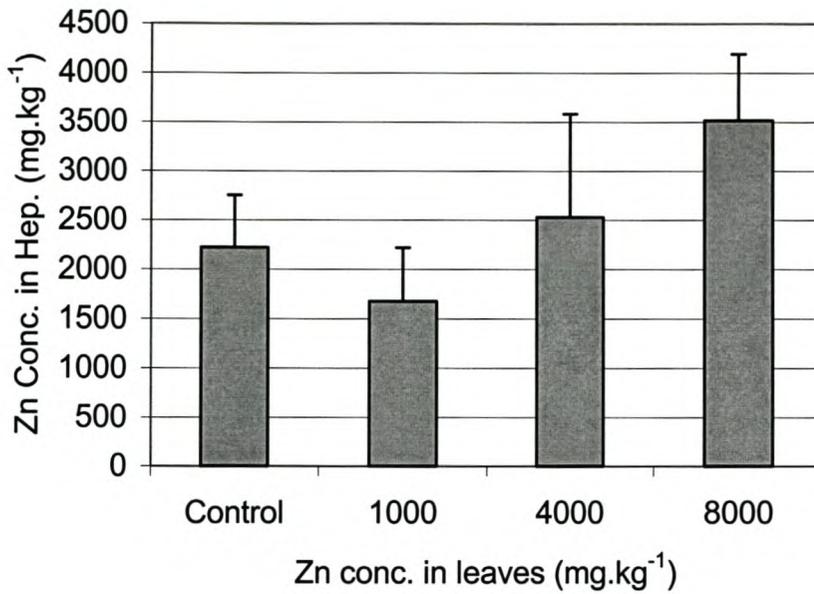


Figure 9: The mean zinc concentrations (mg.kg⁻¹; dry mass) (\pm SD) in the hepatopancreas of *P. laevis* after 6 weeks of exposure to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹). n=5. See Table 9 for statistics.

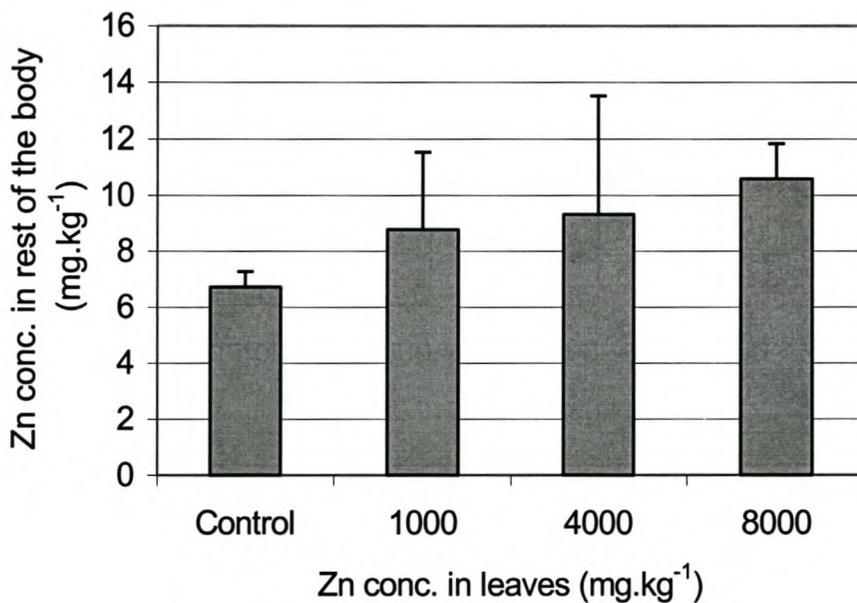


Figure 10: The mean zinc concentrations (mg.kg⁻¹; dry mass) (\pm SD) in rest of the body samples (excluding the hepatopancreas) of *P. laevis* after 6 weeks of exposure to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹). n=5. See Table 9 for statistics.

3.1.2.3. Accumulation of cadmium in *P. laevis* after combined exposure to cadmium- and zinc sulphate

The concentrations of cadmium in the hepatopancreas samples of *P. laevis* after 6 weeks of exposure to mixtures of cadmium- and zinc sulphate are shown in Table 10 and Fig. 11. Cadmium concentrations in the hepatopancreas of woodlice of all exposure groups were significantly higher than in the control group after 6 weeks of exposure ($P < 0.05$). None of the exposure groups differed statistically significantly from each other after the 6 week exposure period, concerning cadmium concentrations in the hepatopancreas ($P > 0.05$).

Cadmium concentrations in the rest of the body samples (excluding the hepatopancreas) of *P. laevis* after 6 weeks of exposure to mixtures of cadmium- and zinc sulphate are shown in Table 10 and Fig. 12. Cadmium concentrations in rest of the body samples of woodlice in all the exposure groups were statistically significantly higher than that of the control group ($P < 0.05$). Cadmium concentrations in rest of the body samples of woodlice exposed to mix1 were significantly lower than the case of woodlice exposed to mix2 and mix3 ($P < 0.05$) (Table 10).

Table 10: The mean cadmium concentrations (mg.kg^{-1} ; dry mass) (\pm SD) in hepatopancreas and rest of the body samples of *P. laevis* after 6 weeks of exposure to mixtures of cadmium- and zinc sulphate. $n=5$. Stat. sign. diff. from: control=a; mix1=b; mix2=c; mix3=d ($P < 0.05$). ND = not detectable. mix1 = 20 mg.kg^{-1} CdSO_4 with 1000 mg.kg^{-1} ZnSO_4 ; mix2 = 80 mg.kg^{-1} CdSO_4 with 4000 mg.kg^{-1} ZnSO_4 ; mix3 = 160 mg.kg^{-1} CdSO_4 with 8000 mg.kg^{-1} ZnSO_4

	Exposure concentrations (mg.kg^{-1})			
	Control	mix1	mix2	mix3
hepatopancreas	ND ^{bcd}	134.2 (\pm 55.1) ^a	217.7 (\pm 91.6) ^a	189.8 (\pm 39.7) ^a
% of total body load	-----	99.4	98.5	98.2
rest of the body	ND ^{bcd}	0.8 (\pm 0.3) ^{acd}	3.4 (\pm 0.6) ^{ab}	3.5 (\pm 0.3) ^{ab}
% of total body load	-----	0.6	1.5	1.8

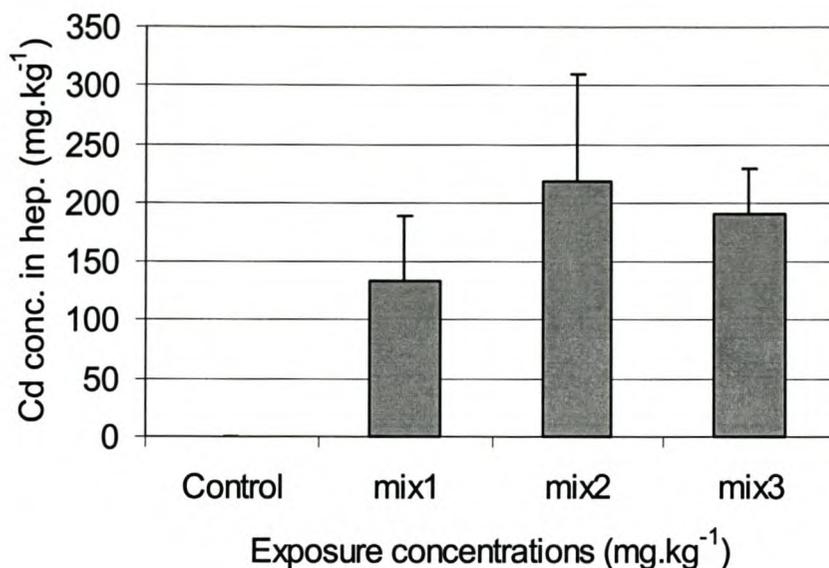


Figure 11: The mean cadmium concentrations (mg.kg⁻¹; dry mass) (\pm SD) in hepatopancreas samples of *P. laevis* after 6 weeks of exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. n=5. See Table 10 for statistics.

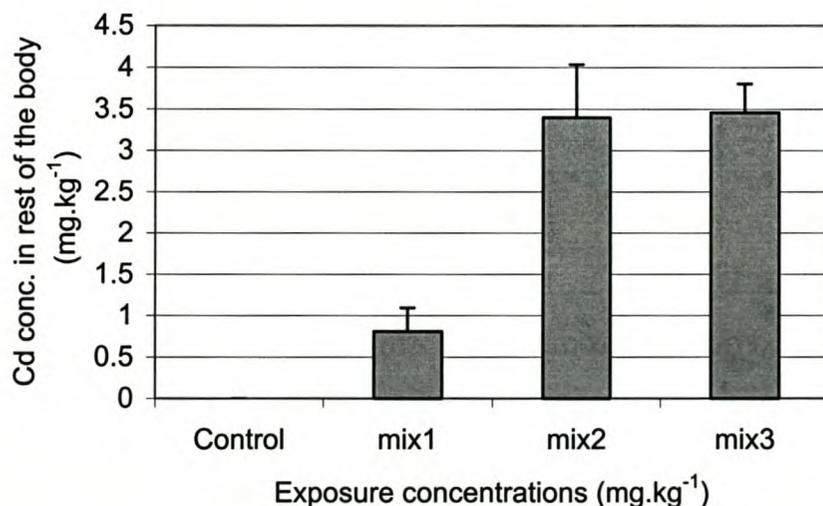


Figure 12: The mean cadmium concentrations (mg.kg⁻¹; dry mass) (\pm SD) in the rest of the body samples (excluding hepatopancreas) of *P. laevis* after 6 weeks of exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. n=5. See Table 10 for statistics.

3.1.2.4. Accumulation of zinc in *P. laevis* after combined exposure to cadmium- and zinc sulphate

The concentrations of zinc in the hepatopancreas of *P. laevis* after 6 weeks of exposure to mixtures of cadmium- and zinc sulphate are shown in Table 11 and Fig. 13. No statistical significant differences were found between the exposure groups and control group when zinc concentrations in the hepatopancreas were compared ($P>0.05$). Pairwise multiple comparison analysis revealed that zinc concentrations in the hepatopancreas were only significantly different between woodlice exposed to mix1 and mix3 ($P<0.05$).

The concentrations of zinc the rest of the body samples of *P. laevis* after 6 weeks of exposure to mixtures of cadmium- and zinc sulphate are shown in Table 11 and Fig. 14. Zinc concentrations in rest of the body samples of woodlice of all the exposure groups differed significantly from that of the control group after six weeks ($P<0.05$). Zinc concentrations in the rest of the body samples of all the exposure groups differed significantly from each other ($P<0.05$).

Table 11: The mean zinc concentrations (mg.kg^{-1} ; dry mass) ($\pm\text{SD}$) in hepatopancreas and rest of the body samples of *P. laevis* after 6 weeks of exposure to mixtures of cadmium- and zinc sulphate. $n=5$. Stat. sign. diff. from: control=a; mix1=b; mix2=c; mix3=d ($P<0.05$). mix1 = $20 \text{ mg.kg}^{-1} \text{ CdSO}_4$ with $1000 \text{ mg.kg}^{-1} \text{ ZnSO}_4$; mix2 = $80 \text{ mg.kg}^{-1} \text{ CdSO}_4$ with $4000 \text{ mg.kg}^{-1} \text{ ZnSO}_4$; mix3 = $160 \text{ mg.kg}^{-1} \text{ CdSO}_4$ with $8000 \text{ mg.kg}^{-1} \text{ ZnSO}_4$

	Exposure concentrations (mg.kg^{-1})			
	Control	mix1	mix2	mix3
hepatopancreas	2221.9 (± 530.9)	1541.9 (± 497.8) ^d	2081.7 (± 176.6)	2464.5 (± 351.9) ^b
% of total body load	99.7	99.6	99.6	99.6
rest of the body	6.7 (± 0.6) ^{bcd}	6.1 (± 0.3) ^{acd}	8.8 (± 1.0) ^{abd}	10.8 (± 1.4) ^{abc}
% of total body load	0.3	0.4	0.4	0.4

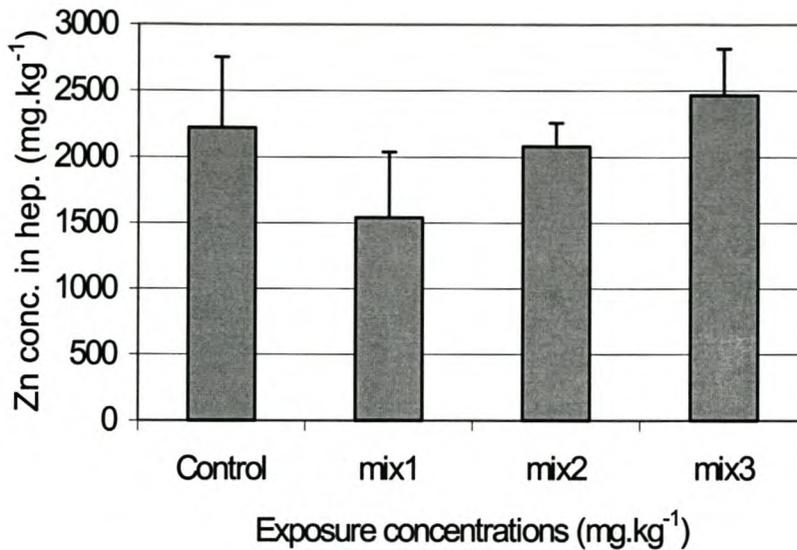


Figure 13: The mean zinc concentrations (mg.kg⁻¹; dry mass) (\pm SD) in hepatopancreas samples of *P. laevis* after 6 weeks of exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. n=5. See Table 11 for statistics.

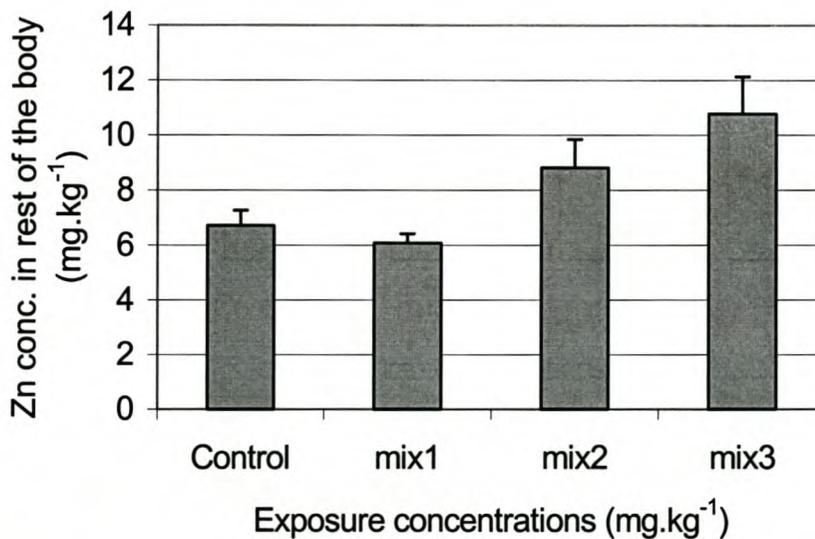


Figure 14: The mean zinc concentrations (mg.kg⁻¹; dry mass) (\pm SD) in the rest of the body samples (excluding hepatopancreas) of *P. laevis* after 6 weeks of exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. n=5. See Table 11 for statistics.

3.1.2.5. Comparison of cadmium concentrations in *P. laevis* after singular exposure to cadmium sulphate and combined exposure to cadmium- and zinc sulphate

The comparisons between the hepatopancreas cadmium concentrations in *P. laevis* after 6 weeks of exposure to various concentrations of cadmium sulphate and mixtures of cadmium- and zinc sulphate are presented in Table 12 and Fig. 15. Pairwise multiple comparisons showed that cadmium concentrations in hepatopancreas samples of woodlice exposed to 20 mg.kg⁻¹ cadmium sulphate were significantly lower than in the case of mix1. Cadmium concentrations in the hepatopancreas of woodlice exposed to 80 mg.kg⁻¹ cadmium sulphate were significantly higher than in the case of mix2. Cadmium concentrations in the hepatopancreas of woodlice exposed to 160 mg.kg⁻¹ cadmium sulphate were significantly higher than in the case of mix3 (P<0.05) (Table 12; Fig. 15).

The comparisons between the cadmium concentrations in rest of the body samples (excluding hepatopancreas) of *P. laevis* after 6 weeks of exposure to cadmium sulphate and mixtures of cadmium- and zinc sulphate are shown in Table 12 and Fig. 16. After pairwise multiple comparison analysis it was shown that cadmium concentrations in the rest of the body samples of woodlice exposed to 160 mg.kg⁻¹ cadmium sulphate were significantly higher than in the case of mix3 (P<0.05).

Table 12: Comparison of cadmium concentrations (mg.kg⁻¹; dry mass) (\pm SD) in the hepatopancreas and rest of the body samples of *P. laevis* after exposure for 6 weeks to cadmium sulphate (20, 80, 160 mg.kg⁻¹) and exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. n=5. See Figures 15 and 16 for statistics.

	Exposure concentrations (mg.kg ⁻¹)						
	Control	20	mix1	80	mix2	160	mix3
Hepato pancreas	ND	75.2 (\pm 7.6)	134.2 (\pm 55.1)	370.9 (\pm 103.7)	217.7 (\pm 91.6)	1004.6 (\pm 239.3)	189.8 (\pm 39.7)
Rest of the body	ND	0.8 (\pm 0.06)	0.8 (\pm 0.3)	4.3 (\pm 0.8)	3.4 (\pm 0.6)	11.2 (\pm 0.9)	3.5 (\pm 0.3)

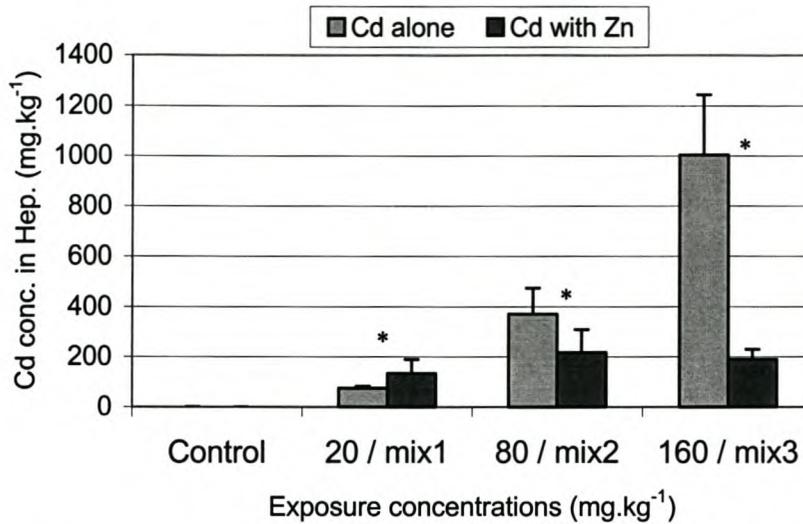


Figure 15: Comparison between cadmium concentrations (mg.kg^{-1} ; dry mass) ($\pm\text{SD}$) in the hepatopancreas of *P. laevis* after exposure to cadmium sulphate (20, 80, 160 mg.kg^{-1}) and exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg^{-1} CdSO_4 with 1000 mg.kg^{-1} ZnSO_4 ; mix2 = 80 mg.kg^{-1} CdSO_4 with 4000 mg.kg^{-1} ZnSO_4 ; mix3 = 160 mg.kg^{-1} CdSO_4 with 8000 mg.kg^{-1} ZnSO_4 . Stat. sign. diff. between single metal exposure and joint exposure indicated by (*) ($P < 0.05$). $n = 5$.

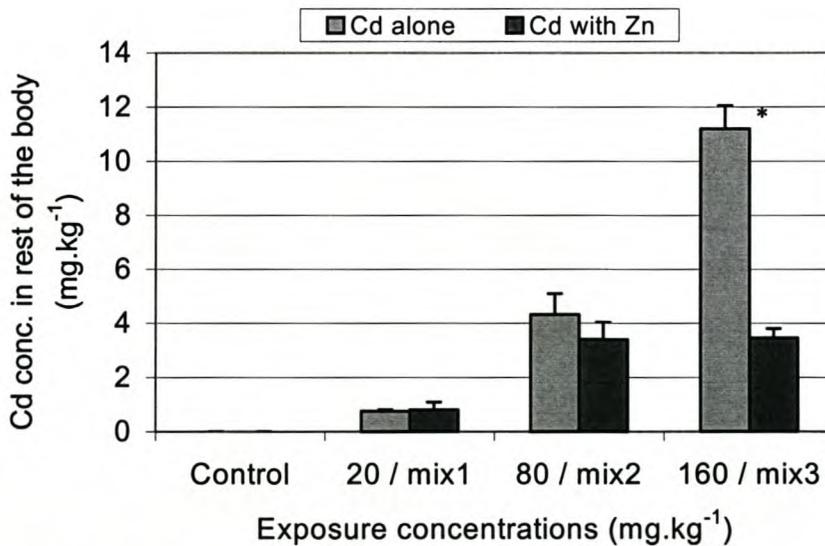


Figure 16: Comparison between cadmium concentrations (mg.kg^{-1} ; dry mass) ($\pm\text{SD}$) in the rest of the body samples of *P. laevis* after exposure to cadmium sulphate (20, 80, 160 mg.kg^{-1}) and exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg^{-1} CdSO_4 with 1000 mg.kg^{-1} ZnSO_4 ; mix2 = 80 mg.kg^{-1} CdSO_4 with 4000 mg.kg^{-1} ZnSO_4 ; mix3 = 160 mg.kg^{-1} CdSO_4 with 8000 mg.kg^{-1} ZnSO_4 . Stat. sign. diff. between single metal exposure and joint exposure indicated by (*) ($P < 0.05$). $n = 5$.

