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Original Research

Metal Bioaccumulation and Oxidative Stress in Millipedes Experimentally Exposed to a Cocktail of Aluminium, Iron and Manganese

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Abstract

Forest pockets adjacent to cities are impacted by atmospheric pollution containing metals arising from urban activities. Elevated concentrations of metals have been found in forest soils and contamination thereof threatens soil biodiversity. Metals are known to induce oxidative stress and metal cytotoxicity in organisms has been linked to oxidative damage, which may threaten the health of forest soil and its biodiversity. Pill millipedes in the Afromontane forest pockets in Cape Town, South Africa are exposed to a combination of metals, arising from various sources of pollution. The objectives of this investigation were to determine the level of metal bioaccumulation and oxidative stress in millipedes experimentally exposed to a cocktail of metals. The millipedes were exposed to a high and low concentration aluminium, iron and manganese cocktail for 6 weeks. The experimental exposure resulted in bioaccumulation of these metals in millipedes in their different exposure groups. The higher tGSH concentrations, indicated activation of the endogenous antioxidant system, and the higher MDA levels, suggests lipid peroxidation by means of the increased generation of free radicals, which suggests that the pill millipedes have experienced induced oxidative stress.

Keywords: bioaccumulation, exposure, metals, millipedes, oxidative stress

Introduction

Metals found in remnant patches, such as forest pockets adjacent to cities arise mostly from emission sources born from urban land usage, inevitably contaminating these forest soils [1].

Soil invertebrates are well known indicators of pollutant levels [2], but the results are often influenced by factors [3] such as their response to metal exposure, as a result of their physiology, mobility, feeding habits and microhabitat preferences [4]. Some diplopod species developed strategies to potentially minimize

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the effects of metals on their survival, which may include a decrease in food intake, a decrease in nutrient assimilation, or both [5].

Soil is fundamental to the delivery of virtually every terrestrial ecosystem service on earth. In fact, critical ecosystem functions and services such as litter decomposition, nutrient cycling and diverse aboveground vegetation processes are sustained by soil biodiversity [6]. Soil community composition is also eminent in processes such as carbon cycling [7] and at the same time drives soil processes and functions [8]. Soil-dwelling groups form a major and significant proportion of total biodiversity [9]. It is therefore imperative to engage in long-term monitoring studies that includes soil fauna to evaluate the environmental stresses on organisms [10], which would certainly work towards ensuring the health of the rich biodiversity contained in forests [11].

The health of forest pockets that is generally rich in biodiversity is vital to human health and well-being, especially in urban cities [11] where the population is ever increasing. In Europe over 50% of the population live in urban areas and is said to increase [12]. South Africa is no different and the population [13], traffic and pollution is increasing at an alarming rate [14, 15], exposing urban dwellers to high levels of environmental pollution that increases health risks [16]. The ecosystem services provided by remnant forests pockets enhances environmental quality [17]. These benefits are economically measurable for urban dwellers [18], of which air purification, mitigation of near-road air pollution impact, urban temperature regulation, run-off mitigation, noise reduction and recreation are the most significant [19]. Nature is instrumental in coping with these environmental problems and is mostly cost effective, while simultaneously providing environmental, social and economic benefits, as well as to help build resilience [20].

Forests in South Africa are of the most species-rich temperate forests worldwide [21], but soil-dwelling organisms have not been sufficiently studied, with regard to conservation or land-use planning in South Africa. This is largely because of the lack of monitoring resources available on which to base indicators of biodiversity [22]. Natural resource management decisions in South Africa are therefore directed with little information on soil biota, which may lead to diminished functionality, a reduction in ecosystem services and in extreme cases, permanent damage to ecosystems [23].

The effects that complex mixtures in atmospheric pollution have on soil arthropods have not been investigated adequately [24]. Likewise, the pill millipedes in the Afromontane forest pockets in Cape Town are exposed to a combination of metals, arising from atmospheric pollution and the effects thereof are unclear

Previous studies, however report that in severe cases species richness and diversity of detritivores have been found to curtail along metal pollution gradients [25]. Metal cytotoxicity in organisms, have also been linked to oxidative damage [26] and have been found to induce oxidative stress [27]. Forest ecosystems are already globally under pressure from diverse environmental stresses and if soil ecosystems, which determine the productivity of forests [28] are contaminated, forest ecosystems may struggle with changes in the environment. A consequence of a decline in detrivore biodiversity may be loss of species or species combinations that play an important role in the maintenance of ecosystem function [29]. Moreover, is an increased risk of secondary poisoning due to soil organisms being a primary food source for many invertebrate and vertebrate predators [30]. Accumulated metals in soil fauna therefore has the potential for the bioconcentration of metals up the food chain [3].