3.1.2.6. Comparison of zinc concentrations in *P. laevis* after singular exposure to zinc sulphate and combined exposure to cadmium- and zinc sulphate

The comparisons between the zinc concentrations in hepatopancreas samples of *P. laevis* after 6 weeks of exposure to zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) and mixtures of cadmium- and zinc sulphate are presented in Table 13 and Fig. 17. Pairwise multiple comparisons showed that the zinc concentrations in the hepatopancreas of woodlice exposed to 8000 mg.kg⁻¹ zinc sulphate were significantly higher than in the case of mix3 (P<0.05) (Table 13; Fig. 17).

The comparisons between zinc concentrations in rest of the body samples (excluding hepatopancreas) of *P. laevis* after 6 weeks of exposure to zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) and mixtures of cadmium- and zinc sulphate are shown in Table 13 and Fig. 18. Pairwise multiple comparisons showed that the zinc concentrations in the rest of the body samples (excluding hepatopancreas) of woodlice exposed to 1000 mg.kg⁻¹ zinc sulphate were significantly higher than in the case of mix1 (P<0.05).

Table 13: Comparison of zinc concentrations (mg.kg⁻¹; dry mass) (\pm SD) in the hepatopancreas and rest of the body samples of *P. laevis* after exposure for 6 weeks to zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) and exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. n=5. See Figures 17 and 18 for statistics.

	Exposure concentrations (mg.kg ⁻¹)						
	Control	1000	mix1	4000	mix2	8000	mix3
Hepato pancreas	2221.9 (\pm 530.9)	1675.7 (\pm 544.1)	1541.9 (\pm 497.8)	2529.6 (\pm 1053)	2081.7 (\pm 176.6)	3516.4 (\pm 675.8)	2464.5 (\pm 351.9)
Rest of the body	6.7 (\pm 0.6)	8.8 (\pm 2.8)	6.1 (\pm 0.3)	9.3 (\pm 4.2)	8.8 (\pm 1.0)	10.6 (\pm 1.3)	10.8 (\pm 1.4)

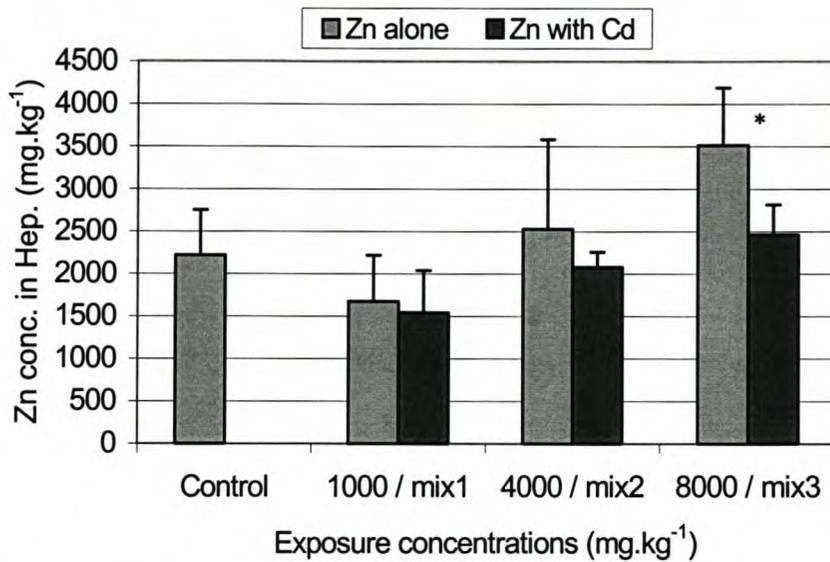


Figure 17: Comparison between zinc concentrations (mg.kg^{-1} ; dry mass) ($\pm\text{SD}$) in the hepatopancreas of *P. laevis* after exposure to zinc sulphate (1000, 4000, 8000 mg.kg^{-1}) and exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg^{-1} CdSO_4 with 1000 mg.kg^{-1} ZnSO_4 ; mix2 = 80 mg.kg^{-1} CdSO_4 with 4000 mg.kg^{-1} ZnSO_4 ; mix3 = 160 mg.kg^{-1} CdSO_4 with 8000 mg.kg^{-1} ZnSO_4 . Stat. sign. diff. between single metal exposure and joint exposure indicated by (*) ($P < 0.05$). $n=5$.

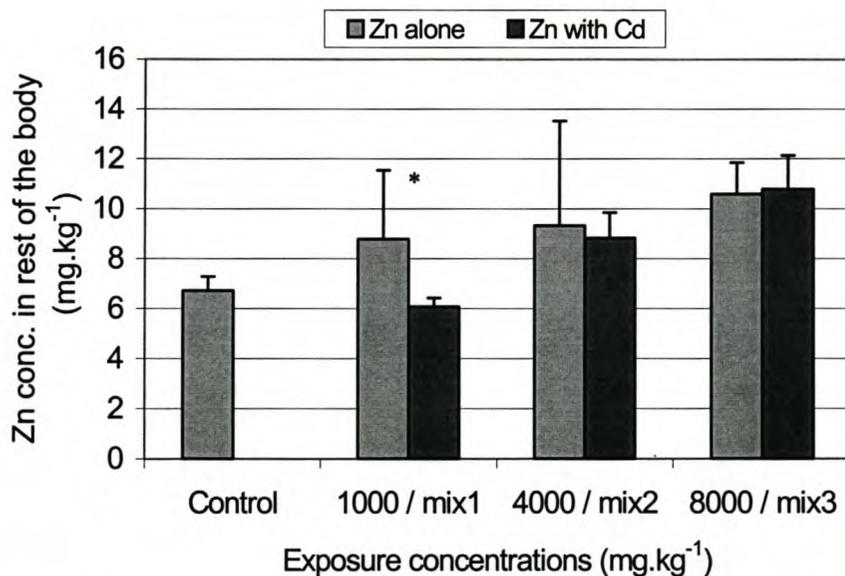


Figure 18: Comparison between zinc concentrations (mg.kg^{-1} ; dry mass) ($\pm\text{SD}$) in the rest of the body samples of *P. laevis* after exposure to zinc sulphate (1000, 4000, 8000 mg.kg^{-1}) and exposure to various mixtures of cadmium- and zinc sulphate. mix1 = 20 mg.kg^{-1} CdSO_4 with 1000 mg.kg^{-1} ZnSO_4 ; mix2 = 80 mg.kg^{-1} CdSO_4 with 4000 mg.kg^{-1} ZnSO_4 ; mix3 = 160 mg.kg^{-1} CdSO_4 with 8000 mg.kg^{-1} ZnSO_4 . Stat. sign. diff. between single metal exposure and joint exposure indicated by (*) ($P < 0.05$). $n=5$.

3.1.3. Histological analysis of the hepatopancreas of *P. laevis*

3.1.3.1. Percentage Cellular Area (PCA) in the hepatopancreas after exposure to cadmium sulphate

Figure 19 photographically illustrates examples of histological sections made in the Z3 zone of hepatopancreas samples of the control group and 20, 80 and 160 mg.kg⁻¹ cadmium sulphate exposure groups.

Table 14 show the PCA measurements in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after exposure to various concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹) for 6 weeks. In this Table interzonal comparisons are made, and also comparisons between the different exposure groups, within a particular zone. In Figure 20 the different zones (Z1-Z4), of each of the exposure groups (control, 20, 80, 160 mg.kg⁻¹), are compared to each other. Statistical analysis of the data revealed that there was significant differences in the PCA measurements among all four zones in the hepatopancreas of the control group and the 20 mg.kg⁻¹ cadmium sulphate exposure group ($P < 0.05$). In the 80 mg.kg⁻¹ exposure group PCA measurements of all the zones, apart from that of Z3 and Z4, differed significantly from each other after pairwise multiple comparison analysis ($P < 0.05$). In the 160 mg.kg⁻¹ exposure group only the PCA measurements of Z1 and Z3 did not differ statistically significantly from each other ($P > 0.05$).

The PCA measurements of hepatopancreas sections of the control group and all cadmium sulphate exposure groups (20, 80 and 160 mg.kg⁻¹), within a particular zone, are shown and compared in Table 14 and Fig. 21. Pairwise multiple comparison analysis revealed the following differences. In the Z1, Z2, Z3 and Z4 zones PCA measurements of all the exposure groups (20, 80, 160 mg.kg⁻¹) differed significantly from that of the control group ($P < 0.05$). In Z1 and Z2 PCA

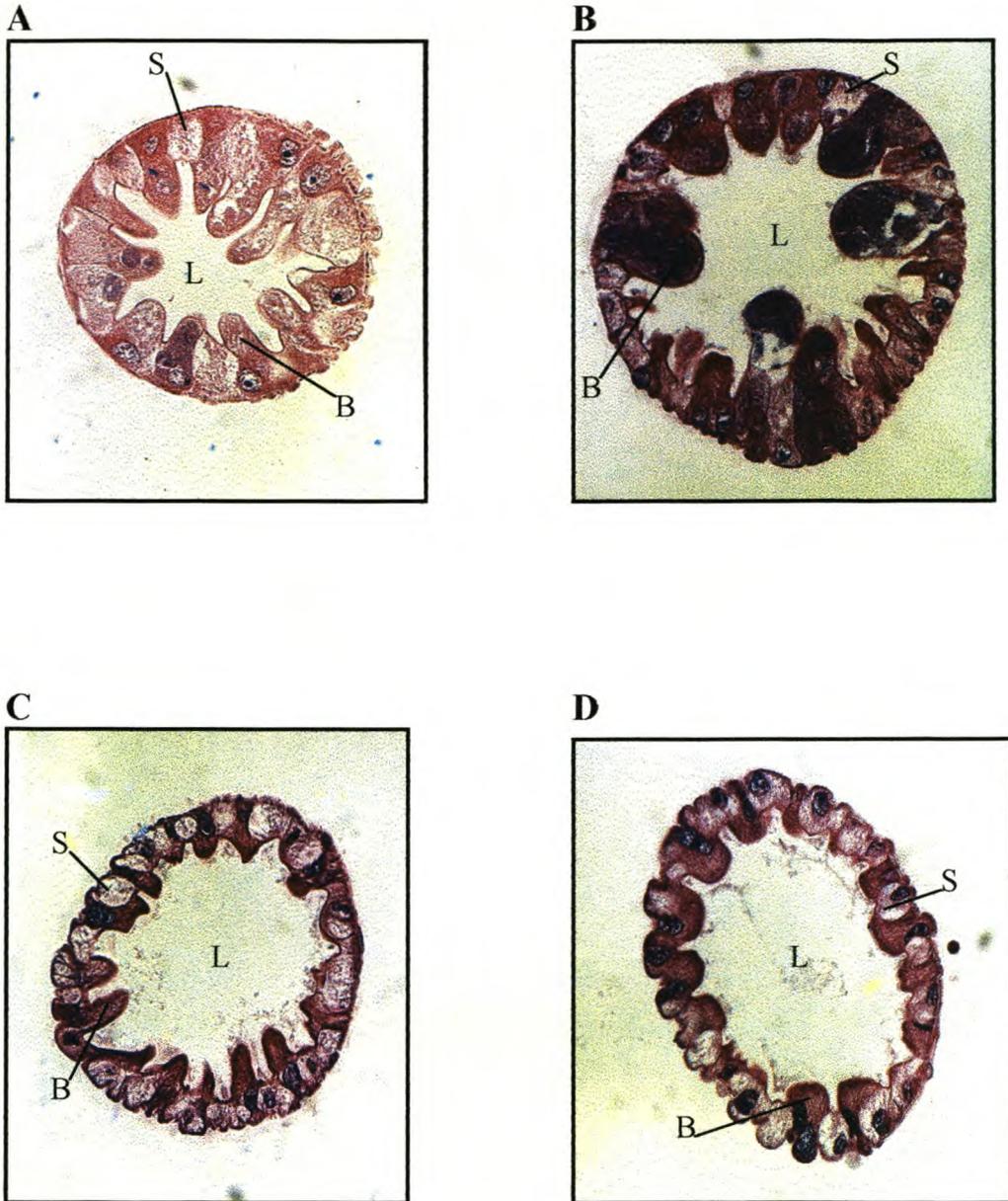


Figure 19: Examples of histological sections, stained with hematoxylin and eosin, in the Z3 zone of hepatopancreas samples of *P. laevis* after six weeks of exposure to various concentrations of cadmium sulphate. **A:** control; **B:** 20 mg.kg⁻¹ CdSO₄ exposure; **C:** 80 mg.kg⁻¹ CdSO₄ exposure; **D:** 160 mg.kg⁻¹ CdSO₄ exposure. Magnification = X250. (L = lumen; B = B-cells; S = S-cells.)

measurements of the 160 mg.kg⁻¹ exposure group were significantly higher than that of the 20 and 80 mg.kg⁻¹ exposure groups (P<0.05). In the Z3 zone PCA measurements of all the cadmium sulphate exposure groups differed significantly from each, with PCA decreasing with increasing exposure concentration (P<0.05). In the Z4 zone the PCA measurements of the 80 mg.kg⁻¹ exposure group was significantly lower than that of the 20 and 160 mg.kg⁻¹ exposure groups (P<0.05).

Table 14: PCA measurements (\pm SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after 6 weeks of exposure to various concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹). n=40. Left superscript show interzonal differences: stat. sign. diff. from: Z1=a; Z2=b; Z3=c; Z4=d (P<0.05). Right superscript show differences between exposure groups, within a particular zone: stat. sign. diff. from: control=a; 20=b, 80=c; 160=d (P<0.05).

Zones in Hepatop.	Exposure concentrations (mg.kg ⁻¹)			
	Control	20	80	160
Z1	^{bcd} 59.5 (\pm 11.0) ^{bcd}	^{bcd} 32.5 (\pm 5.1) ^{ad}	^{bcd} 34.5 (\pm 2.9) ^{ad}	^{bd} 46.0 (\pm 11.1) ^{abc}
Z2	^{acd} 73.5 (\pm 2.9) ^{bcd}	^{acd} 41.0 (\pm 9.6) ^{ad}	^{acd} 47.1 (\pm 11.6) ^{ad}	^{acd} 57.4 (\pm 4.2) ^{abc}
Z3	^{abd} 81.9 (\pm 1.2) ^{bcd}	^{abd} 67.7 (\pm 7.0) ^{acd}	^{ab} 56.4 (\pm 9.3) ^{abd}	^{bd} 48.5 (\pm 1.9) ^{abc}
Z4	^{abc} 78.5 (\pm 3.6) ^{bcd}	^{abc} 64.5 (\pm 4.2) ^{ac}	^{ab} 58.7 (\pm 7.8) ^{abd}	^{abc} 64.5 (\pm 6.8) ^{ac}

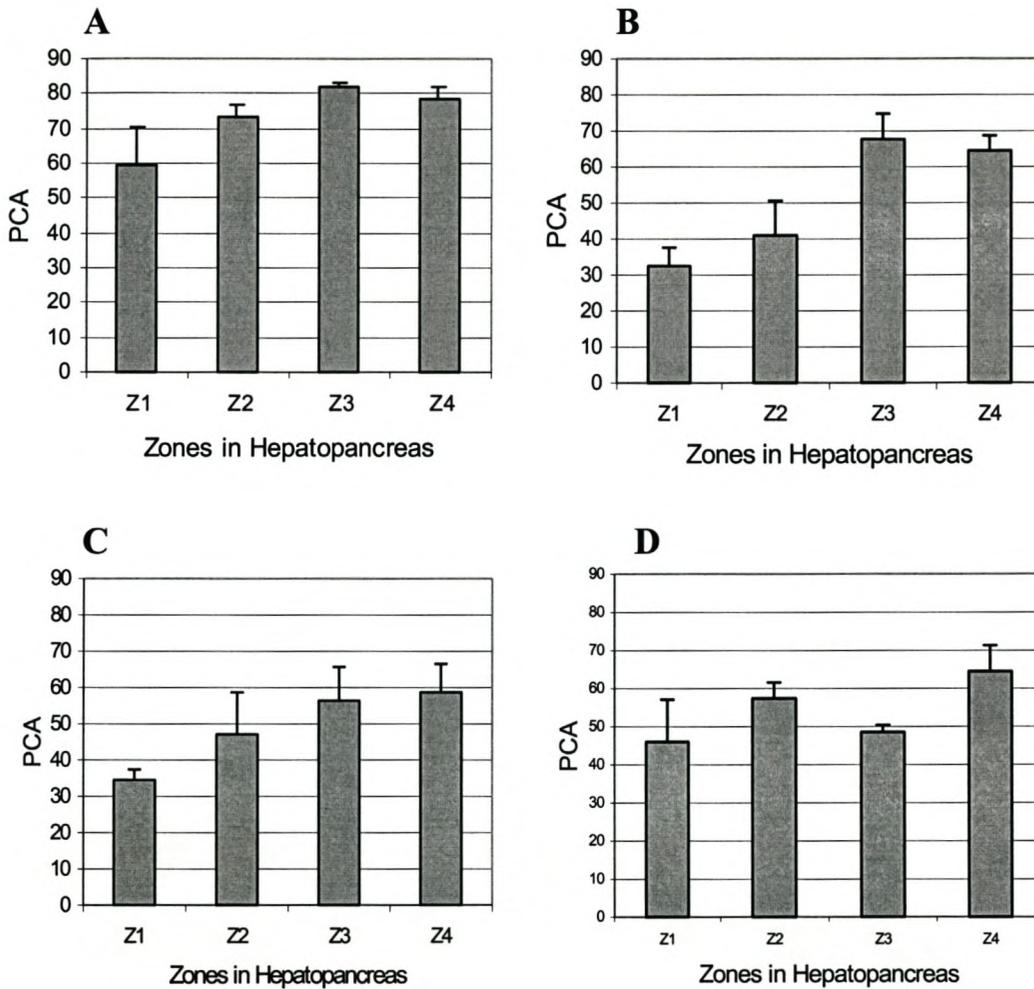


Figure 20: Percentage Cellular Area (PCA) measurements (\pm SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after 6 weeks of exposure to various concentrations of cadmium sulphate, with **A:** control; **B:** 20 mg.kg⁻¹; **C:** 80 mg.kg⁻¹; **D:** 160 mg.kg⁻¹. n=40. See Table 14 for statistics.

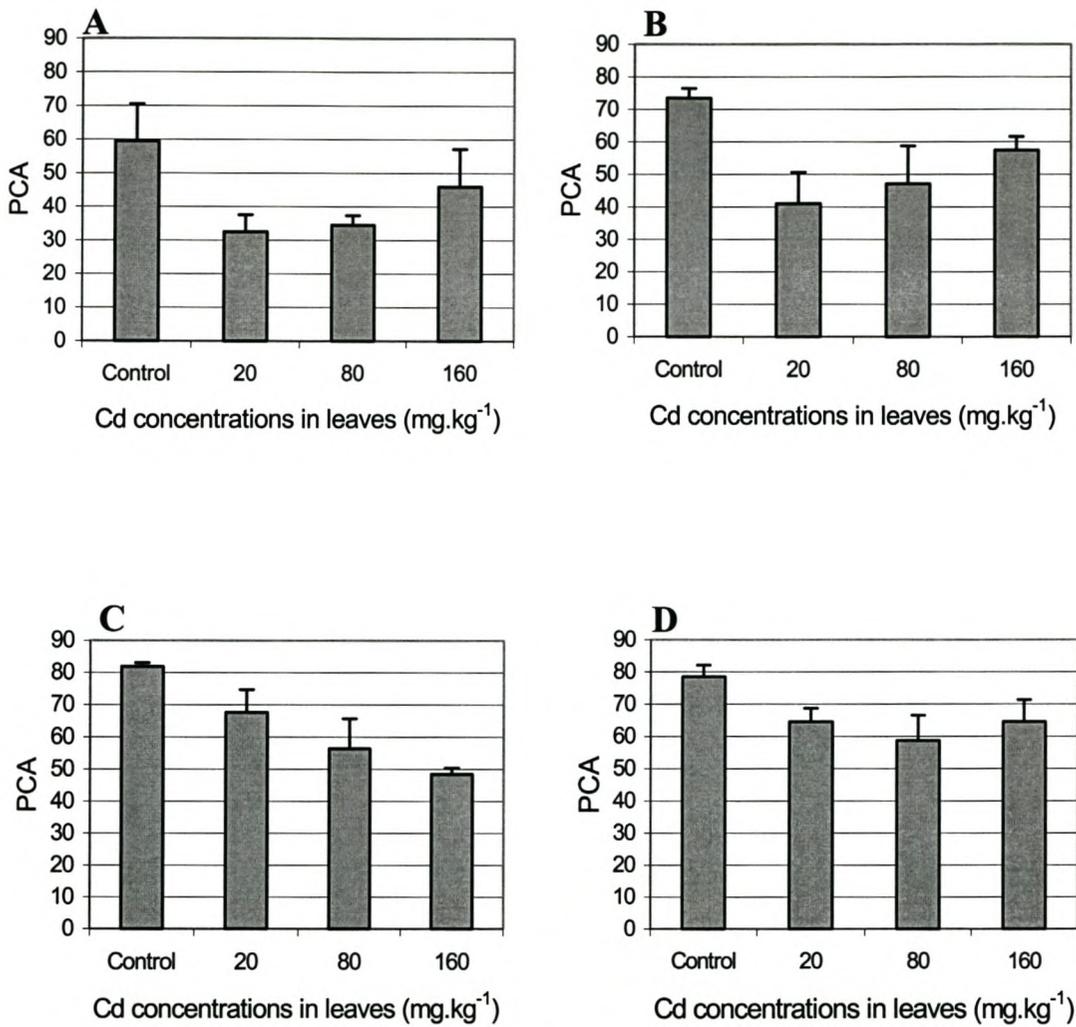


Figure 21: PCA measurements (\pm SD) of hepatopancreas sections, within a particular zone (Z1-Z4), of cadmium sulphate exposed groups (20, 80 and 160 mg.kg⁻¹), with A: Z1; B: Z2; C: Z3; D: Z4. n=40. See Table 14 for statistics.