The synergy between metals and the constituents of the antioxidant defence systems is essential in the ecotoxicological response of an organism to its environment [31]. Studies on these interactions are imperative for the identification of biomarkers that can serve as early warning systems for environmental monitoring [32]. Organisms have developed complex antioxidant defence mechanisms of both enzymatic and non-enzymatic nature in an attempt to minimize ROS-induced damage. The non-enzymatic antioxidant defences contain molecules of low molecular weight that act as free radical scavengers such as ascorbic acid, tocopherols and glutathione. Conversely, lipid peroxidation is a common mechanism of cellular injury in invertebrates, and acts as an indicator of oxidative damage in cells and tissues [33].

In the current study the forest situation was mimicked in a laboratory experiment through exposure of the pill millipedes to a combination of the metals, (Al, Fe and Mn) at high, low and control concentrations, as it would naturally occur in the environment. These particular metals were chosen, because of the higher concentrations found in the millipedes, soil and the leaf litter in a field investigation conducted by the authors of this investigation [34].

The overall aim of the exposure study was to determine the health of forest pockets associated with a city. It is thus critical to know at which metal concentrations the organisms react negatively in order to ensure the survival and effective management of forests. The objectives of the exposure experiment were: 1) to determine the metal accumulation in the pill millipede *Spaerotherium compressum,* after exposure to a cocktail on metals after a period of 6 weeks and 2) to assess whether responses linked to oxidative stress measured in the millipedes may be utilized as potential biomarkers of exposure to the metals Al, Fe and Mn. Two markers associated with oxidative stress, i.e. MDA (measured as TBARS) as marker for oxidative lipid damage, which is a product formed between MDA and thiobarbituric acid (TBA) [35] and glutathione (tGSH) levels, a non-enzymatic endogenous antioxidant, which performs an essential role in neutralizing and or

detoxifying the oxidative damage caused by ROS will be used [36].

Materials and Methods

Exposure Medium

Soil, leaf litter and decaying wood from the ancient, indigenous Orange Kloof forest on Table Mountain, Cape Town, South Africa, where the pill millipedes were collected, served as the control medium (C). The decomposing leaf litter contained leaves from the various Afromontane tree species and the decaying wood was covered partially in the moss *Hypnum cupressiforme* and lichen *Parmotrema* sp. The soil was classified loam sandy after a 5 fraction analysis. Decaying wood and branches covered in the moss and lichen were added to the tanks as food, but more so to create the most natural environment possible for the millipedes, thus minimizing unnecessary stress [37].

Pill Millipede Species Used in the Study

Adult millipedes (*Sphaerotherium compressum)* similar in size were hand collected underneath the litter layer on the soil from the indigenous Afromomtane Orange Kloof forest, approximately 22.4 km from the city centre, which is relatively secluded from urban activities. These pill millipedes were selected due to their high population density on the mountain notwithstanding the fact that they have, to the best of our knowledge, not been used in similar studies. Other millipede species were however previously shown to be indicators of pollution and have been used to assess the quality of soils [24]. Extra care was taken when they were transported to the laboratory, i.e. in separate plastic containers filled with forest soil in order to prevent unnecessary stress.

Exposure Procedure

The pill millipedes were acclimatized in the laboratory for fifteen days, which is advised in order for these organisms to return to a relatively stress free condition after being handled and before being exposed to contaminated soil for six weeks [38].

Soil pH (7.21 to 7.28) as well as moisture percentage of the soil (20.39% to 21.89%) and leaf litter (64.39 % to 65.38%) was measured before the three tanks (20 cm wide x 25 cm long x 45 cm high) with perforated lids for aeration were filled with the forest soil, leaf litter, decaying branches and thirty millipedes per tank. The environmental conditions of the collection site such as photoperiod of 12 h light/12 h dark, relative humidity (30-50%) and temperature of ±21 to 22ºC, which included a daily mist spray of distilled water was adhered to in the laboratory [24, 38].