3.1.3.2. Percentage Cellular Area (PCA) in the hepatopancreas after exposure to zinc sulphate

Examples of histological sections of hepatopancreas samples of *P. laevis* after six weeks of exposure to various concentrations of zinc sulphate are shown in Fig. 22. All sections illustrated were made in the Z3 zone of the hepatopancreas.

Table 15 shows the PCA measurements in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after exposure to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) for 6 weeks. In this table interzonal comparisons are made, and also comparisons between the different exposure groups, within a particular zone. In Figure 23 the different zones (Z1-Z4), of each of the exposure groups (control, 1000, 4000, 8000 mg.kg⁻¹), are compared to each other. Analysis of the data by pairwise multiple comparisons revealed the following patterns. In the control group and 1000 mg.kg⁻¹ exposure group PCA measurements in all the zones differed statistically significantly from each other ($P < 0.05$). Only the PCA's in the Z3 and Z4 zones in the 4000 mg.kg⁻¹ exposure group did not differ from each other ($P > 0.05$). In the 8000 mg.kg⁻¹ exposure group PCA's measured in Z1 were significantly lower than that of Z2, Z3 and Z4 ($P < 0.05$).

The PCA measurements of hepatopancreas sections of the control group and all zinc sulphate exposure groups (1000, 4000 and 8000 mg.kg⁻¹), within a particular zone, are shown in Table 15 and Fig. 24. Concerning PCA measurements in the Z1, Z2, Z3 and Z4 zones all the exposure groups differed significantly from the control group ($P < 0.05$.) PCA measurements in the Z1 zone of none of the exposure groups differed significantly from each other ($P > 0.05$). PCA values in the Z2 zone of the 8000 mg.kg⁻¹ exposure group were significantly higher than those in the 1000 and 4000 mg.kg⁻¹ exposure groups ($P < 0.05$). PCA values in the Z3 and Z4 zones of the

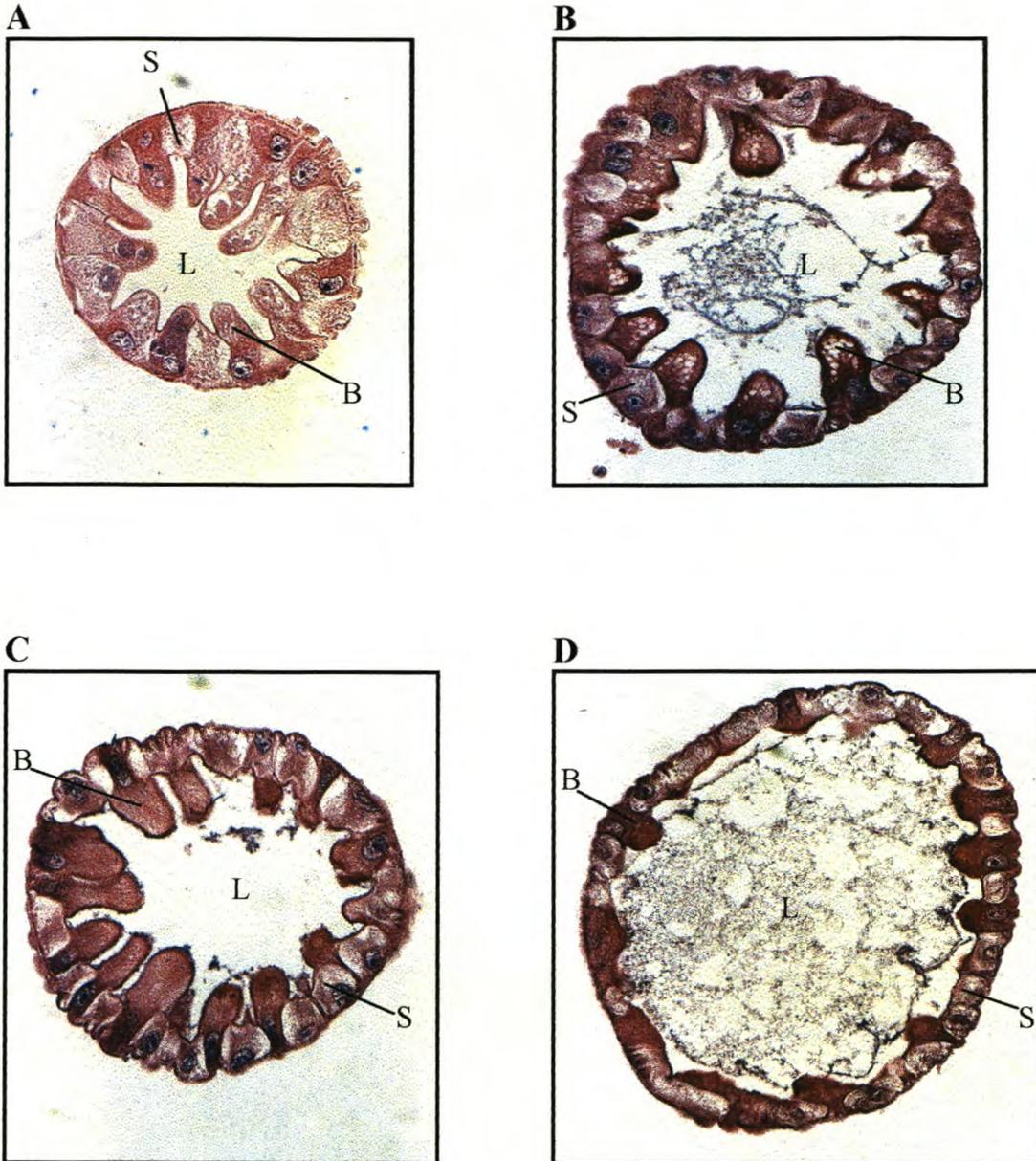


Figure 22: Examples of histological sections, stained with hematoxylin and eosin, in the Z3 zone of hepatopancreas samples of *P. laevis* after six weeks of exposure to various concentrations of zinc sulphate. **A:** control; **B:** 1000 mg.kg⁻¹ ZnSO₄ exposure; **C:** 4000 mg.kg⁻¹ ZnSO₄ exposure; **D:** 8000 mg.kg⁻¹ ZnSO₄ exposure. Magnification = X250. (L = lumen; B = B-cells; S = S-cells.)

8000 mg.kg⁻¹ exposure group were significantly lower than those in the 1000 and 4000 mg.kg⁻¹ exposure groups (P<0.05).

Table 15: PCA measurements (\pm SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after 6 weeks of exposure to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹). n=40. Left superscript show interzonal differences: stat. sign. diff. from Z1=a; Z2=b; Z3=c; Z4=d (P<0.05). Right superscript show differences between exposure groups, within a particular zone: stat. sign. diff. from control=a; 1000=b, 4000=c; 8000=d (P<0.05).

Zones in Hepatop.	Exposure concentrations (mg.kg ⁻¹)			
	Control	1000	4000	8000
Z1	^{bcd} 59.5 (\pm 11.0) ^{bcd}	^{bcd} 30.2 (\pm 7.8) ^a	^{bcd} 29.2 (\pm 5.4) ^a	^{bcd} 28.2 (\pm 4.9) ^a
Z2	^{acd} 73.5 (\pm 2.9) ^{bcd}	^{acd} 39.5 (\pm 7.2) ^{ad}	^{acd} 39.9 (\pm 9.3) ^{ad}	^a 48.8 (\pm 7.9) ^{abc}
Z3	^{abd} 81.9 (\pm 1.2) ^{bcd}	^{abd} 51.9 (\pm 7.5) ^{ad}	^{ab} 55.6 (\pm 11.8) ^{ad}	^a 45.1 (\pm 11.8) ^{abc}
Z4	^{abc} 78.5 (\pm 3.6) ^{bcd}	^{abc} 60.4 (\pm 10.7) ^{ad}	^{ab} 59.1 (\pm 11.3) ^{ad}	^a 46.2 (\pm 9.7) ^{abc}

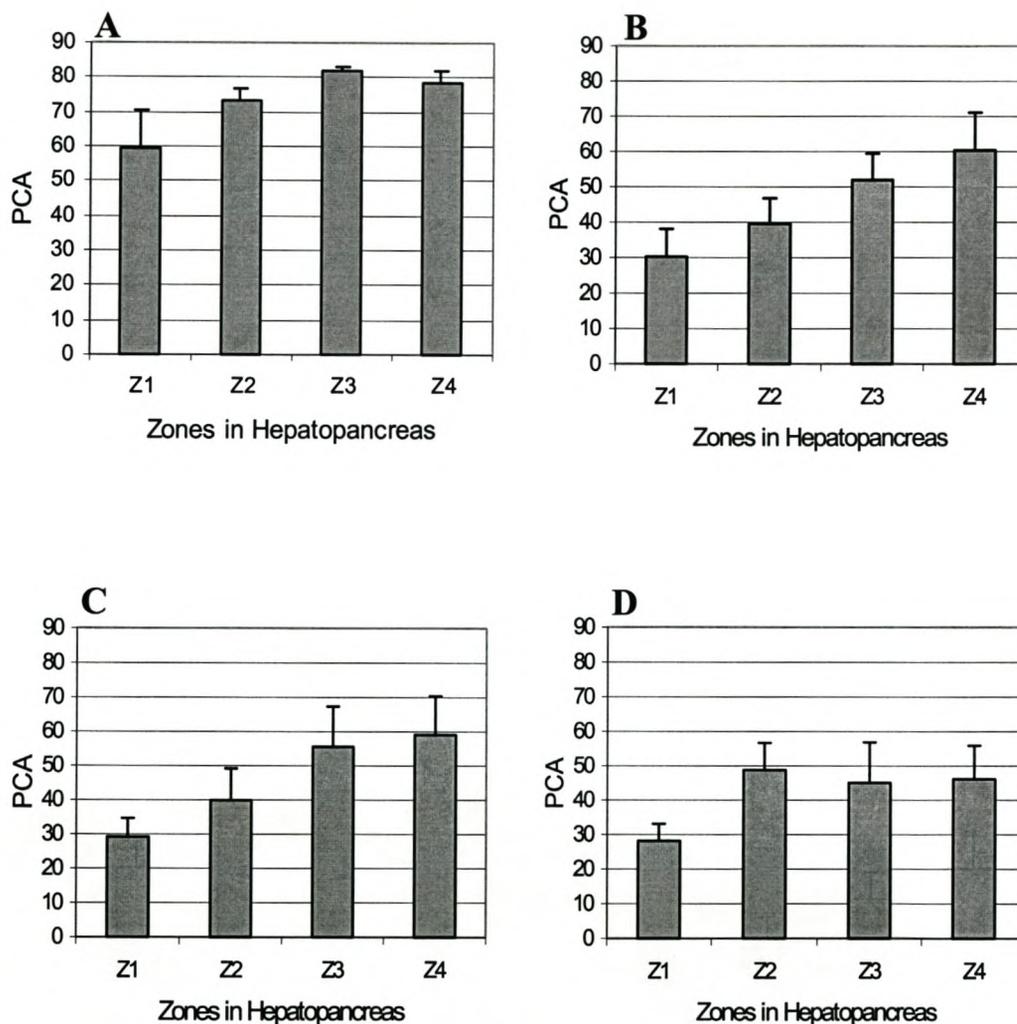


Figure 23: Percentage Cellular Area (PCA) measurements (\pm SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after 6 weeks of exposure to various concentrations of zinc sulphate, with **A:** control; **B:** 1000 mg.kg⁻¹; **C:** 4000 mg.kg⁻¹; **D:** 8000 mg.kg⁻¹. n=40. See Table 15 for statistics.

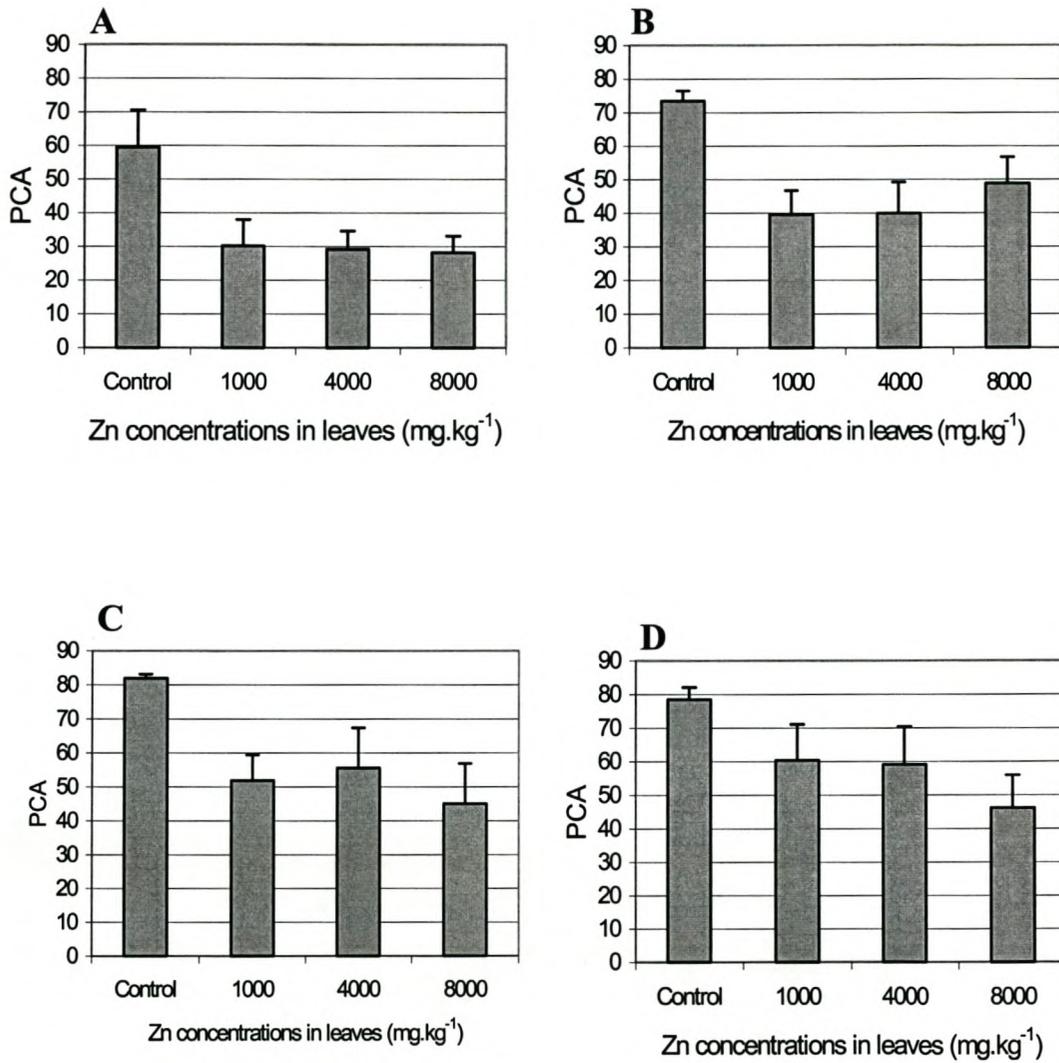


Figure 24: PCA measurements (\pm SD) of hepatopancreas sections, within a particular zone (Z1-Z4), of zinc sulphate exposed groups (1000, 4000 and 8000 mg.kg⁻¹), with **A:** Z1; **B:** Z2; **C:** Z3; **D:** Z4. n=40. See Table 15 for statistics.

3.1.3.3. Percentage Cellular Area (PCA) in the hepatopancreas after combined exposure to cadmium- and zinc sulphate

Examples of histological sections of hepatopancreas samples of *P. laevis* after six weeks of exposure to various mixtures of cadmium- and zinc sulphate are shown in Fig. 25. All sections illustrated were made in the Z3 zone.

Table 16 show the PCA measurements in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after exposure to various mixtures cadmium- and zinc sulphate (mix1, mix2, mix3) for 6 weeks. In this Table interzonal comparisons are made, and also comparisons between the different exposure groups, within a particular zone. In Figure 26 the different zones (Z1-Z4), of each of the exposure groups (control, mix1, mix2, mix3), are compared to each other. Analysis of the data by pairwise multiple comparisons revealed the following patterns. PCA measurements in all the zones differed statistically significantly from each other in the control group ($P < 0.05$). Interzonal comparisons of PCA measurements in mix1, mix2 and mix3 all differed from each other, apart from Z3 and Z4 ($P < 0.05$).

The PCA measurements of hepatopancreas sections of the control group and all groups exposed to mixtures of cadmium- and zinc sulphate (mix1, mix2 and mix3), within a particular zone, are shown in Table 16 and Fig. 27. PCA measurements in all four zones of all exposure groups (mix1, mix2 and mix3) differed significantly from that of the control group ($P < 0.05$.) Comparisons of PCA's in the Z1, Z2 and Z3 zones of all exposure groups showed no differences ($P > 0.05$). PCA measurements in the Z4 zone of mix2 were significantly lower than that of mix1 and mix3 ($P < 0.05$).

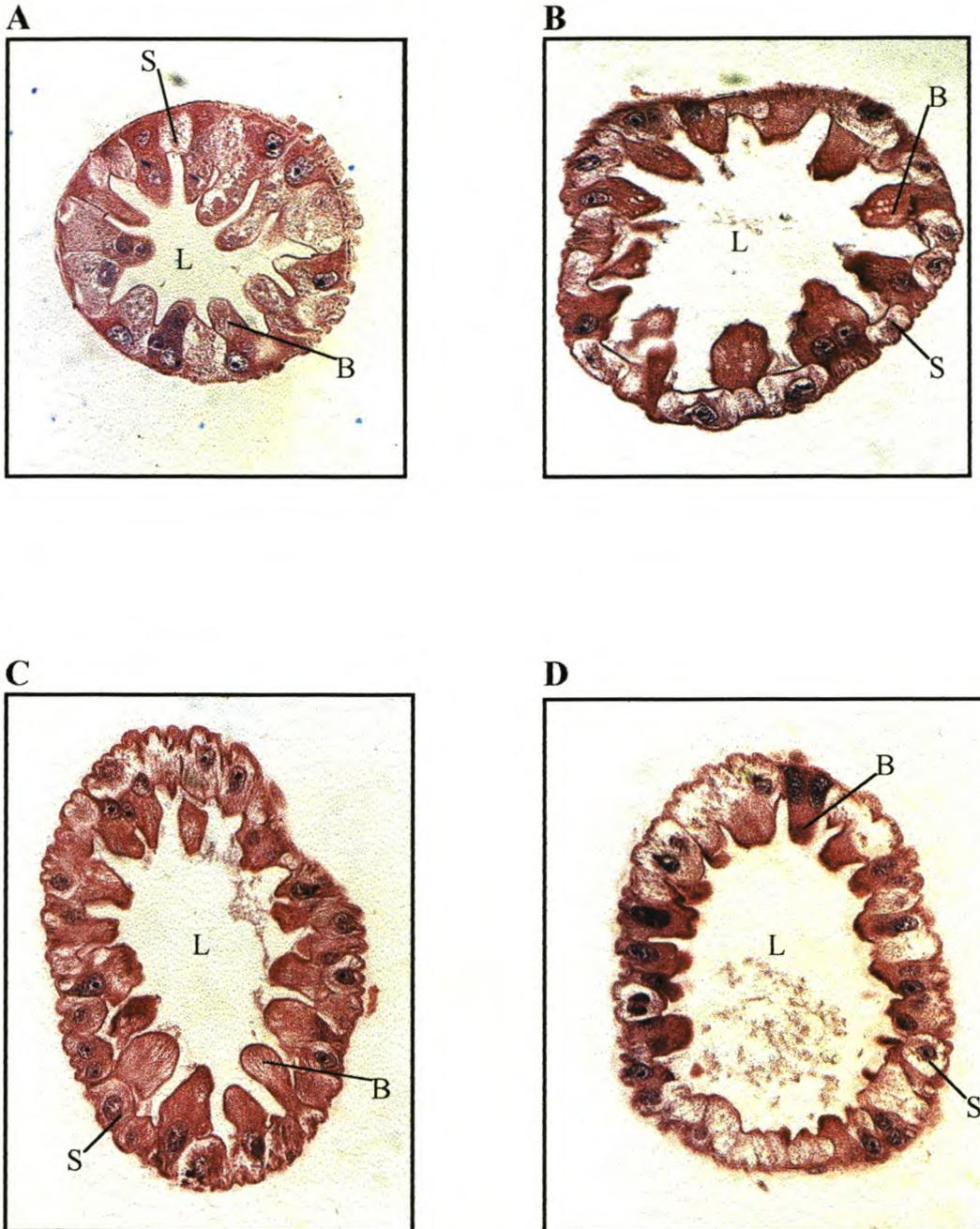


Figure 25: Examples of histological sections, stained with hematoxylin and eosin, in the Z3 zone of hepatopancreas samples of *P. laevis* after six weeks of exposure to various mixtures of cadmium- and zinc sulphate. **A:** control; **B:** mixture of $20 \text{ mg.kg}^{-1} \text{ CdSO}_4$ and $1000 \text{ mg.kg}^{-1} \text{ ZnSO}_4$; **C:** mixture of $80 \text{ mg.kg}^{-1} \text{ CdSO}_4$ and $4000 \text{ mg.kg}^{-1} \text{ ZnSO}_4$; **D:** mixture of $160 \text{ mg.kg}^{-1} \text{ CdSO}_4$ and $8000 \text{ mg.kg}^{-1} \text{ ZnSO}_4$. Magnification = X250. (L = lumen; B = B-cells; S = S-cells.)

Table 16: PCA measurements (\pm SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after 6 weeks of exposure to various mixtures of cadmium- and zinc sulphate (mix1, mix2, mix3). n=40. Left superscript show interzonal differences: stat. sign. diff. from Z1=a; Z2=b; Z3=c; Z4=d ($P<0.05$). Right superscript show differences between exposure groups, within a particular zone: stat. sign. diff. from control=a; mix1=b, mix2=c; mix3=d ($P<0.05$). mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

Zones in Hepatop.	Exposure concentrations (mg.kg ⁻¹)			
	Control	mix1	mix2	mix3
Z1	^{bcd} 59.5 (\pm 11.0) ^{bcd}	^{bcd} 25.4 (\pm 2.5) ^a	^{bcd} 22.6 (\pm 10.6) ^a	^{bcd} 21.3 (\pm 3.7) ^a
Z2	^{acd} 73.5 (\pm 2.9) ^{bcd}	^{acd} 46.1 (\pm 7.0) ^a	^{acd} 45.7 (\pm 12.7) ^a	^{acd} 43.1 (\pm 10.1) ^a
Z3	^{abd} 81.9 (\pm 1.2) ^{bcd}	^{ab} 61.7 (\pm 4.1) ^a	^{ab} 58.2 (\pm 8.8) ^a	^{ab} 61.8 (\pm 5.7) ^a
Z4	^{abc} 78.5 (\pm 3.6) ^{bcd}	^{ab} 62.6 (\pm 5.3) ^{ac}	^{ab} 56.6 (\pm 8.0) ^{abd}	^{ab} 60.0 (\pm 5.6) ^{ac}

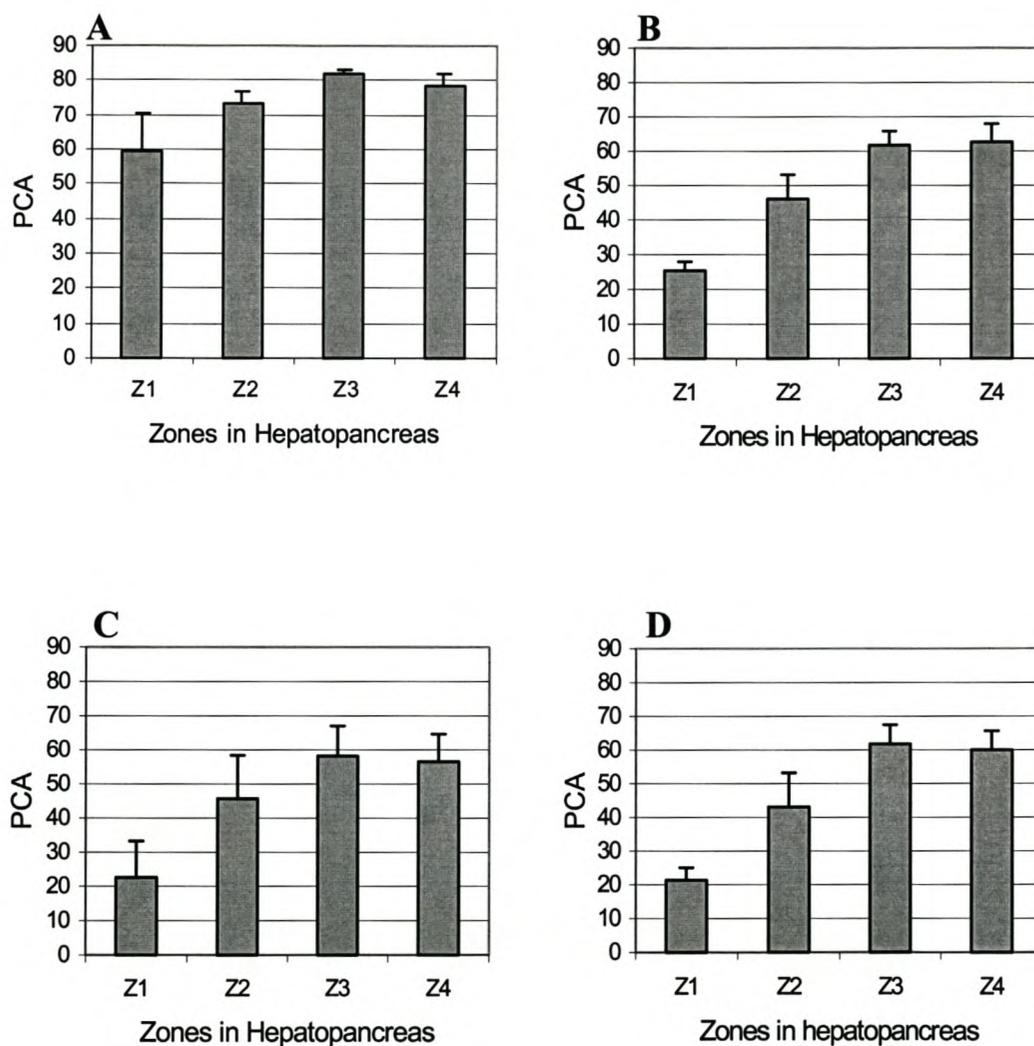


Figure 26: Percentage Cellular Area (PCA) measurements (\pm SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after 6 weeks of exposure to various mixtures cadmium- and of zinc sulphate, with **A:** control; **B:** mix1; **C:** mix2; **D:** mix3. $n=40$. See Table 16 for statistics. mix1 = $20 \text{ mg.kg}^{-1} \text{ CdSO}_4$ with $1000 \text{ mg.kg}^{-1} \text{ ZnSO}_4$; mix2 = $80 \text{ mg.kg}^{-1} \text{ CdSO}_4$ with $4000 \text{ mg.kg}^{-1} \text{ ZnSO}_4$; mix3 = $160 \text{ mg.kg}^{-1} \text{ CdSO}_4$ with $8000 \text{ mg.kg}^{-1} \text{ ZnSO}_4$.

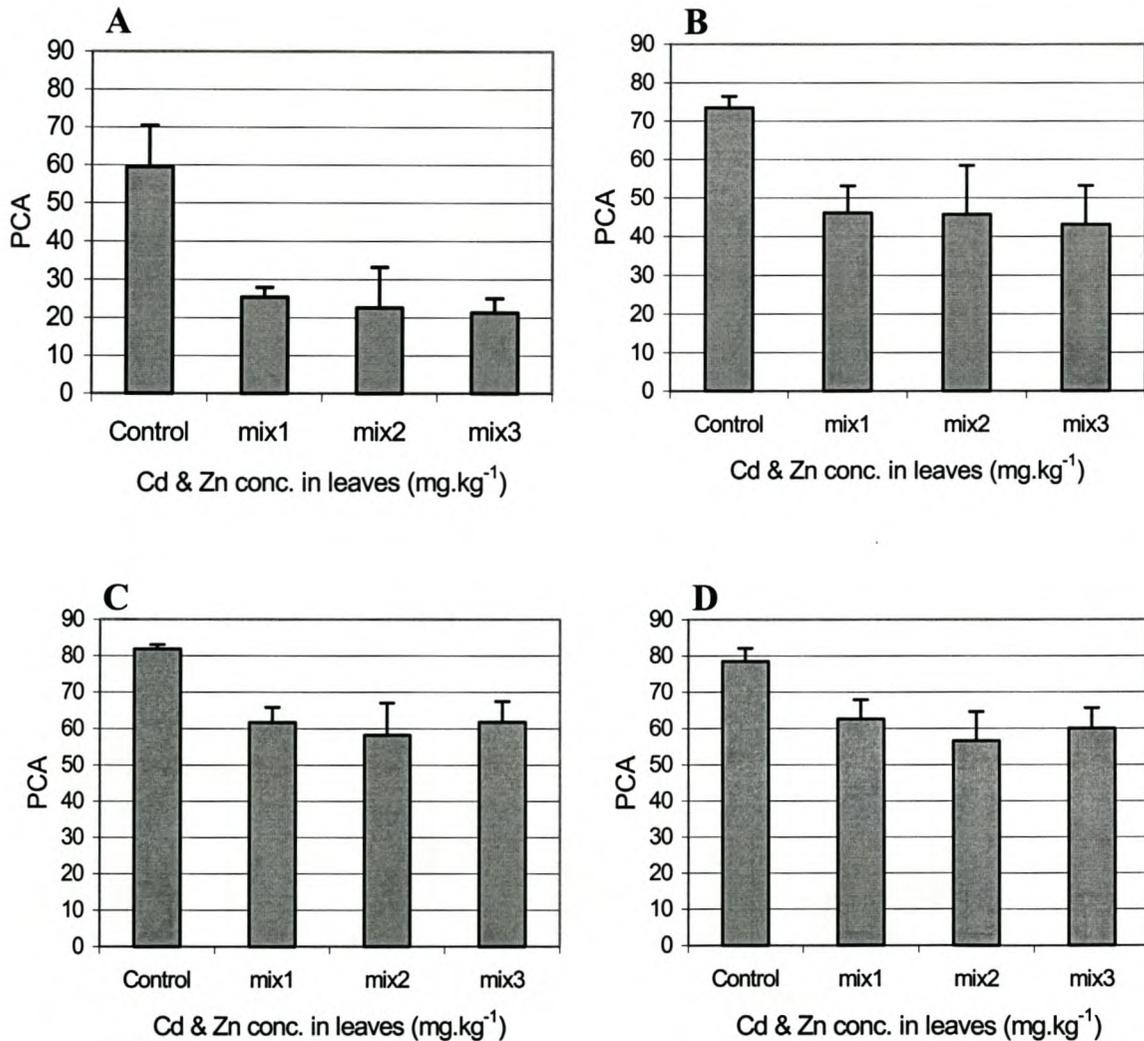


Figure 27: PCA measurements (\pm SD) of hepatopancreas sections, within a particular zone (Z1-Z4), of groups exposed to mixtures of cadmium- and zinc sulphate (mix1, mix2 and mix3), with **A:** Z1; **B:** Z2; **C:** Z3; **D:** Z4. $n=40$. See Table 16 for statistics. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

3.1.3.4. Comparison of PCA measurements in the hepatopancreas after exposure of *P. laevis* to cadmium sulphate and combined exposure to cadmium- and zinc sulphate

The PCA measurements in the hepatopancreas of *P. laevis* after six weeks of exposure to various concentrations of cadmium sulphate and combined exposure various mixtures of cadmium- and zinc sulphate are compared in Table 17 and Fig. 28. Pairwise multiple comparisons of PCA measurements in the Z1 zone showed that the PCA's of exposure groups where cadmium sulphate was administered alone were significantly higher than that of the groups exposed to mixtures of cadmium- and zinc sulphate ($P < 0.05$). PCA's in the Z2 zone of the 160 mg.kg⁻¹ cadmium sulphate group was significantly higher than that of mix3 ($P < 0.05$). In Z3 PCA measurements in the 20 mg.kg⁻¹ exposure groups were significantly higher than that in mix1 ($P < 0.05$). PCA's in the 160 mg.kg⁻¹ exposure group were significantly lower than that in mix3 ($P < 0.05$). PCA measurements in the Z4 zone in the 160 mg.kg⁻¹ exposure groups were significantly higher than that in mix3 ($P < 0.05$).

Table 17: Comparison of Percentage Cellular Area (PCA) measurements (\pm SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after six weeks of exposure to various concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹) and combined exposure to various mixtures of cadmium- and zinc sulphate (mix1, mix2, mix3). $n=40$. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. See Figure 28 for statistics.

Zones in hepatopancreas	Exposure concentrations (mg.kg ⁻¹)						
	Control	20	mix1	80	mix2	160	mix3
Z1	59.5 (\pm 11.0)	32.5 (\pm 5.1)	25.4 (\pm 2.5)	34.5 (\pm 2.9)	22.6 (\pm 10.6)	46.0 (\pm 11.1)	21.3 (\pm 3.7)
Z2	73.5 (\pm 2.9)	41.0 (\pm 9.6)	46.1 (\pm 7.0)	47.1 (\pm 11.6)	45.7 (\pm 12.7)	57.4 (\pm 4.2)	43.1 (\pm 10.1)
Z3	81.9 (\pm 1.2)	67.7 (\pm 7.0)	61.7 (\pm 4.1)	56.4 (\pm 9.3)	58.2 (\pm 8.8)	48.5 (\pm 1.9)	61.8 (\pm 5.7)
Z4	78.5 (\pm 3.6)	64.5 (\pm 4.2)	62.6 (\pm 5.3)	58.7 (\pm 7.8)	56.6 (\pm 8.0)	64.5 (\pm 6.8)	60.0 (\pm 5.6)

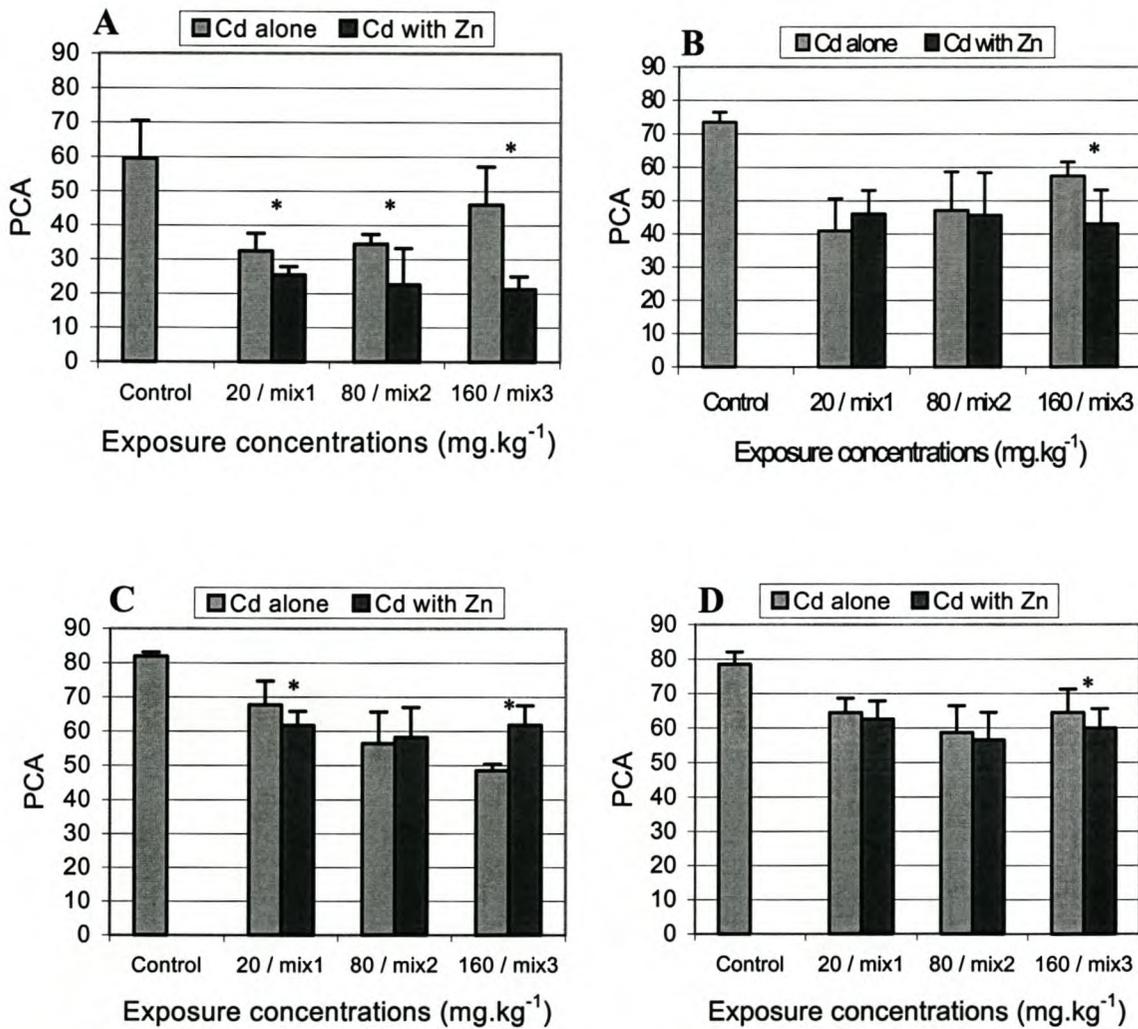


Figure 28: Comparison of Percentage Cellular Area (PCA) measurements (\pm SD) in the different zones (A: Z1, B: Z2, C: Z3, D: Z4) of the hepatopancreas of *P. laevis* after six weeks of exposure to cadmium sulphate (20, 80, 160 mg.kg^{-1}) and combined exposure to various mixtures of cadmium- and zinc sulphate (mix1, mix2, mix3). $n=40$. Stat. sign. diff. between single metal exposure and combined exposure indicated by (*). mix1 = 20 mg.kg^{-1} CdSO_4 with 1000 mg.kg^{-1} ZnSO_4 ; mix2 = 80 mg.kg^{-1} CdSO_4 with 4000 mg.kg^{-1} ZnSO_4 ; mix3 = 160 mg.kg^{-1} CdSO_4 with 8000 mg.kg^{-1} ZnSO_4 .

3.1.3.5. Comparison of PCA measurements in the hepatopancreas after exposure of *P. laevis* to zinc sulphate and combined exposure to cadmium- and zinc sulphate

The PCA measurements in the hepatopancreas of *P. laevis* after six weeks of exposure to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) and combined exposure to various mixtures of cadmium- and zinc sulphate (mix1, mix2, mix3) are compared in Table 18 and Fig. 29. Pairwise multiple comparisons of PCA measurements in Z1 showed that PCA's in the exposure groups where zinc sulphate was administered alone were significantly higher than that from the exposure groups where cadmium- and zinc sulphate was administered together (P<0.05). PCA measurements in the Z2 zone from the 1000 and 4000 mg.kg⁻¹ exposure groups were significantly lower than that from the mix1 and mix2 groups, respectively (P<0.05). PCA measurements in the Z3 zone from the 1000 and 8000 mg.kg⁻¹ exposure groups were significantly lower than that from the mix1 and mix3 groups, respectively (P<0.05). PCA's in the Z4 zone from the 8000 mg.kg⁻¹ exposure group were significantly lower than that from the mix3 group (P<0.05).

Table 18: Comparison of Percentage Cellular Area (PCA) measurements (\pm SD) in the different zones (Z1-Z4) of the hepatopancreas of *P. laevis* after six weeks of exposure to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) and combined exposure to various mixtures of cadmium- and zinc sulphate (mix1, mix2, mix3). n=40. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄. See Figure 29 for statistics.