After the acclimation period, twelve millipedes were randomly removed from the three tanks and weighed. Six millipedes were killed and frozen to be digested, as were samples of the soil and leaf litter. This procedure marked week 0 of the six-week exposure period which followed.

The six-week exposure period commenced by preparing three tanks with 2 kg of fresh, moist (20-23%) forest soil, 200 g of leaf litter, as well as decaying wood. Two separate cocktails of the metals (Al, Fe and Mn) were prepared for two exposure tanks (exposure group one and exposure group two). The remaining tank served as the control tank. The mixture of exposure group one consisted of 1.4 g of aluminium sulphate, 1.4 g of iron sulphate and 0.06 g of manganese sulphate. The powders were mixed with distilled water and sprayed onto the soil. The same method was used to spike the soil of exposure group two, which consisted of 20 g of aluminium sulphate, 20 g of iron sulphate and 1.2 g of manganese sulphate. These exposure concentrations were based on concentrations that were measured of these metals in the forest soils during a previous field study [34]. The purpose of exposure groups one and two was to simulate a once off "pollution event" in a laboratory-based experimental setting.

The pill millipedes from the acclimation period were divided between the three tanks (26 per tank) and the same procedure with regards to photoperiod of 12 h light and 12 h dark, relative humidity (30-50%) and temperature of ±21 to 22ºC, including a daily mist spray of distilled water [38] was adhered to in the laboratory during the exposure period of six weeks. Fresh leaf litter was collected from the control field site on a weekly basis and a thin layer added to the tanks to replenish the millipedes' food source.

After the six-week exposure period, the pH of the soil (6.76 to 7.29) was taken, as well as the moisture percentage of the soil (22.35 to 23.11%) and leaf litter (54.35 to 55.93%), after which the samples were frozen before metal analysis. Twelve millipedes per tank were removed and six were frozen for subsequent digestion purposes, each stored in a separate labelled 10 ml plastic vial. The remaining six millipedes per group were gut-cleared and frozen at -80ºC for oxidative stress analysis.

Acid Digestion

The soil, leaf litter and millipede samples were dried at 60ºC for 48 hours in an oven and ground into powder, homogenized and weighed to approximately 0.2 to 0.3 g per sample. The weighed samples were digested with 10 ml 65 % nitric acid, in accordance with the method used by [39], after which it was filtered through Whatman no 6 filter paper, followed by Whatman 0.45 µm cellulose nitrate membrane filter paper. The soil, leaf litter and millipedes were analysed for the three metals used in the cocktail (Al,

Fe and Mn). The metal concentrations in the samples was determined with an Inductively Coupled Plasma Mass Spectrophotometer (ICP-MS) and calculated using the following formula:

$$\frac{\text{(ICP reading–Blank) x [200 dilution factor]}}{\text{Dry mass of sample (g)}}$$

Metal concentration is expressed as mg/kg.

Biochemical Analysis

The exoskeleton of the pill millipedes was removed and freeze dried for 48 hours. The preparation for tGSH and lipid peroxidation analysis was done by using approximately 2 g of millipede samples, respectively. Each of the samples was weighed using a Sartorius 2006 MP Balance and homogenized in ice cold 10 ml Sodium Phosphate monobasic buffer (7.5) (Sigma Aldridge). EDTA (Ethylenediamine-tetraacetic acid disodium salt-dihidrate) (99.1%), distilled water and NAOH (Sodium hydroxide), as well as Triton (0.2%) x 100 was added to the buffer solution The homogenate was stored in Eppendorf microfuge tubes at -80ºC until further analysis.

Lipid Peroxidation

The concentration of malondialdehyde (MDA) as a marker of lipid peroxidation (LPO) was determined [40] and based on the reaction with thiobarbituric acid (TBA). A mixture of 3 ml of TBA reagent made up of 1:3 by volume of 0.8% TBA and 20% trichloroacetic acid (TCA) was prepared and mixed well with a 0.33 ml of the homogenate. A boiling water bath was used to incubate the mixture for 20 min, whereafter it was cooled on ice. The mixture was centrifuged at 4200 rpm for 20 min and the MDA level was measured spectrophotometrically at 532 nm. The results were expressed as nM of MDA mg^{-1} of wet tissue.