Zones in hepatopancreas	Exposure concentrations (mg.kg ⁻¹)						
	Control	1000	mix1	4000	mix2	8000	mix3
Z1	59.5 (\pm 11.0)	30.2 (\pm 7.8)	25.4 (\pm 2.5)	29.2 (\pm 5.4)	22.6 (\pm 10.6)	28.2 (\pm 4.9)	21.3 (\pm 3.7)
Z2	73.5 (\pm 2.9)	39.5 (\pm 7.2)	46.1 (\pm 7.0)	39.9 (\pm 9.3)	45.7 (\pm 12.7)	48.8 (\pm 7.9)	43.1 (\pm 10.1)
Z3	81.9 (\pm 1.2)	51.9 (\pm 7.5)	61.7 (\pm 4.1)	55.6 (\pm 11.8)	58.2 (\pm 8.8)	45.1 (\pm 11.8)	61.8 (\pm 5.7)
Z4	78.5 (\pm 3.6)	60.4 (\pm 10.7)	62.6 (\pm 5.3)	59.1 (\pm 11.3)	56.6 (\pm 8.0)	46.2 (\pm 9.7)	60.0 (\pm 5.6)

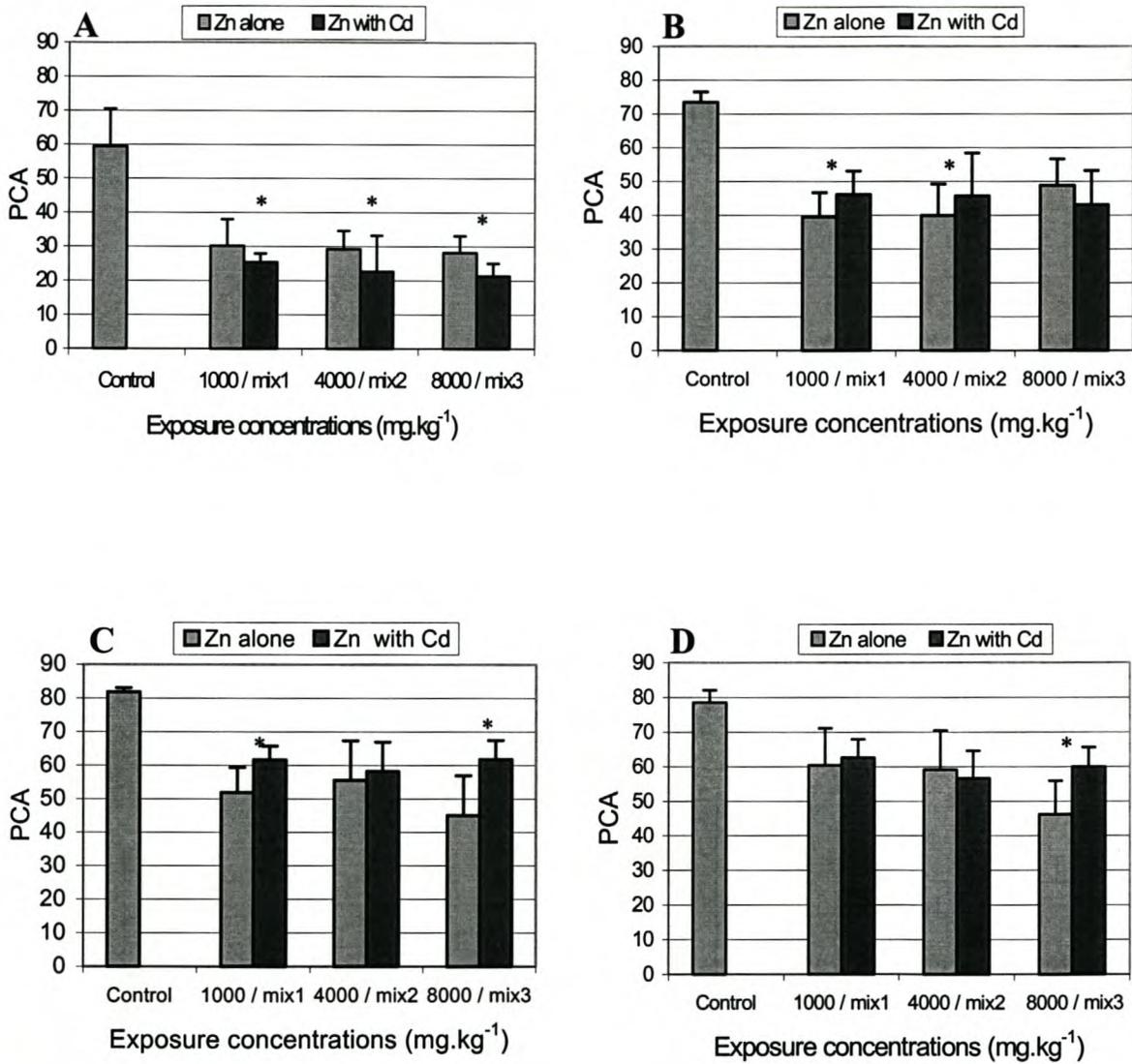


Figure 29: Comparison of Percentage Cellular Area (PCA) measurements (\pm SD) in the different zones (A: Z1, B: Z2, C: Z3, D: Z4) of the hepatopancreas of *P. laevis* after six weeks of exposure to zinc sulphate (1000, 4000, 8000 mg.kg^{-1}) and combined exposure to various mixtures of cadmium- and zinc sulphate (mix1, mix2, mix3). $n=40$. Stat. sign. diff. between single metal exposure and combined exposure indicated by (*). mix1 = 20 mg.kg^{-1} CdSO_4 with 1000 mg.kg^{-1} ZnSO_4 ; mix2 = 80 mg.kg^{-1} CdSO_4 with 4000 mg.kg^{-1} ZnSO_4 ; mix3 = 160 mg.kg^{-1} CdSO_4 with 8000 mg.kg^{-1} ZnSO_4 .

3.1.4. Relationships between Percentage Cellular Area (PCA) of the hepatopancreas and measured parameters of *P. laevis*

3.1.4.1. Relationship between PCA in the Z3 zone of the hepatopancreas and exposure concentrations

When the PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, exposed to various concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹) for six weeks, were plotted against their corresponding cadmium exposure concentrations, linear regression analysis revealed a strong negative correlation ($r = -0.93$) (Fig. 30).

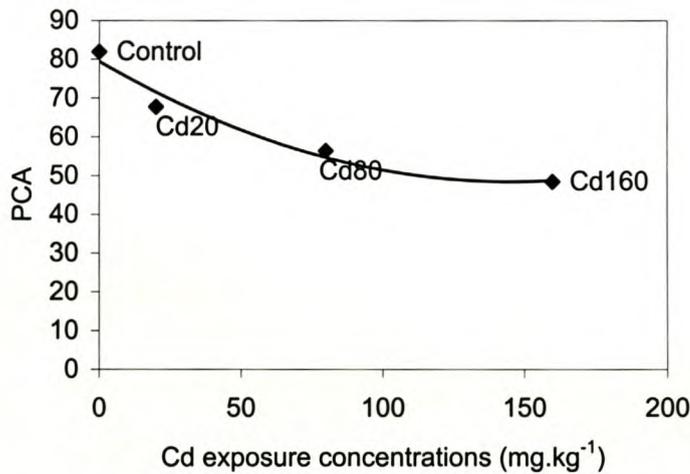


Figure 30: Relationship ($r = -0.93$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to cadmium sulphate (20, 80, 160 mg.kg⁻¹), and cadmium exposure concentrations.

When the PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, exposed to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) for six weeks, were plotted against their corresponding zinc exposure concentrations, linear regression analysis revealed a negative correlation ($r = -0.73$) (Fig. 31).

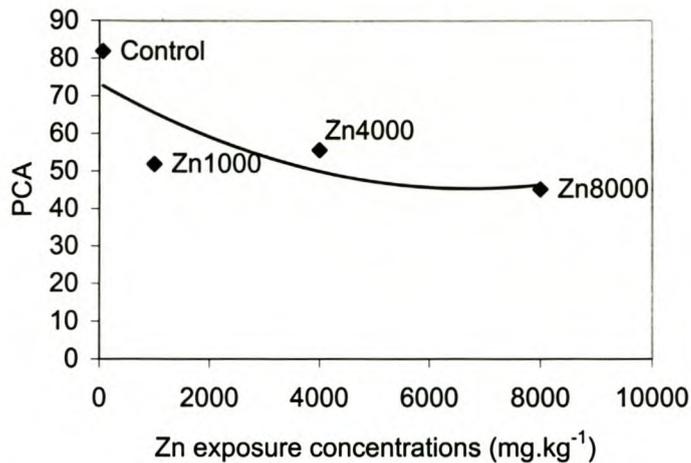


Figure 31: Relationship ($r = -0.73$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to zinc sulphate (1000, 4000, 8000 mg.kg⁻¹), and zinc exposure concentrations.

When the PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, exposed to various mixtures of cadmium- and zinc sulphate for six weeks, were plotted against their corresponding exposure concentrations, linear regression analysis revealed a relatively weak negative correlation ($r = -0.58$) (Fig. 32).

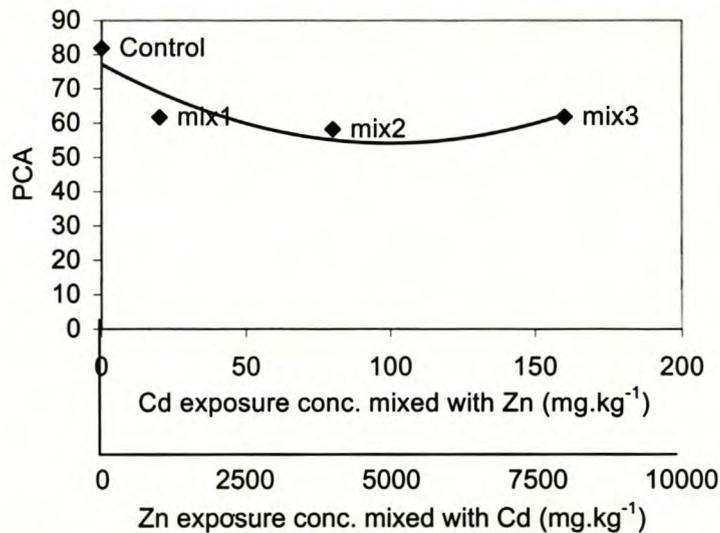


Figure 32: Relationship ($r = -0.58$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to various mixtures of cadmium- and zinc sulphate, and cadmium and zinc exposure concentrations. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

3.1.4.2. Relationship between PCA and cadmium and zinc concentrations in the hepatopancreas

When the PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, exposed to various concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹) for six weeks, were plotted against their corresponding cadmium concentrations in the hepatopancreas, linear regression analysis revealed a very good negative correlation ($r = -0.88$) (Fig. 33).

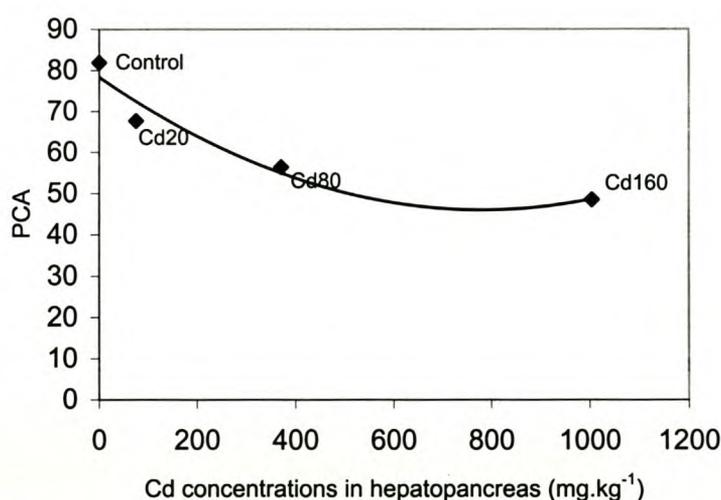


Figure 33: Relationship ($r = -0.88$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to cadmium sulphate (20, 80, 160 mg.kg⁻¹), and cadmium concentrations in the hepatopancreas.

When the PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, exposed to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) for six weeks, were plotted against their corresponding zinc concentrations in the hepatopancreas, linear regression analysis revealed a very weak negative correlation ($r = -0.40$) (Fig. 34).

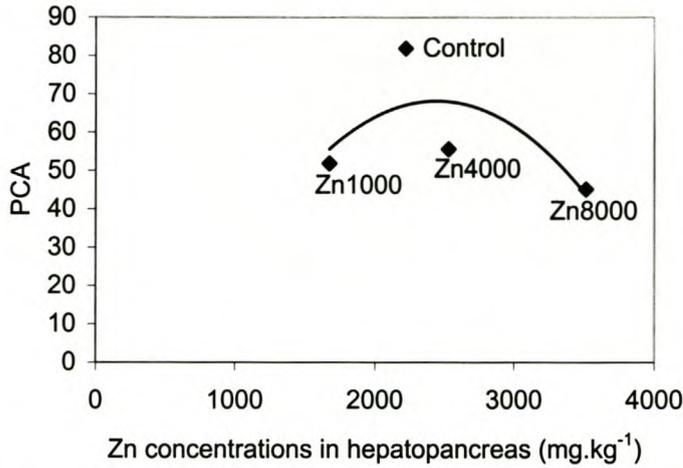


Figure 34: Relationship ($r = -0.40$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to zinc sulphate (1000, 4000, 8000 mg.kg⁻¹), and zinc concentrations in the hepatopancreas.

When the PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, exposed to various mixtures of cadmium- and zinc sulphate for six weeks, were plotted against their corresponding cadmium concentrations in the hepatopancreas, linear regression analysis revealed a strong negative correlation ($r = -0.96$) (Fig. 35).

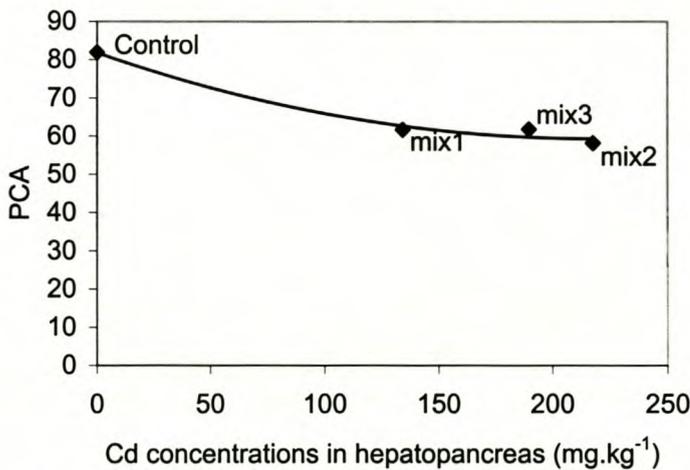


Figure 35: Relationship ($r = -0.96$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to mixtures of cadmium- and zinc sulphate, and cadmium concentrations in the hepatopancreas. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

When the PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, exposed to various mixtures of cadmium- and zinc sulphate for six weeks, were plotted against their corresponding zinc concentrations in the hepatopancreas, linear regression analysis revealed a very weak positive correlation ($r = 0.23$) (Fig. 36).

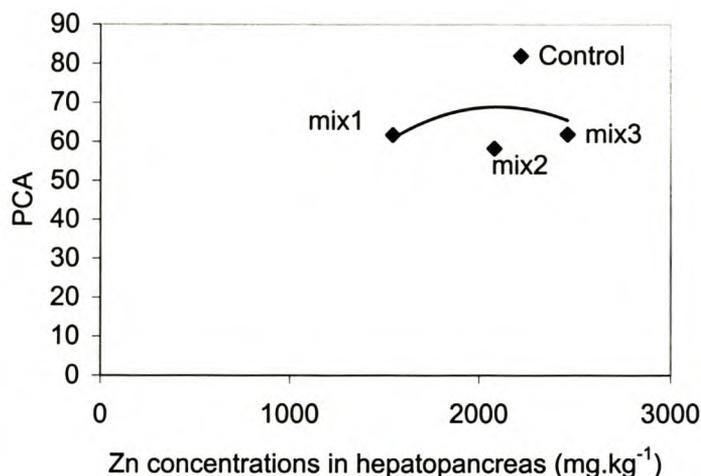


Figure 36: Relationship ($r = 0.23$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to mixtures of cadmium- and zinc sulphate, and zinc concentrations in the hepatopancreas. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

3.1.4.3. Relationship between PCA and percentage mass change

When the PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, exposed to various concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹) for six weeks, were plotted against their corresponding percentage mass change, linear regression analysis revealed a very strong positive correlation ($r = 0.99$) (Fig. 37).

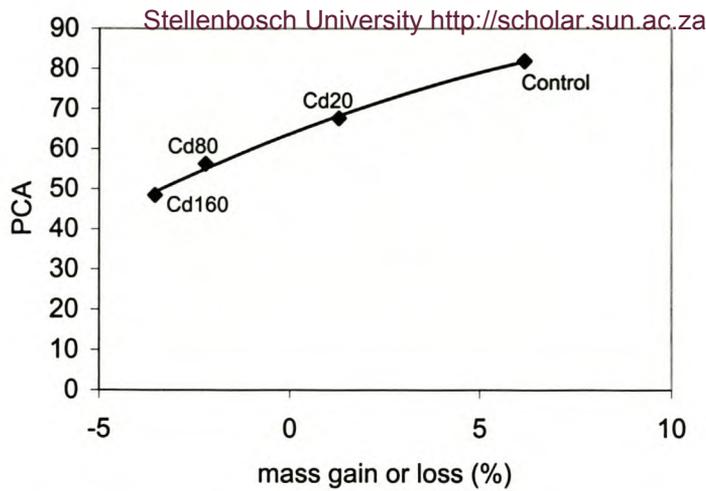


Figure 37: Relationship ($r = 0.99$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to various concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹), and percentage mass change.

When the PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, exposed to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) for six weeks, were plotted against their corresponding percentage mass change, linear regression analysis revealed a relatively good positive correlation ($r = 0.80$) (Fig. 38).

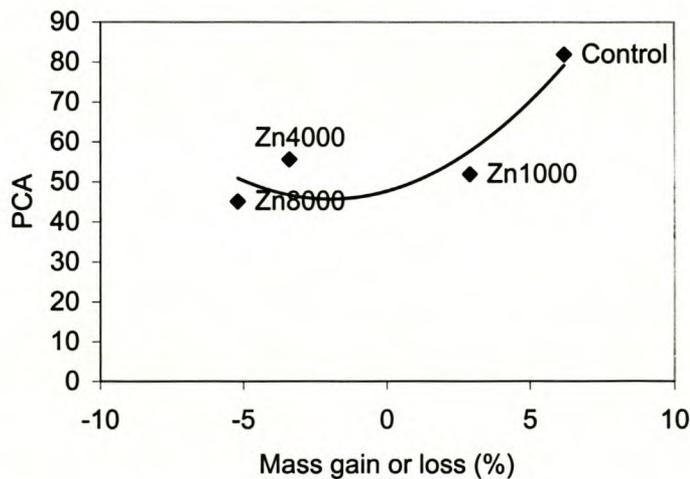


Figure 38: Relationship ($r = 0.80$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹), and percentage mass change.

When the PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, exposed to various mixtures of cadmium- and zinc sulphate for six weeks, were plotted against their corresponding percentage mass change, linear regression analysis revealed a extremely weak negative correlation ($r = -0.08$) (Fig. 39).

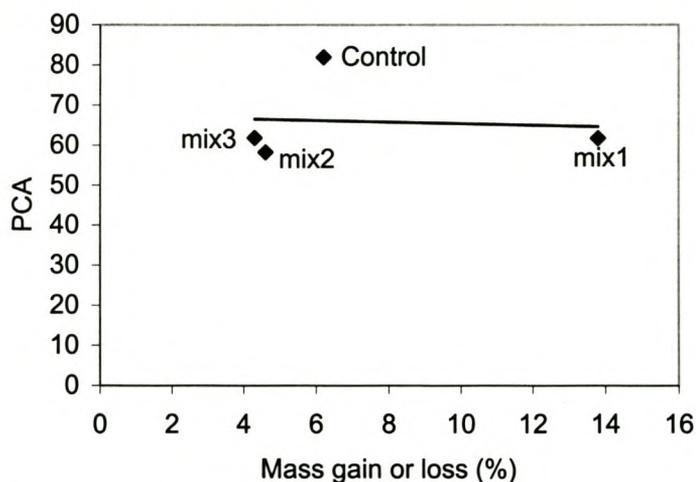


Figure 39: Relationship ($r = -0.08$) between PCA measurements in the Z3 zone of the hepatopancreas of *P. laevis*, after six weeks of exposure to various mixtures of cadmium- and zinc sulphate, and percentage mass change. mix1 = 20 mg.kg⁻¹ CdSO₄ with 1000 mg.kg⁻¹ ZnSO₄; mix2 = 80 mg.kg⁻¹ CdSO₄ with 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ with 8000 mg.kg⁻¹ ZnSO₄.

3.2. Field survey

3.2.1. Cadmium and zinc concentrations in soil and leaf litter from field sites

Concentrations of cadmium and zinc in the soil and leaf litter at the uncontaminated- and contaminated sites are shown in Table 19. The soil and leaf litter from the smelting works had statistically significantly higher concentrations of cadmium and zinc than those from the botanical gardens ($P < 0.05$).

Table 19: Cadmium and zinc concentrations (mg.kg^{-1} ; dry mass) (\pm SD) in soil and leaf litter samples from the uncontaminated- and contaminated sites. Range of concentrations in [brackets]. $n=5$. ND = not detectable.

	Cadmium (mg.kg^{-1})		Zinc (mg.kg^{-1})	
	Soil	Leaf litter	Soil	Leaf litter
Uncontam. site	ND	ND	79.4 (± 4.8) [74.9-84.5]	67.7 (± 10.7) [55.5-81.2]
Contam. Site	4.2 (± 2.9) [2.0-7.5]	6.7 (± 1.4) [5.9-8.3]	1292.4 (± 354.7) [947.5-1788.4]	1167.2 (± 277.9) [857.9-1605.5]

3.2.2. Bioaccumulation of cadmium and zinc in *P. pruinus* collected from field sites

The concentrations of cadmium in the hepatopancreas and rest of body samples of *P. pruinus* from the uncontaminated site and contaminated site are shown in Table 20. The cadmium concentrations in hepatopancreas samples were statistically significantly lower in woodlice collected from the uncontaminated site than those from the contaminated site ($P=0.008$). Cadmium concentrations in the rest of the

body samples of woodlice collected from the uncontaminated site were also significantly lower than those from the contaminated site ($P=0.008$).

The concentrations of zinc in the hepatopancreas and rest of body samples of *P. pruinus* from the uncontaminated site and contaminated site are shown in Table 20. The zinc concentrations in hepatopancreas samples were statistically significantly lower in woodlice collected from the uncontaminated site than those from the contaminated site ($P=0.008$). Zinc concentrations in the rest of the body samples of woodlice collected from the uncontaminated site were also significantly lower than those from the contaminated site ($P=0.016$).

Table 20: Cadmium and zinc concentrations (mg.kg^{-1} ; dry mass) ($\pm\text{SD}$) in hepatopancreas and rest of the body samples of *P. pruinus* collected from an uncontaminated site and contaminated site. $n=5$. Range of concentrations in [brackets]. ND = not detectable.

		Hepatopancreas	Rest of the body
Cd	Uncontam. Site	ND	ND
	Contam. Site	89.9 (± 7.6) [81.6-98.4]	2.9 (± 0.57) [2.2-3.6]
Zn	Uncontam. Site	313.7 (± 61.8) [243.9-411.7]	14.7 (± 2.0) [11.7-17.1]
	Contam. Site	10537.5 (± 4035.5) [6408.2-15913.0]	37.0 (± 7.8) [28.8-46.2]

3.2.3. Histological analysis of the hepatopancreas of *P. pruinus*

Examples of histological sections made in the Z3 zone of hepatopancreas samples from the uncontaminated- and contaminated sites are shown in Fig. 40. PCA measurements in the Z3 zone of hepatopancreas samples of *P. pruinus* collected from these sites are given in Table 21 and illustrated in Fig. 41. Statistical analysis

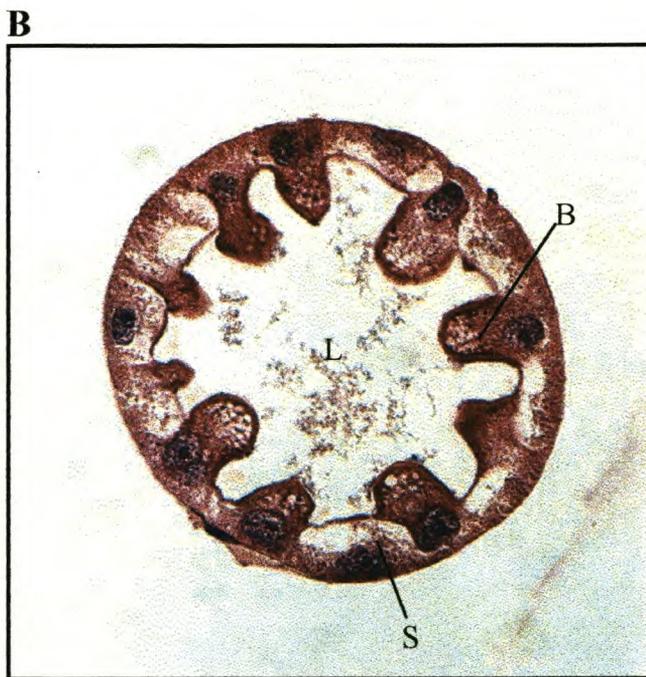
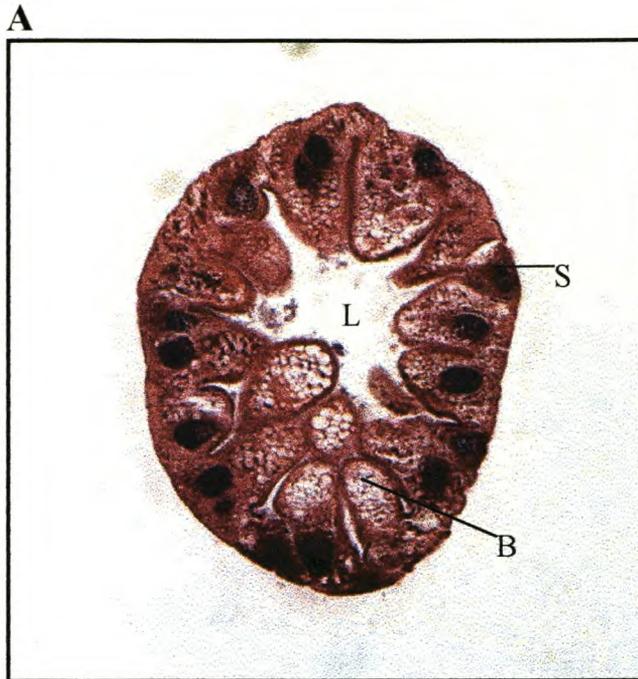


Figure 40: Examples of histological sections, stained with hematoxylin and eosin, in the Z3 zone of hepatopancreas samples of *P. pruinosis* collected from an **A**: uncontaminated site and **B**: contaminated site. Magnification = X250. (L = lumen; B = B-cells; S = S-cells)

showed that there was a highly significant difference between the uncontaminated site and contaminated site, concerning PCA measurements in the hepatopancreas ($P < 0.001$).

Table 21: Percentage Cellular Area (PCA) measurements (\pm SD) in the Z3 zone of the hepatopancreas of *P. pruinosis* collected from the uncontaminated- and contaminated sites. The range is shown in [brackets]. $n=40$. $P < 0.001$.

Uncontaminated site	Contaminated site
84.2 (\pm 5.3)	55.9 (\pm 11.4)
[76.6-94.9]	[33.2-74.9]

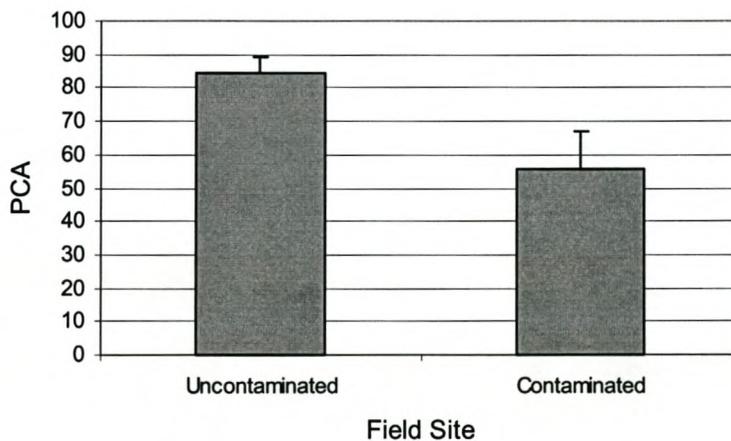


Figure 41: Percentage Cellular Area's (PCA) (\pm SD) in the Z3 zone of the hepatopancreas of *P. pruinosis* collected from the uncontaminated- and contaminated sites. $n=40$. $P < 0.001$.

4.

Discussion

4.1. Influence of cadmium- and zinc sulphate exposure on body mass changes of *P. laevis*

4.1.1. Effects of separately administered cadmium- and zinc sulphate on mass

The weekly mass change showed quite large variations, as evidenced by the large standard deviations (Tables 3 & 4; Figs. 2 & 3). It has previously been documented by various authors that terrestrial isopods' growth rate over several weeks is rather variable (Van Straalen & Van Gestel 1993; Van Gestel & Van Straalen 1994; Drobne 1997). The variation in mass change in this study is therefore not unnatural or a result of defective methodology.

The mean mass of the unexposed isopods in the control group increased over the 6 weeks of observation (Table 3; Fig. 2; Appendix 1). Mass changes of woodlice of the 80 mg.kg⁻¹ cadmium sulphate exposure group were significantly lower than that of the control after the first and second weeks, and their masses decreased in the fifth and sixth weeks of exposure (Table 3; Fig. 2; Appendix 1) indicating a detrimental effect. Woodlice of the 160 mg.kg⁻¹ cadmium sulphate exposure group's mass changes were also significantly lower than that of the control after the first, second, third and fifth weeks, and their masses decreased after the sixth week of exposure (Table 3; Fig. 2; Appendix 1). In contrast to the findings on the mass changes of *P. laevis* by Odendaal & Reinecke (1999), findings in the present study on the mass changes of woodlice exposed to 20 mg.kg⁻¹ cadmium sulphate were not significantly different from that of the control group (Table 3; Fig. 2; Appendix 1). This could be

attributed to the fact that growth rate or mass gain during the present study was lower than in the study by Odendaal & Reinecke (1999) as evidenced by comparing the growth rates of the woodlice in the controls of the two studies. Nevertheless, the observed significantly lower mean mass of exposed woodlice relative to that of the control woodlice and even a decrease in mass suggest that cadmium sulphate had a negative effect on the mass of *P. laevis* after six weeks of exposure. Severe inhibition of growth was observed in *P. scaber* exposed to cadmium in previous studies (Donker & Bogert 1991; Jones & Hopkin 1994; Khalil et al. 1995). The growth of terrestrial snails were also found to be negatively impacted on by cadmium (Gomot 1997; Swaileh & Ezzughayyar 2000).

Mass changes of woodlice exposed to 1000 mg.kg⁻¹ zinc sulphate were only significantly different from those of the control woodlice in the second week of exposure. No differences were observed in any of the other weeks of exposure although the mean mass changes of woodlice of the 1000 mg.kg⁻¹ group were mostly lower than those of the control woodlice (Table 4; Fig. 3; Appendix 2). The well known ability of woodlice to resist metal contamination (Donker & Bogert 1991), may have caused the woodlice exposed to 1000 mg.kg⁻¹ zinc sulphate not to have significantly lower mass changes than those of the control. The mass of woodlice exposed to 4000 mg.kg⁻¹ zinc sulphate significantly decreased from the third week until the end of the experiment (Table 4; Fig. 3; Appendix 2). The mass of woodlice exposed to 8000 mg.kg⁻¹ zinc sulphate were lower than that of the control woodlice, which gained in mass throughout the exposure period, whilst the mass of these woodlice decreased during the experimental period (Table 4; Fig. 3; Appendix 2). It can thus be concluded that zinc sulphate, especially the two higher concentrations, had a negative effect on the mass of *P. laevis*. Previous studies also showed that zinc

had a negative impact on the growth of woodlice (Joosse et al. 1981; Beyer et al. 1984; Witzel 2000). Zinc was also found to be toxic, in terms of growth, for other taxa such as the collembole *Folsomia candida* (Smit & Van Gestel 1996).

According to Alikhan (1995) chronic exposure to metals produces pervasive stress manifestations with likely long-term effects on growth. The physiological processes of the isopods are impaired (Donker & Bogert 1991) due to energy used to resist the contaminant (Donker 1992), thereby leaving less energy for growth. Donker (1992) found in a previous study that the lowest energy reserves at a contaminated site coincided with the highest zinc concentrations. More energy may be used to resist the contaminant by avoidance and/or detoxification. Lowered food intake and consumption due to zinc contamination was found in woodlice by Drobne & Hopkin (1995) and Bibic et al. (1997), indicating avoidance of contaminated food.

A dose-dependent differentiation in mass change was found in this study for *P. laevis* exposed to zinc sulphate (Table 4; Fig. 3). Almost throughout the exposure period differences were found between the three zinc sulphate exposure groups. The higher the zinc concentrations that the woodlice were exposed to the lower was the mass gain and the higher the mass loss observed. More energy was used to resist the zinc as the exposure concentrations of zinc increased, with less available for growth.

4.1.2. Combined effects of mixtures of cadmium and zinc on mass

Mass changes (gain or loss) of *P. laevis* after exposure to various mixtures of cadmium- and zinc sulphate, were different from the mass changes of woodlice when Cd and Zn were administered separately (Table 5; Fig. 4; Appendix 3). Combination of the lowest concentrations of Cd and Zn (20 and 1000 mg.kg⁻¹) produced a stimulating effect in terms of mass change compared to the control. This stimulation

may be explained by hormesis, described by Stebbing (1982) as an over-correction from biosynthetic control mechanisms, resulting in increased growth (Donker & Bogert 1991). Mass changes of the other two combinations of Cd and Zn (80 and 4000 mg.kg⁻¹ and 160 and 8000 mg.kg⁻¹) showed no differences from that of the control (Table 5; Fig. 4; Appendix 3) and mass changes of woodlice exposed to Cd and Zn mixtures were mostly the same as that of the control over the six week exposure period. Comparisons of mass changes of woodlice exposed to singly administered cadmium sulphate and combinations of Cd and Zn (Fig. 5) show that the presence of Zn neutralised the negative effect of Cd on mass change. This was true for all three the combinations used (Fig. 5A, B, C). Likewise, Cd also neutralised the negative effect of Zn on the mass of *P. laevis*. Woodlice exposed to combinations of cadmium- and zinc sulphate mostly showed an increase in mass compared to those exposed to Zn only (Fig. 6A, B, C). It can thus be concluded that mixtures of Cd and Zn had an antagonistic effect on mass of *P. laevis*.

In a study by Khalil et al. (1996b) on the combined effects of metals (Cd, Cu, Zn) on the growth of the earthworm *Aporrectodea caliginosa* a non-significant tendency towards antagonism was detected. The effect of a mixture of Cd and Zn on the growth of the springtail *Folsomia candida* was found to be antagonistic by Van Gestel & Hensbergen (1997). This is in agreements with the findings in the present study for isopods.

In the literature contradictory findings on the effect of metals on growth of terrestrial isopods occur. Odendaal & Reinecke (1999) found that cadmium, applied singly, impaired the growth of *P. laevis*. However, Hopkin (1990b) found that metals did not impair the growth of *P. scaber*. The woodlice used in Hopkin's (1990b) study were collected from a contaminated site containing significant concentrations of

cadmium and zinc in leaf litter. It may be possible that the mixture of Cd and Zn in that study acted antagonistically, resulting in no reduction in growth, as was observed in the present study (Table 5; Fig 4).

Conventional ecotoxicological test procedures are designed to evaluate the relative toxicities of a range of chemicals singly. However, animals are mostly exposed to mixtures of chemicals in the real field situations, where these chemicals may act upon the accumulation and toxicity of each other in animals. Very few studies on the toxicity of mixtures of chemicals have been done with terrestrial invertebrates (eg. Khalil et al. 1996b; Van Gestel & Hensbergen 1997; Witzel 2000). As evident from the present study, the effects of mixtures of metals (and indeed other chemicals) upon their toxicities to terrestrial invertebrates is very important and needs further attention from researchers.

4.2. Bioaccumulation and compartmentalization of cadmium and zinc in *P. laevis*

4.2.1. Accumulation of cadmium and zinc after exposure to separately administered cadmium- and zinc sulphate

It can be seen from the results (Table 8; Figs. 7 & 8) that cadmium accumulated in the body of *P. laevis*. Cadmium also accumulates in other terrestrial isopods, such as *P. scaber* (Martin et al. 1976; Coughtrey et al. 1977; Hopkin & Martin 1982; Hopkin et al. 1986; Hopkin 1990b; Hames & Hopkin 1991a; Hopkin et al. 1993). In the present study it was shown that accumulation of cadmium in the hepatopancreas and rest of the body samples increased with increasing exposure concentrations (Table 8; Fig. 7

& 8). This may be due to the fact that woodlice have no natural ability to regulate cadmium and will accumulate it in concentrations relative to that of the exposure concentrations. As in a previous study by Odendaal & Reinecke (1999) it was shown that *P. laevis* is a macroconcentrator (Dallinger 1993) of cadmium. *P. scaber* and *Oniscus asellus* were also shown to be macroconcentrators of cadmium (Coughtrey et al. 1980; Hopkin & Martin 1982; Martin & Coughtrey 1982; Hopkin 1990b).

From the results of the present study it can be seen that zinc accumulated in the body of *P. laevis* (Table 9; Figs. 9, 10). Other studies also showed that zinc accumulates in other terrestrial isopods previously studied, such as *P. scaber* and *Oniscus asellus* (Hopkin & Martin 1982a; Hopkin 1990b; Hames & Hopkin 1991a; Donker et al. 1993; Hopkin & Hames 1994). In the present study no differences were found between zinc concentrations in the hepatopancreas of control woodlice and those of woodlice exposed to 1000 and 4000 mg.kg⁻¹ zinc sulphate (Table 9; Figs. 9, 10). Only the zinc concentrations in the hepatopancreas samples of woodlice exposed to 8000 mg.kg⁻¹ zinc sulphate were significantly higher than that found in the hepatopancreases of control woodlice (Table 9; Figs. 9, 10). Relatively high zinc concentrations (2221.9 mg.kg⁻¹) were measured in hepatopancreases of control *P. laevis*. In other studies it was also found that zinc accumulated to a much higher degree than other metals in control *P. scaber* (Köhler et al. 1996). The concentration factor of control *P. scaber* was found to be much higher than for zinc exposed woodlice (Witzel 2000). According to Dallinger (1993) *Porcellio* is a facultative concentrator of zinc. The concentration factors will decrease with increasing exposure concentrations. The same trend can be seen in this study (Table 9; Fig. 9 & 10). It thus seems as if these animals can regulate the intake of zinc depending on the physiological need (Witzel 2000). This explains the lack of differences in zinc

accumulation in hepatopancreases of the different zinc sulphate exposure groups in this study. Only the zinc concentrations in hepatopancreas samples of the 8000 mg.kg⁻¹ group were significantly higher than those of the other exposure groups.

Digestive tissues such as gut epithelia or digestive glands, which play a role in nutrition physiology, are the predominant sites of metal accumulation (Dallinger 1993). In this study more than 98% of the cadmium (Table 8) and 99% of the zinc (Table 9) in *P. laevis* accumulated in the hepatopancreas. These animals thus exhibit the phenomenon of compartmentalization, whereby substances are stored in specific organs or structures of the animal. Compartmentalization is a means of detoxification of potentially toxic substances like cadmium and zinc. Terrestrial isopods, similar to other terrestrial invertebrates, have developed a strategy which allows them to inactivate and retain toxic metals or excessive amounts of essential trace metals by intracellular compartmentalization (Dallinger 1993). The advantage of compartmentalization is that it prevents other organs and tissues from being contaminated (Hopkin 1989). In terrestrial isopods metals can be intracellularly compartmentalized in 2 types of metal-containing granules. Type B granules may contain large amounts of sulphur in association with class B and borderline metals such as cadmium, copper, lead mercury and zinc. Type C granules are composed almost exclusively of iron but may also contain lead and zinc. The epithelium of the hepatopancreas is composed of two types of cells, namely B-cells and S-cells (Clifford & Witkus 1971; Vernon et al. 1974). The main function of the S-cells is storage (Prosi et al. 1983). Type B granules occur in the S-cells of the hepatopancreas (Wieser 1968; Wieser & Klima 1969; Dallinger & Prosi 1988; Prosi & Dallinger 1988). Type C granules occur in the B-cells. The cadmium accumulated in *P. laevis* in the present study was probably compartmentalized in type B granules in the S-cells

of the hepatopancreas as cadmium is not stored in type C granules that occur in B-cells. It has been shown previously that, in contrast to the B-cells, the metal-containing S-cells are permanent and never void their contents. Thus, cadmium deposited in them remains there until the animal dies (Hopkin 1990a). It was also shown in a decontamination experiment that *P. scaber* was unable to excrete accumulated cadmium after exposure to singly applied cadmium (Witzel 2000). Zinc can be stored in both types of granules (Hopkin 1990a). Zinc can thus be found in S-cells and B-cells. It has been shown that S-cells never void their content (Hopkin 1990a), while B-cells undergo apocrine secretion every 24 hours, voiding their content (Hames & Hopkin 1991b). In the literature contradictory conclusions are made concerning the question whether woodlice can excrete zinc or not. According to results obtained by Hopkin (1990b) *P. scaber* is unable to excrete zinc. In other studies the same species was able to excrete zinc (Bibic et al. 1997). This contradictory results may be explained by the fact that zinc is stored in both cell types of the hepatopancreas. When the zinc is stored in the S-cells its not excreted, but when its stored in the B-cells zinc is lost through the daily apocrine secretion (Hames & Hopkin 1991b).

The percentage zinc in the hepatopancreas in this study is much higher than those found in some other studies. Donker (1992) found that between 70 and 85% of accumulated zinc in *P. scaber* were in the hepatopancreas. The amount of zinc stored in the hepatopancreas may be connected to the storage capacity of the hepatopancreas. If the storage capacity of the hepatopancreas is exceeded more zinc tend to accumulate in the rest of the body (Hopkin & Martin 1984).

High concentrations of cadmium accumulated in the hepatopancreas of *P. laevis* in this study. A review of literature on the accumulation of metals in terrestrial

invertebrates by Heikens et al. (2001) revealed that isopods accumulate zinc in their bodies at higher levels than other taxa tested. In the present study it was shown that *P. laevis* accumulated zinc at levels above 1500 mg.kg^{-1} . This could have negative consequences for animals in higher trophic levels that are unable to handle these levels of cadmium and zinc. If an animal that accumulates these metals such as *P. laevis* is preyed upon, the predator population may suffer more serious effects than the prey (Hopkin 1994). Terrestrial isopods have a large range of predators, such as centipedes, spiders, coleopterans, lizards, little owls and shrews (Avery 1966; Sunderland & Sutton 1980; Avery et al. 1983), which could be at risk of being contaminated. Acute poisoning may take place if they consume a single toxic dose from a highly contaminated individual prey item (Depledge 1990; Hopkin 1993a; b).