Total Glutathione

The total glutathione level at 412 nm was measured [41] using 50, 5-dithio-bis-(2-nitrobenzoic acid) (DTNB). The assay mixture comprised 0.1 ml of the homogenate, 1.5 ml of 0.5 M phosphate buffer, pH 8.0 followed by 0.4 ml of 3% metaphosphoric acid and 30 l L DTNB (0.01 M). The total glutathione present in the sample in terms of 1 g g^{-1} wet weight tissue was calculated after calibration against the standard curve of GSH.

Statistical Analysis of Data

Kruskal-Wallis One-Way Analysis of Variance on Ranks was carried out to compare the concentrations of metals in soil, leaf litter and millipede samples, as well as the tGSH and MDA concentrations in millipedes between the different exposure tanks. The Student-Newman-Keuls method was used to do pairwise multiple comparisons. T-tests were used to compare soil, leaf litter and millipede metal concentrations between week 0 and week 6, as well as to compare the tGSH and MDA concentrations of millipeds between week 0 and week 6 in their respective exposure groups. The values are presented as the mean ±SD and the probability levels used for statistical significance were $P<0.05$. Statistical analysis was done using the Sigmaplot 13.0 software package.

Results and Discussion

Metal Bioaccumulation

Comparisons of Metal Concentrations in Soil, Leaf Litter and Millipedes between Different Exposure Groups

The mean metal concentrations in soil, leaf litter and millipedes of the control group, as well as exposure groups one and two are presented in Table 1 and Fig 1. Metal concentrations are expressed in mg/kg.

There were no statistically significant differences found in terms of soil between the exposure groups in the concentrations of the metals aluminium ($P = 0.067$), iron ($P = 0.067$) and manganese ($P = 0.067$) at week 0 or at week 6: aluminium ($P = 0.333$), iron ($P = 0.067$) and manganese ($P = 0.333$). Nor were there any significant differences in terms of leaf litter between the exposure groups at week 0 aluminium ($P = 0.067$), iron ($P = 0.067$) and manganese concentrations ($P = 0.067$) or week 6 aluminium ($P = 0.333$), iron ($P = 0.333$) and manganese ($P = 0.067$). There were no statistical significant differences when week 0 and week 6 were compared for soil and leaf litter (Table 1).

The soil from the control group, taken from the collection site in the forest contained elevated metal concentrations, of which its crustal origin may have contributed to the metal load [42]. The lack of statistically significant differences found in the metal concentrations in soil and leaf litter, respectively after week 6 may be due to leaching of the elements to the surface of the tank, as a result of the daily mist spray to accommodate the moisture requirement of the millipedes. This may have resulted in lower metal concentrations in the topsoil and is a natural occurrence in the highly porous soils [43] used in this experiment [44]. Metals are similarly released from contaminated litter [45] and may in all probability also serve as clarification for the same results. Notwithstanding such metal releases, the use of the forest soil, already contaminated by metals and further contaminated in this experiment, illustrated a pollution event as it may occur naturally in the forest environment. The results found in this study may therefore be a more realistic view of pollution deposition, accumulation and contamination as it occurs naturally in the environment.

Table 1. The mean metal concentrations (mg/kg) (± SD) in soil, leaf litter and millipedes at the start (week 0) and the end (week 6) of the experimental exposure period for all three exposure groups (control, exposure group one and exposure group two).

WEEK 0		SOIL			LEAF LITTER			MILLIPEDES		
		Control	One	Two	Control	One	Two	Control	One	Two
Al	Mean	[a]4008.40	[a]4414.21	[a]6699.92	[a]825.44	[a]946.06	[a]1408.78	[a]585.62	[a]366.65	[a]307.55
	SD	0.01	0.00	0.00	0.00	0.01	0.01	315.77	139.56	57.78
Fe	Mean	[a]3166.60	[a]3492.28	[a]3554.70	[a]640.96	[a]740.37	[a]875.97	[a]301.86	[a]256.10	[a]216.27
	SD	0.00	0.00	0.00	0.00	0.00	0.00	158.05	57.86	44.61
Mn	Mean	[a]24.45	[a]50.99	[a]174.93	[a]97.64	[a]134.63	[a]132.20	[a]0.00	[a]16.77	[a]19.07
	SD	0.00	0.00	0.00	0.00	0.00	0.00	9.43	7.47	8.40
WEEK 6		**SOIL**			**LEAF LITTER**			**MILLIPEDES**		
		Control	One	Two	Control	One	Two	Control	One	Two
Al	Mean	N/A	[a]5014.40	[a]6228.80	N/A	[a]1305.42	[a]1448.71	[a]391.84	[a]471.69	[a]368.72
	SD	N/A	0.00	0.00	N/A	0.00	0.00	310.54	30.41	178.71
Fe	Mean	N/A	[a]4286.38	[a]5431.27	N/A	[a]882.29	[a]909.62	[a]228.88	[a]310.11	[a]254.53
	SD	N/A	0.00	0.00	N/A	0.00	0.00	164.12	33.07	84.38
Mn	Mean	N/A	[a]77.59	[a]80.83	N/A	[a]120.45	[a]338.10	[a]0.00	[a]9.15	[a]12.68
	SD	N/A	0.00	0.00	N/A	0.00	0.00	16.16	3.57	4.47

Statistical significant differences between exposure groups are indicated with different superscripted letters. Comparisons were done separately for the soil, leaf litter, millipedes and the three metals. N/A: Control soil and leaf litter was not analysed after week 6, as the control substrate was not exposed to the metal cocktail. Note for SD of 0.00: Rounded down as a result of very low values. N = 6.

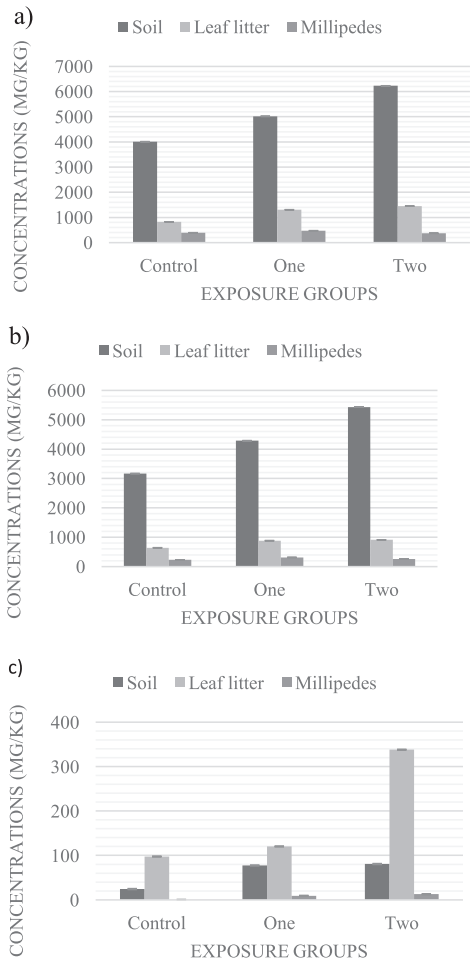

Fig. 1. The mean metal concentrations (mg/kg) (±SD) in soil, leaf litter and millipedes at week 6 of the experimental exposure period for all three exposure groups (control, exposure group one and exposure group two) are shown in Figs 1: a) Al, b) Fe and c) Mn. N = 6.

Aluminium concentrations in millipedes displayed no statistically significant differences between any of the three exposure groups at the start (P = 0.361) or the end (P = 0.829) of the exposure period.

No statistically significant differences in iron concentrations at the onset (P = 0.829) or end (P = 0.629) of the exposure period in the millipedes were measured between any of the three exposure groups. Millipede manganese concentrations showed no statistically significant differences between the three groups at week 0 (P = 0.050) or at week 6 (P = 0.071). The metals, Al, Fe & Mn also showed no statistical significant differences between the week 0 and week 6 comparison for millipedes.