4.2.2. Accumulation of cadmium and zinc after exposure to mixtures of cadmium- and zinc sulphate

After exposure to various mixtures of cadmium- and zinc sulphate the hepatopancreas was still the main storage organ for Cd and Zn as in the case when these metals were administered separately (Tables 10 & 11). In the mixture toxicity experiments accumulation of cadmium in the hepatopancreas did not differ significantly from each other in any of the exposure groups (Table 10; Fig. 11). This is in contrast to the single metal exposures of cadmium, where accumulation of cadmium in the hepatopancreas of all the exposure groups differed significantly from each other (Table 8; Fig. 7). The concentrations of cadmium in *P. laevis* were also influenced by the presence of zinc in the mixture toxicity experiments (Table 12; Figs. 15 & 16). Cadmium concentrations in the hepatopancreases of woodlice exposed to mix1 ($20 \text{ mg.kg}^{-1} \text{ CdSO}_4$ and $1000 \text{ mg.kg}^{-1} \text{ ZnSO}_4$) were significantly higher than that found in

hepatopancreases of woodlice exposed to cadmium alone. However, cadmium concentrations in hepatopancreases of woodlice exposed to the two higher mixture concentrations (mix2 = 80 mg.kg⁻¹ CdSO₄ and 4000 mg.kg⁻¹ ZnSO₄; mix3 = 160 mg.kg⁻¹ CdSO₄ and 8000 mg.kg⁻¹ ZnSO₄) were much lower than that in hepatopancreases of woodlice exposed to cadmium alone (Fig. 15). Here the presence of zinc seemed to have a clear antagonistic effect on the accumulation of cadmium. It is also clear that the influence of zinc on cadmium accumulation was dependent on zinc concentration in the food, since the higher the food concentration of zinc the bigger was its impact on the accumulation of cadmium in the animals (Figs. 15 & 16). This is in agreement with the findings of Beyer et al. (1982) that high zinc concentrations in soil substantially reduced cadmium concentrations in earthworms.

The higher concentrations of cadmium in the hepatopancreases of woodlice exposed to mix1, relative to that found in the 20 mg.kg⁻¹ cadmium sulphate single metal exposure may be explained by the fact that the food zinc concentration (1000 mg.kg⁻¹) in mix1 not being high enough to reduce the cadmium concentrations as in the study of Beyer et al. (1982) but low enough to enhance cadmium concentrations in the hepatopancreas. Elliott et al. (1986) also found that cadmium accumulation in *Mytilus edulis* was increased in the presence of relatively low concentrations of zinc. Similar results were found in a study on shrimps by Ahsanullah et al. (1981).

It is widely accepted that woodlice cannot excrete cadmium (Witzel 1998). However, contradictory results have been found by Hopkin (1990b) and Hames and Hopkin (1991a). In these studies cadmium was excreted by *P. scaber*. In both these cases where cadmium was excreted, significant concentrations of zinc, together with cadmium, were found in the diet of the woodlice. Witzel (2000) provided experimental evidence that cadmium can be excreted by woodlice in the presence of

zinc. Cadmium is normally only stored in the S-cells of the hepatopancreas. They are permanent and never void their content (Hopkin 1990a). It could therefore be possible for cadmium, in the presence of zinc, to be stored together with a certain fraction of the accumulated zinc in the B-cells, from where it can be excreted. This could explain the lower concentrations of cadmium in the hepatopancreas of *P. laevis* after combined exposure to cadmium and zinc in mix2 and mix3 in this study (Fig 15). The possibility that cadmium, in the presence of zinc, could be stored in the rest of the body of woodlice to explain the lower cadmium concentrations in the hepatopancreas of woodlice exposed to mix2 and mix3 in the present study is not plausible. Firstly, cadmium concentrations in the rest of the body of woodlice was not significantly higher in the presence of zinc (Fig. 16), and secondly cadmium in the rest of the body would have affected mass changes of the woodlice negatively. This was not the case in the mixture experiments.

In the mixture toxicity tests none of the zinc concentrations in the hepatopancreases of *P. laevis* of any of the Cd/Zn mixture exposure groups differed from that in the control (Table 11; Fig. 13). Only the zinc concentrations in hepatopancreases of woodlice exposed to mix3 was significantly higher than that of mix1. No other differences were observed. Cadmium did not seem to influence the concentrations of hepatopancreas zinc in the mix1 and mix2 mixture toxicity tests, when hepatopancreas zinc concentrations were compared to those found in the corresponding single metal zinc exposures (Fig. 17). However, cadmium had an antagonistic effect on hepatopancreas zinc concentrations in the mix3 mixture toxicity tests (Fig. 17). The excretion rate of zinc could have been increased in the presence of relatively high concentrations of cadmium in the food, like in mix3, or the uptake of zinc by the woodlice might have been affected by the relatively high cadmium

concentrations. In mixture toxicity tests by Witzel (2000) cadmium had no influence on the zinc concentrations in *P. scaber* in the experiments where lower concentrations of these metals were used but cadmium enhanced zinc concentrations in these animals where higher exposure concentrations were used. This is contrary to the findings of the present study. In a study by Bryan (1969) on brown seaweed, cadmium reduced the accumulation of zinc. This is in agreement with the findings of the present study for woodlice exposed to mix3 (Fig. 17).

In studies on other invertebrates where the influence of cadmium and zinc upon each other was investigated, varying results were reported. Van Gestel & Hensbergen (1997) found that these two metals did not affect each other's uptake in the springtail *Folsomia candida*. In *Helix pomatia* the concentrations of cadmium and zinc were not affected by the presence of the other metal (Berger et al. 1993). The concentrations of cadmium were reduced by the presence of zinc in the freshwater clam *Anodonta cygnea* (Hemelraad et al. 1987). Cadmium concentrations were enhanced in the presence of relatively low concentrations of zinc in the mussel, *Mytilus edulis* (Elliot et al. 1986). Ahsanullah et al. (1981) also found that cadmium accumulation was increased in the presence of zinc in the shrimp, *Callinassa australiensis*.

The inconsistency in the literature regarding the influence of cadmium and zinc on the accumulation of each other in animals could be attributed to a variety of reasons. The ratio of the concentrations of cadmium and zinc used may at least partially be responsible for the interaction or lack of interaction of these metals. It is known that for zinc to have the same impact as cadmium on organisms its concentration in the diet should usually be ten times higher than that of cadmium. Consequently, if the concentration of zinc is ten times that of cadmium in the diet then

toxicity will be due to both metals equally (Walker et al. 1996). It can then be assumed that the one metal will not influence the toxicity of the other. Berger et al. (1993) found no interaction between cadmium and zinc in *Helix pomatia*. This could partly be because the Cd:Zn ratio was much lower than that used in the present study. Beyer et al. (1982) also suggested that Cd:Zn ratio plays a role in the interaction of these two metals. However, ratio cannot be the only factor of importance in metals interaction. As suggested by Beyer et al. (1982), the actual concentrations of cadmium and zinc in the diet may also be of importance. The interaction of cadmium and zinc was not the same in all the mixture exposure concentrations of cadmium and zinc used in the present study, although the ratio remained the same. The same phenomenon can be seen in the study by Witzel (2000). The duration of exposure may also be a contributing factor in metal interaction. The metals in a mixture may initially not interact because they are handled differently by the animal and are stored in different compartments (Berger et al. 1994) but as exposure time passes these storage compartments may become saturated (Hopkin 1989) and only then will the metal interact.

4.3. Histological analysis of the hepatopancreas of *P. laevis* after cadmium- and zinc sulphate exposure

4.3.1. Histological toxicity of separately administered cadmium- and zinc sulphate

Figs. 19 & 22 provide examples of the of the clearly observable qualitative effect of cadmium and zinc exposure on the hepatopancreas. The epithelium layers of hepatopancreases of woodlice exposed to cadmium- and zinc sulphate were thinner in

comparison with those of the control specimens. It can also be seen that the B-cells show most of the obvious change/damage (Figs. 19 & 22). This could be due to various factors. The cadmium and zinc in the food of the woodlice, when present in the lumen of the hepatopancreas, may affect the apical membrane of nutrient absorbing B-cells, resulting in a reduction of these cells (Köhler et al. 1996). In the case of cadmium it may also be that the capacity of cadmium storing S-cells is exceeded at a certain level of contamination (Hopkin 1989), which somehow causes the cadmium to accumulate in the B-cells. This cadmium may affect the B-cells negatively as these cells do not usually store cadmium, but this is not certain because B-cells do not have the capacity (type B granules) to store cadmium (Hopkin & Martin 1982b), unless the cadmium accumulates in the cytoplasm of B-cells. It is also possible that there is a replacement of cell types due to the cadmium burden. B-cells may be sacrificed to accommodate more cadmium storing S-cells. It is known that zinc can accumulate in granules in the S-cells and the B-cells (Hopkin & Martin 1982b). However, if the storage capacity of zinc storing granules in the B-cells are exceeded, the excess zinc may 'spil over' into the cytoplasm of these cells damaging the integrity of the B-cells. Replacement of cell types (like in the case of cadmium), to accommodate the extra zinc burden, is unlikely as both S-cells and B-cells have the necessary capability to store zinc (Hopkin & Martin 1982b). It must also be taken into consideration that indirect factors such as starvation due to avoidance of cadmium and zinc contaminated food may play a role (Drobne et al. 1995; Odendaal & Reinecke 1999).

Similar qualitative observations showing thinning of the epithelial layer of the digestive gland after exposure to various contaminants were studied in molluscs (Lowe et al. 1981; Sunila 1986; Lowe 1988; Jonnalagadda & Rao 1996).

Histopathological damage to digestive glands of molluscs due to cadmium exposure was demonstrated by other authors. Cells in the digestive glands of *Littorina littorea* (Marigomez et al. 1990), *Mytilus sp.* (Da Ros et al. 1995) and antarctic limpets (Najle et al. 2000) were negatively affected by cadmium exposure. Qualitative damage to the hepatopancreas of the marine isopod *Idotea baltica*, after cadmium exposure, was reported by De Nicola et al. (1993). Very few studies have been conducted in the past to assess the impact of zinc on the digestive glands of aquatic or terrestrial invertebrates. Cells of the digestive gland of the grey garden slug, *Deroceras reticulatum* were negatively impacted on by zinc exposure (Triebkorn & Köhler 1996). Qualitative damage to the hepatopancreas of the terrestrial isopod *P. scaber*, due to zinc exposure, was reported by Drobne & Strus (1996).

In the present study possible change (damage) to the hepatopancreas of *P. laevis* after exposure to various concentrations of separately administered cadmium- and zinc sulphate were quantified, using Percentage Cellular Area (PCA) (Tables 14 & 15; Figs. 20, 21, 23 & 24). The sectioned hepatopancreas tubules were divided into four different zones, as described in the Materials and Methods chapter. The PCA measurements through the length of the hepatopancreas tubes in all four zones (Z1, Z2, Z3, Z4) clearly show differentiation in PCA through the length of the tube (Tables 14 & 15; Figs. 20 & 23). This can be seen in the control hepatopancreases (Table 14 & 15; Fig. 20A & 23A) and the other hepatopancreases of cadmium and zinc exposed *P. laevis* (Tables 14 & 15; Fig. 20 B, C, D & Fig. 23 B, C). However, in the 8000 mg.kg⁻¹ zinc sulphate exposure group there was only a difference observed between Z1 and the other zones (Table 15; Fig. 23D), probably because the PCA's in Z2, Z3 and Z4 were near or at their lowest levels. These results indicate that caution should be taken if such measurements as PCA or any other measurements in the

hepatopancreas of woodlice are to be used as biomarkers. For example, a false interpretation can be made if measurements in Z1 of the control hepatopancreas of woodlice are compared to those in Z3 in other woodlice. It is thus important to have relevant information for the whole organ and not to draw conclusions on the basis of measurements or observations in only a small part.

Analysis of the PCA data of hepatopancreases of *P. laevis* exposed to various concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹) and zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) show that there were declines in PCA measurements, in all four zones, compared to those in the control organisms (Tables 14 & 15; Figs. 21 & 24). It has also been shown that area of digestive gland epithelia of *Helix aspersa*, expressed as a percentage of total tubule area, was also significantly smaller than those in the control snails after exposure to copper oxychloride (Snyman 2001). Percentage Cellular Area (PCA) is therefore also useful for other contaminants and organisms.

PCA's in Z1 and Z2 of the hepatopancreas showed an unclear trend, when the different cadmium sulphate exposure groups (20, 80, 160 mg.kg⁻¹) were compared (Table 14; Figs. 21 A, B) because PCA's in these zones increased with increasing exposure concentrations. Measurements in Z3 in hepatopancreases of all three cadmium sulphate exposure groups suggest a dose-dependency, with PCA's in this zone decreasing with increasing exposure concentrations. PCA's of Z3 in all three cadmium sulphate exposure groups also differed significantly from each other ($P < 0.05$) (Table 14; Figs. 21C). Few statistical differences were found between the Z4 zones of the different cadmium exposure groups (Table 14; Fig. 21D).

PCA's in the Z1 zone of hepatopancreases of *P. laevis* exposed to various concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹) did not differ significantly when compared to each other (Table 15; Fig. 24A), indicating that a dose-related

effect could not be established in this zone. PCA measurements in Z2 of the different zinc exposure groups showed statistical differences, but PCA's increased with increasing exposure concentrations (Table 15; Fig. 24B). Measurements in the Z3 zone were mostly significantly different from each other, when the different zinc sulphate exposure groups (1000, 4000, 8000 mg.kg⁻¹) were compared. Only the PCA's in Z3 of hepatopancreases of woodlice exposed to 1000 and 4000 mg.kg⁻¹ zinc sulphate did not differ from each other significantly. PCA's in Z3 of the highest exposure group (8000 mg.kg⁻¹) were the lowest of the three exposure groups (Table 15; Fig. 24C). PCA measurements in Z4 zone were in most cases significantly different from each other, when the different zinc sulphate exposure groups (1000, 4000, 8000 mg.kg⁻¹) were compared (Table 15; Fig. 24D). Only the PCA's in Z4 of hepatopancreases of woodlice exposed to 1000 and 4000 mg.kg⁻¹ zinc sulphate did not differ significantly from each other. PCA's in Z4 of the highest exposure group (8000 mg.kg⁻¹) were the lowest of the three exposure groups (Table 15; Fig. 24D).

In a real field situation it seems unpractical and a needless exercise to section a whole hepatopancreas and measure PCA's over the total length of the organ. The Z1 and Z4 zones are not advisable for use as they are the front and terminal ends of the lobe. These ends are usually damaged to some degree because of handling with tweezers in the preparation process for histological analysis. Pieces of these ends can also break off. Reliability of measurements in these zones are thus not high. The Z2 zone can possibly be used but PCA values in this zone were not the highest, compared to other zones, in the control hepatopancreas (Fig. 20A). The potential for reduction in PCA is thus not at its highest in Z2, compared to Z3 and Z4. The full picture of possible damage done by cadmium or zinc will thus not be seen. Z3, on the other hand, exhibited the highest PCA measurements of all zones in the control

hepatopancreas (Fig. 20A). Little danger of handling damage existed in Z3. As mentioned earlier, PCA's in Z3 decreased with increasing cadmium sulphate exposure concentrations, suggesting that PCA measurements in Z3 was dose-dependent (Fig. 21C). In contrast to the case with cadmium, zinc did not give a linear dose-dependent relationship, regarding PCA's in Z3 after zinc sulphate exposure (Fig. 24C). However, a general downward trend can be seen. Zinc is also, other than cadmium, an essential metal that can generally be regulated (Bibic et al. 1997), making dose-response relationships less straightforward. All these factors suggest that the Z3 zone (from middle to three quarters to the back of tubules) in the hepatopancreas of *P. laevis* is, relative to Z1, Z2 and Z4, best suited for use if PCA's are used as a biomarker in these animals.

4.3.2. Combined histological toxicity of mixtures of cadmium and zinc

From the examples in Fig. 25 it can be seen that the epithelium layer of the hepatopancreases of *P. laevis* exposed to various mixtures of cadmium- and zinc sulphate (mix1 = 20 mg.kg⁻¹ CdSO₄ and 1000 mg.kg⁻¹ ZnSO₄ [Fig. 25B]; mix2 = 80 mg.kg⁻¹ CdSO₄ and 4000 mg.kg⁻¹ ZnSO₄ [Fig. 25C]; mix3 = 160 mg.kg⁻¹ CdSO₄ and 8000 mg.kg⁻¹ ZnSO₄) [Fig. 25D] is thinner in comparison with the control (Fig. 25A).

As discussed previously, PCA measurements are not constant through the length of the hepatopancreatic tubules of *P. laevis*. This is true for the hepatopancreatic tubules of woodlice of the control as well as the exposed woodlice groups (Table 16; Fig. 26). The four zones, as used in the present study, have been discussed earlier in this chapter. The Z3 zone was proposed as the most suited zone for PCA measurements, and various reasons for this were previously put forward.

It is clear that exposure to the mixtures of cadmium and zinc had a negative effect on PCA values of *P. laevis* (Fig. 27). PCA's in all three the mixture exposure groups were significantly lower than that of the control, in all four the zones of the hepatopancreas. However, very little differences were observed between PCA's of hepatopancreases of the different mixture exposure groups (Table 16), compared to when these metals were administered separately.

For the sake of simplicity only the Z3 zone will be considered for the next part of the discussion. In Fig. 28C it can be seen that the PCA values in mix1 were significantly lower than those when cadmium sulphate (20 mg.kg^{-1}) were administered separately. This could be attributed to the fact that cadmium accumulation (as discussed previously) was increased in the presence of zinc in mix1. PCA values in mix2 was not different from those measured for the separately administered cadmium exposure. However, in mix3 PCA measurements were significantly higher than those found in the 160 mg.kg^{-1} cadmium sulphate exposure. This can be attributed to much lower concentrations of cadmium in the hepatopancreases of *P. laevis* exposed to mix3 ($189.8 \pm 39.7 \text{ mg.kg}^{-1}$) than in the 160 mg.kg^{-1} cadmium sulphate group ($1004.6 \pm 239.3 \text{ mg.kg}^{-1}$).

In Fig. 29C it can be seen that the simultaneous presence of cadmium and zinc in the diet influenced PCA's in Z3, compared to those where zinc was administered alone. In mix1 the PCA value was higher than in the single 1000 mg.kg^{-1} zinc sulphate exposure. An explanation for this is difficult because zinc concentrations in the hepatopancreases of woodlice exposed to mix1 were not influenced by cadmium. The presence of cadmium in the diet must somehow have impacted on the effect of zinc on the PCA. In mix2 the presence of cadmium in the diet did not have an impact on the effect of zinc on the PCA. The concentrations of zinc in the hepatopancreas

were also the same as in the 4000 mg.kg⁻¹ zinc sulphate exposure. In mix3 the PCA values were much higher than those measured in the 8000 mg.kg⁻¹ zinc sulphate single exposure group. This may be attributed to much lower concentrations of zinc in the hepatopancreases of woodlice exposed to mix3 (2464.5 ±351.9 mg.kg⁻¹) than those exposed to 8000 mg.kg⁻¹ zinc sulphate (3516.4 ±675.8 mg.kg⁻¹).

Since PCA values for all the mixture exposure groups were significantly lower than those obtained for the control woodlice, PCA could have a potential as a biomarker for woodlice exposed to mixtures. The importance of PCA to measure toxic effect of metal mixtures is further emphasized by the finding in the present study that cadmium and zinc also "neutralised" each others effect in terms of mass change. Mass changes of woodlice mostly showed no differences from those of the control animals after exposure to cadmium and zinc mixtures. In mix1 the mass of woodlice in fact increased at a higher rate than those in the control group. Still, PCA values showed that these cadmium and zinc mixtures had measurable toxic effects on the metabolically important hepatopancreas of *P. laevis*. This means that measurements of mass of woodlice in medium term mixture toxicity experiments, like in the present study, could give a false impression by not revealing the toxic effects of metal mixtures as the PCA measurements, as employed in the present study, clearly showed. Negative effects on the mass of *P. laevis* could have been forthcoming if the exposure period was longer. Kammenga et al. (2000) suggested that biomarkers could be sensitive indicators of chemical stress before sublethal effects, such as inhibition of growth or effects on reproduction, become apparent. It seems to be the case for the cadmium-zinc mixtures in the present study. Walker (1998) also stated that biomarkers are valuable for detecting and quantifying toxicity where organisms are exposed to mixtures of compounds.

4.3.3. Advantages of Percentage Cellular Area (PCA) measurements

Quantitative techniques had been increasingly utilised, particularly in aquatic organisms. Quantification also avoid the subjectivity associated with descriptive histology (Lowe & Clarke 1989). Parameters usually used in such quantitative studies are lumen width/area, tubule width/area and epithelial cell height (Lowe & Clarke 1989). The parameter used in the present study, namely Percentage Cellular Area (PCA), has certain advantages above the afore mentioned parameters. A degree of subjectivity may still be a problem if you measure cell heights because you have to choose the cells to be measured, or you must measure the cell heights of all the cells. This will make the parameter laborious to use. With PCA you take all the cells in a section into account without having to make a choice. Tubule width/area together with lumen width/area may vary along the length of a single tubule or vary with size of the animal. PCA is a percentage which takes into account variations in the whole tubule area.

4.3.4. PCA-Index

A comparison of PCA's of control woodlice and exposed woodlice could provide a ratio, expressed as the PCA-index. Such an index ($PCA_i = PCA_{control} / PCA_{exposed}$) could be used to provide an indication of the toxic stress experienced as a result of exposure. Example: the PCA-index values for the 20, 80 and 160 mg.kg⁻¹ cadmium sulphate exposed woodlice are 1.21, 1.45 and 1.68, respectively. The bigger the value the more stress were experienced by the woodlice and *vice versa*. If, for instance, a PCA-index value is determined to be 1.00 no stress is experienced.

4.4. Relationships between Percentage Cellular Area (PCA) of the hepatopancreas and measured parameters in *P. laevis*

The response of biomarkers to toxic stress only has ecological relevance if the response can be linked to the exposure levels of the toxicant or effects at higher organisation levels (Kammenga et al. 2000). Van Gestel & Van Brummelen (1996) stated that biomarkers can only be sensibly used in ecological risk assessment if the relation between the biomarker response and exposure levels, on the one hand, and effects on life-history parameters, on the other hand are determined. Therefore, relationships between the PCA values of the hepatopancreas (only Z3) of *P. laevis*, the biomarker in this study, and the exposure concentrations, hepatopancreas cadmium and zinc levels, and mass change were determined.

There seems to be a clear dose-response relationship (Fig. 30) between PCA and cadmium exposure concentrations ($r = -0.93$); PCA measurements decreasing with increasing cadmium exposure concentrations. This suggests that PCA can be a very good general biomarker of exposure (Kammenga et al. 2000) for cadmium in *P. laevis*. PCA can not only be used to determine whether woodlice are under cadmium induced stress but can be used to determine the degree of stress, depending on cadmium dose. The dose-response relationship (Fig. 31) between PCA and zinc exposure concentrations ($r = -0.73$) were not as strong as in the case of cadmium. This may be due to the well known fact that zinc is normally required in the metabolism of animals and can therefore be regulated (Miller 1983), while cadmium is a foreign substance to isopods, and cannot be regulated to desirable concentrations (Hiatt & Huff 1975). In the mixture toxicity experiments (cadmium-zinc mixtures) the relationship (Fig. 32) between PCA and exposure concentrations of cadmium and

zinc tended to be weak ($r = -0.58$). This is probably due to the interaction of these two metals with each other, as demonstrated in this study.

Relationships between PCA and concentrations of cadmium and zinc in the hepatopancreas, were also calculated to determine if accumulated cadmium and zinc played any role in the intensity of the response of the measured biomarker. A good relationship (Fig. 33) was found between PCA and cadmium concentrations in the hepatopancreas after exposure to cadmium sulphate ($r = -0.88$). The response can thus be causally linked to cadmium accumulation in the hepatopancreas. Thus, the response (decreasing PCA of the hepatopancreas) cannot be attributed solely to indirect factors, such as starvation. No reliable linkage (Fig. 34) could be made between PCA (the biomarker response) and zinc concentrations in the hepatopancreas after exposure of *P. laevis* to zinc sulphate ($r = -0.40$). Again, the ability of isopods to regulate zinc can be put forward as a probable reason. After exposure of *P. laevis* to various mixtures of cadmium and zinc a very strong relationship (Fig. 35) was found between PCA and cadmium concentrations in the hepatopancreas ($r = -0.96$) but a very weak relationship (Fig. 36) was displayed between PCA and hepatopancreas zinc levels ($r = 0.23$). From this it can be concluded that the biomarker response (PCA) in *P. laevis*, after exposure to mixtures of cadmium and zinc, can be attributed to cadmium accumulation rather than zinc accumulation.

A very strong response-effect relationship (Fig. 37) was found between PCA and mass change of *P. laevis* after exposure to cadmium sulphate ($r = 0.99$). A relatively good response-effect relationship (Fig. 38) was found between PCA and mass change of *P. laevis* after exposure to zinc sulphate ($r = 0.80$). Although, the relationship between PCA and hepatopancreas zinc dose was weak, the effect of zinc on *P. laevis* was shown to be related to the biomarker response measured. The

negative effect on hepatopancreas cells after cadmium and zinc exposure, especially the B-cells, could influence the ability of these cells to perform their digestive related function (Storch 1984) adequately, which can in turn influence mass change. The lowered increase or decrease in mass of woodlice exposed to cadmium and zinc relative to those in the control woodlice can thus be causally linked to the degeneration of B-cells, expressed as PCA in the present study. These results therefore link this cellular response to a ecologically relevant life-cycle trait such as mass change, and could have predictive value in assessing toxic impact of cadmium and zinc at the higher organisational level. These results suggest that the PCA of the hepatopancreas could be regarded as a useful biomarker of effect (Kammenga et al. 2000) for cadmium and zinc in *P. laevis*. No relationship (Fig. 39) whatsoever could be found between PCA (the biomarker response) and mass change (the effect) of *P. laevis* after exposure to mixtures of cadmium and zinc ($r = -0.08$). However, this does not mean that PCA is less useful as a biomarker of effect if woodlice are exposed to mixtures of cadmium and zinc. It is reasonable to assume that the observed change in hepatopancreas structure in the present study, after exposure of *P. laevis* to mixtures of cadmium and zinc, should eventually result in the impairment of the mass of *P. laevis* if the observation period is extended. Köhler et al. (1998) noted that alterations in life-cycle parameters may be long-term responses, and that biomarker responses may be measurable in the short-term. In these cases biomarker responses may act as early warning systems, predicting changes in life-cycle parameters prior to their manifestation.

Although it is generally accepted that biomarkers have ecological relevance if they can be correlated with effects at higher organisational levels, very little research has been conducted to demonstrate causal relationships between such biomarker

responses and damage at higher levels of biological organisation in soil invertebrates (Kammenga et al. 2000). The results and findings of the present study forms part of a very small body of research results aiming to link biomarker responses to effects at a higher organisational level. Kammenga et al. (2000) only noted a few existing examples of this type of research (Köhler et al. 1998; Migula 2000).

4.5. Field survey

4.5.1. Cadmium and zinc concentrations in soil and leaf litter from field sites

Metal analysis of soil and leaf litter showed that the area surrounding the smelting works is contaminated by cadmium and zinc (Table 19). The concentrations of cadmium and zinc measured in soil and leaves collected from the unpolluted botanical gardens are typical control values. Hopkin (1990b) found 0.72 mg.kg^{-1} Cd and 96.4 mg.kg^{-1} Zn in leaf litter from an uncontaminated site. In a study by Beyer et al. (1982) Cd concentrations of between 0.06 and 0.18 mg.kg^{-1} and Zn concentrations between 51 and 56 mg.kg^{-1} were found in uncontaminated soil.

Beyer et al. (1982) found concentrations of up to 8.2 mg.kg^{-1} Cd and 175 mg.kg^{-1} Zn in soil amended with sewage sludge. At heavily contaminated sites near a zinc smelting works concentrations of Cd and Zn were found at a maximum of 1300 mg.kg^{-1} and 35000 mg.kg^{-1} , respectively (Beyer et al. 1984). Leaf litter collected from a metal contaminated site in another study had Cd concentrations of 26 mg.kg^{-1} and Zn concentrations of 1430 mg.kg^{-1} (Hopkin 1990b). The site around the smelting works in the present study could be regarded as moderately contaminated with Cd, and reasonably heavily contaminated with Zn.

Given the feeding preferences of woodlice, the Cd and Zn concentrations in the leaf litter are more important than those in the soil because it is generally accepted that the main route of metal uptake in woodlice is through the diet (Hopkin & Martin 1984).

4.5.2. Accumulation of cadmium and zinc in *P. pruinus*

In the laboratory based experiments the hepatopancreas was shown to be the target organ for cadmium and zinc compartmentalization in *Porcellio laevis*. Metal analysis of the hepatopancreas and rest of the body samples of *Porcellionides pruinus* collected from the two field sites (Table 20) showed that at least 95% of the cadmium and zinc compartmentalized in the hepatopancreas, making the hepatopancreas an important target organ for compartmentalization of cadmium and zinc in the field. The accumulation of cadmium and zinc in *P. pruinus*, relative to the exposure concentrations, in the field seem to be higher than those in *P. laevis* exposed in the laboratory. This could, amongst others, be due to differences in the two species' abilities to accumulate metals (Hopkin 1990b), or differences in the bioavailability of the metals (Lock & Janssen 2001). The variability in metal concentrations in hepatopancreas samples, especially zinc at the smelting works, may be due to genetic variability, feeding behaviour, physiological- and reproductive status (Depledge 1990). The high concentrations of cadmium and especially zinc recorded in *P. pruinus* at the smelting works is not only hazardous for the woodlice but also for their predators. Woodlice have a large range of predators (Avery 1966; Sunderland & Sutton 1980; Avery et al. 1983), which could be at risk of being contaminated with high levels of cadmium and zinc.

4.5.3. *Histological analysis of the hepatopancreas of P. pruinus*

From the examples in figure 40 and table 21 it can be seen that the hepatopancreas of *P. pruinus* from the smelting works differed substantially from those from the botanical gardens. The B-cells were reduced, as was also found in the laboratory study with *P. laevis*. The reduction in size of the B-cells could have negative implications for the maintenance of mass and growth of the woodlice, as discussed previously in this chapter, since the B-cells are involved in the digestive and absorptive processes (Storch 1984) and their impairment is likely to influence the growth of woodlice negatively.

Similar to the findings in the laboratory study, the PCA values of hepatopancreases of *P. pruinus* from the smelting works (contaminated site) were significantly lower than those measured for woodlice from the botanical gardens (uncontaminated site). PCA values of the hepatopancreas may therefore be suitable to be used as a general biomarker to measure metal induced stress in woodlice, provided that the necessary controls are available.

The considerable variability, as evident in the range, in PCA values in the woodlice from the smelting works (Table 21) may be explained by the genetic variability of individuals and differential feeding of contaminated leaf litter (Depledge 1990).

Cadmium is ten times more toxic than zinc (Walker et al. 1996) but according to Lock & Janssen (2001) the cadmium-zinc ratio in the field is usually so high that the risk of zinc ecotoxicity for terrestrial invertebrates will usually be much greater in comparison to cadmium ecotoxicity. The cadmium-zinc ratio in the leaf litter from the smelting works is Cd:Zn 1:174, meaning that the change in the hepatopancreases,

quantitatively expressed as PCA, in the woodlice from the smelter works could be mostly due to the toxic effect of zinc, rather than cadmium.

5.

Conclusions

➤ Experimental exposure of *P. laevis* to various concentrations of cadmium sulphate revealed that the mass of woodlice exposed to the higher concentrations increased at a lower rate than those of the control group and even decreased after six weeks of exposure. It is concluded that cadmium had a negative effect on the mass of these woodlice. The mass of specimens of *P. laevis* exposed to zinc sulphate were also negatively affected, especially in woodlice exposed to the higher concentrations of 4000 and 8000 mg.kg⁻¹. A dose-dependent differentiation in mass change was also found in this study for *P. laevis* exposed to zinc sulphate. Comparisons of mass changes of woodlice exposed to singly administered cadmium sulphate and mixtures of cadmium and zinc showed that the presence of zinc "neutralized" the negative effect of cadmium on mass change. Likewise, cadmium also "neutralized" the negative effect of zinc on the mass of *P. laevis*. It could thus be concluded that cadmium and zinc interacted in an antagonistic manner. Usually polluted field sites are contaminated with mixtures of metals. The findings of the present study suggest that results from single metal exposure experiments on the effect of metals on woodlice, can in most cases, not be directly extrapolated to field situations where mixtures of metals may occur.

➤ After exposure to singly administered cadmium sulphate, specimens of *P. laevis* accumulated cadmium in their bodies, predominantly in the hepatopancreas. More than 98% of the cadmium accumulated in the hepatopancreas. These animals thus exhibited the phenomenon of compartmentalization. Woodlice exposed to zinc

sulphate also accumulated zinc in their bodies. More than 99% of the accumulated zinc was in their hepatopancreases. The woodlice also exhibited compartmentalization of zinc. After exposure of *P. laevis* to mixtures of cadmium- and zinc sulphate, the hepatopancreas remained the main organ of accumulation for cadmium and zinc. The presence of zinc influenced the accumulation of cadmium in the hepatopancreases of the woodlice. Cadmium accumulation in woodlice exposed to the lowest concentrations of mixtures of cadmium and zinc (mix1) was increased in the presence of zinc, while those of the other two cadmium-zinc mixture groups (mix2 and mix3) were markedly lowered in the presence of zinc. Cadmium antagonised the accumulation of zinc in the hepatopancreas in the highest mixture group (mix3) but had no influence on the accumulation of zinc in mix1 and mix2. The ability of animals to accumulate pollutants in their bodies is one of the criteria to be fulfilled if they are to be used as biomonitors. The findings of this study suggest that *P. laevis* fulfills this criterion and may therefore have potential to be utilized in this respect.

➤ Cadmium- and zinc sulphate, administered separately and as mixtures, changed the structure of the main organ of metal accumulation, the hepatopancreas. Quantification of this change in hepatopancreas structure (expressed as PCA) showed that there was a dose-related change in Percentage Cellular Area (PCA) of the hepatopancreases of woodlice exposed to various concentrations of singly administered cadmium sulphate. Zinc sulphate also impacted the PCA of hepatopancreases negatively. Exposure to mixtures of cadmium and zinc had a negative effect on the PCA of the hepatopancreas of *P. laevis*. Cadmium and zinc also influenced each other's effect on PCA. PCA was shown to be a potential general biomarker of exposure to cadmium and zinc in *P. laevis*. PCA values could also be

related to cadmium concentrations in the hepatopancreas in the singly administered cadmium sulphate exposures and the cadmium-zinc mixture exposures. It is concluded that PCA is also a good general biomarker of effect (in terms of mass of woodlice) of cadmium and zinc in *P. laevis*. Although no definitive relationship could be shown between PCA and mass change of the woodlice exposed to mixtures of cadmium and zinc, the observed and measured change in hepatopancreas structure of these woodlice could similarly be seen as an early warning of impending impairment of growth or mass maintenance of *P. laevis* because of impairment of the metabolic and digestive functioning of the hepatopancreas.

➤ Findings of this study showed that PCA varied over the length of the hepatopancreas in the control- and metal exposed woodlice. Care should therefore be taken when using PCA as a biomarker in these animals. For consistency, it is suggested that the part from the middle to three quarters to the back of the hepatopancreas tubule (the Z3 zone) is best suited for this purpose.

➤ PCA values of specimens of *P. pruinosus* collected from the smelting works, were significantly lower than those measured in woodlice from the unpolluted botanical gardens. The indications therefore are that PCA may be suitable to be used as a general biomarker to measure metal induced stress in woodlice from the field, provided that conditions and other possible stress factors operative on exposed and control specimens are similar.

➤ Terrestrial isopods are ecologically important organisms in soil ecosystems because of their role as fragmentors of decaying leaf litter (Hassall et al. 1987).

Changes in their feeding rate due to the contamination (eg. metals) may lead to the reduction in decomposition rate and nutrient cycling. As indicated by this study the ability of terrestrial isopods to accumulate metals, such as cadmium and zinc, to high concentrations in their bodies may affect their growth. It may also be hazardous for their predators (Sunderland & Sutton 1980). The mere measurement of metal residues in the environment gives no indication of the effects of metals on ecologically important biota. The use of biomarkers in ecologically important organisms, such as terrestrial isopods, have the advantage that information on the effects of the bioavailable fraction of pollutants can be obtained (Walker et al. 1996). Biomarkers may be used to answer the following question: are environmental pollutants present at sufficiently high concentrations to cause negative effects? (Walker et al. 1996). Damage to the metabolically important hepatopancreas is a response/change that is likely to cause an impact on parameters that are ecologically relevant and important for the normal functioning of the organisms in question. However, biomarkers can ideally have maximal ecological relevance if the response can be linked causally to either exposure levels of a specific contaminant or effects at higher organizational levels. Since effects on populations are mediated through effects on individuals, biomarker responses could be linked to parameters at the individual level (Kammenga et al. 2000). Cadmium and zinc concentrations in the leaf litter and isopods, and a toxicological endpoint (mass) correlated well with the biomarker response used in this study (histological changes in the hepatopancreas). This change (PCA) of the hepatopancreas emerged as a potential general biomarker of exposure and effect. It is also suggested that this biomarker has the ability to "predict" negative impact on an ecological relevant endpoint (mass) after exposure of isopods to mixtures of cadmium and zinc. PCA of the hepatopancreas of terrestrial

isopods, together with a suite of other biomarkers and soil invertebrate toxicity tests, could form part of ecological risk assessment investigations in soil ecosystems. Histological level biomarkers, such as PCA, is easy to detect, measure and follow in routine toxicity tests and risk assessment investigations. They can provide information on toxic stress in soil ecosystems. Their eventual application in biomonitoring surveys, or in soil management practices, requires serious consideration. It will be dependant on the establishment of further linkages with other ecologically relevant parameters such as reproduction and higher population level responses.

➤ The development and validation of a PCA-index as suggested here, which relates PCA measurements of control and exposed woodlice requires further attention, but could in future provide a useful method of characterizing toxic stress in woodlice.

6.

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* Original not seen.

7.

Appendices

Appendix 1: The mean mass in gram (\pm SD) of *P. laevis* over 6 weeks of exposure to various nominal concentrations of cadmium sulphate (20, 80, 160 mg.kg⁻¹). Numbers in [brackets] indicate the number of woodlice per sample.

Exposure time (weeks)	Exposure concentrations (mg.kg ⁻¹)			
	Control	20	80	160
0	0.0733 (\pm 0.0105) [100]	0.0644 (\pm 0.002) [100]	0.0761 (\pm 0.0014) [100]	0.0828 (\pm 0.0012) [100]
1	0.0753 (\pm 0.0101) [99]	0.0666 (\pm 0.0026) [100]	0.0763 (\pm 0.0008) [100]	0.0831 (\pm 0.0026) [98]
2	0.0762 (\pm 0.0104) [98]	0.0661 (\pm 0.0022) [100]	0.0764 (\pm 0.0006) [100]	0.0840 (\pm 0.0022) [98]
3	0.0760 (\pm 0.0110) [96]	0.0659 (\pm 0.0025) [97]	0.0767 (\pm 0.0008) [99]	0.0840 (\pm 0.0030) [94]
4	0.0763 (\pm 0.0119) [91]	0.0650 (\pm 0.0040) [95]	0.0761 (\pm 0.0011) [97]	0.0842 (\pm 0.0028) [91]
5	0.0773 (\pm 0.0117) [81]	0.0646 (\pm 0.0038) [88]	0.0760 (\pm 0.0017) [90]	0.0844 (\pm 0.0032) [89]
6	0.0792 (\pm 0.0134) [80]	0.0645 (\pm 0.0039) [78]	0.0754 (\pm 0.0023) [86]	0.0817 (\pm 0.0047) [82]

Appendix 2: The mean mass in gram (\pm SD) of *P. laevis* over 6 weeks of exposure to various nominal concentrations of zinc sulphate (1000, 4000, 8000 mg.kg⁻¹). Numbers in [brackets] indicate the number of woodlice per sample.

Exposure time (weeks)	Exposure concentrations (mg.kg ⁻¹)			
	Control	1000	4000	8000
0	0.0733 (\pm 0.0105) [100]	0.0683 (\pm 0.0023) [100]	0.0754 (\pm 0.0022) [100]	0.0636 (\pm 0.0002) [100]
1	0.0753 (\pm 0.0101) [99]	0.0696 (\pm 0.0027) [96]	0.0773 (\pm 0.0014) [94]	0.0626 (\pm 0.0007) [100]
2	0.0762 (\pm 0.0104) [98]	0.0699 (\pm 0.0025) [91]	0.0769 (\pm 0.0016) [93]	0.0616 (\pm 0.0008) [99]
3	0.0760 (\pm 0.0110) [96]	0.0701 (\pm 0.0016) [88]	0.0752 (\pm 0.0025) [86]	0.0615 (\pm 0.0011) [96]
4	0.0763 (\pm 0.0119) [91]	0.0693 (\pm 0.0024) [76]	0.0739 (\pm 0.0017) [84]	0.0608 (\pm 0.0008) [94]
5	0.0773 (\pm 0.0117) [81]	0.0699 (\pm 0.0025) [70]	0.0736 (\pm 0.0028) [76]	0.0609 (\pm 0.0009) [91]
6	0.0792 (\pm 0.0134) [80]	0.0693 (\pm 0.0037) [64]	0.0737 (\pm 0.0028) [72]	0.0604 (\pm 0.0012) [88]

Appendix 3: The mean mass in gram (\pm SD) of *P. laevis* after combined exposure to mixtures (Cd/Zn) of cadmium- and zinc sulphate. Numbers in [brackets] indicate number of woodlice per sample.

Exposure time (weeks)	Exposure concentrations (mg.kg^{-1})			
	Control	20/1000	80/4000	160/8000
0	0.0733 (\pm 0.0105) [100]	0.0684 (\pm 0.0013) [100]	0.0700 (\pm 0.0014) [100]	0.0718 (\pm 0.0009) [100]
1	0.0753 (\pm 0.0101) [99]	0.0716 (\pm 0.0009) [100]	0.0716 (\pm 0.0021) [99]	0.0737 (\pm 0.0013) [99]
2	0.0762 (\pm 0.0104) [98]	0.0723 (\pm 0.0008) [100]	0.0731 (\pm 0.0018) [95]	0.0750 (\pm 0.0016) [97]
3	0.0760 (\pm 0.0110) [96]	0.0734 (\pm 0.0011) [99]	0.0736 (\pm 0.0012) [92]	0.0754 (\pm 0.0020) [97]
4	0.0763 (\pm 0.0119) [91]	0.0752 (\pm 0.0022) [98]	0.0741 (\pm 0.0023) [89]	0.0759 (\pm 0.0018) [92]
5	0.0773 (\pm 0.0117) [81]	0.0769 (\pm 0.0029) [93]	0.0747 (\pm 0.0027) [85]	0.0753 (\pm 0.0018) [89]
6	0.0792 (\pm 0.0134) [80]	0.0779 (\pm 0.0026) [89]	0.0732 (\pm 0.0028) [82]	0.0753 (\pm 0.0009) [83]