However, a general trend of increased concentrations with increased exposure time and exposure concentration was observed. Aluminium concentrations in the control group (585.62 mg/kg) were the higher in millipedes at the start of the exposure period, but lower in this same group (391.84 mg/kg) after six weeks, which may have been a case of detoxification of unwanted elements and consequent inactivation, storage, and/or excretion [46]. Aluminium concentrations in millipedes were higher in exposure groups one and two after six weeks of exposure, which is to be expected with the addition of metals to the diet of the millipedes. [47]. The millipedes in exposure group one (471.69 mg/kg) showed the highest aluminium concentrations after six weeks, even though exposure group two had the higher application of the metal. The difference between the two exposure groups may be explained by a reduction in food uptake in the millipedes of exposure group two [47] to avoid further uptake of the toxins and is a normal response to metal contaminated food [5] (Table 1 and Fig. 1).

The control group (301.86 mg/kg) of millipedes showed the highest iron concentrations at the onset of the exposure experiment, but the millipedes decreased in iron concentrations in this same group (228.88 mg/kg) after the experiment. Detoxification of the undesirable elements, inactivation, storage, and/or excretion may have been instrumental in the lower concentrations [46]. Both the exposure groups showed higher iron concentrations in millipedes after six weeks of exposure, most likely by cause of a higher diffusion rate, due to the application of metals to the millipedes' diet. Consequent increases in the concentration of the respective metals in the bodies of the millipedes usually occurs as a result thereof [47]. The highest iron concentrations were observed in exposure group one (310.11 mg/kg) after six weeks, although exposure group two received the higher addition of metals. This difference between the two exposure groups may be clarified by the millipedes in the exposure group two reducing their food intake [47] in an attempt to avoid further uptake of the toxins, which is an excepted response to metal contaminated food [5] (Table 1 and Fig. 1).

The control group of millipedes displayed manganese concentrations that was too low to be detected in millipedes at the start and end of the period. In exposure groups one and two, on the other hand, manganese showed higher concentrations in millipedes at the onset of the experiment, in comparison with the lower manganese concentrations in millipedes after the exposure period. The highest manganese concentrations were found in exposure group two on week 0 (19.07 mg/kg), but also after the exposure period in the same group (12.68 mg/kg). When the metal concentrations decrease with the addition of metals it can be as a consequence of an accelerated saturation of the uptake mechanisms in animals subjected to extremely high metal burdens. They may also have reduced their food intake [47] (Table 1 and Fig. 1).

Bioaccumulation of aluminium, iron and manganese in millipedes in their individual exposure groups have occurred, indicating ingestion of the contaminated litter in the metal spiked soil [48] (Table 1 and Fig. 1). Leaf litter, being the millipedes' major food source is known to accumulate an exceeding amount of metals [49]. Not only did the leaf litter contain metals from the site in the forest where it was collected from, it also increased in concentrations with the additional exposure to the aluminium, iron and manganese cocktail (Table 1 and Fig 1). The large quantities of metals contained in decaying leaves alone, exposing millipedes to excessive metal concentrations is as startling as it is concerning [5], considering that they digest just about half of the bacteria and fungal hyphae in the litter [50].

The millipedes, however displayed varied concentrations of the metals when analyzed after the exposure period, which was expected, as the impact of toxicants on invertebrates largely depend on the bioavailability of the individual substances for the organism. A number of other factors are also at play with regard to the bioavailability of a toxicant, such as the specific toxicant, characteristics of the environment, the organism itself [51] and the particular composition of the intestinal microflora of the invertebrate [52]. In addition, the concentration present in the diet is predominantly dependent on the rate of resorption of these substances [53]. However, there is still a need for further research with regards to metals in their biology [5]. The concentrations of metals in the bodies of millipedes also vary with different species [54] and depending on the metal content in the food, their assimilability also differs [55]. Their metal concentration levels can also be elevated remarkably when they have been exposed to heavily metal-contaminated soils over a long period of time [56]. This is reiterated in the metal concentrations measured in the millipedes sampled from the collection site, that were not all that different from the concentrations measured in them after a period of six weeks (Table 1 and Fig. 1). Terrestrial invertebrates do not have much control over the uptake of metals from the food pulp [57], but millipedes have shown to discriminate their diet and regulate the rate of consumption in an effort to avoid the uptake of metal contaminated food [55], which is evidence of avoidance behavior [47]. In this study the millipedes seemed to have exerted this behavior in their natural forest soil and leaf litter at concentrations of: Al (4000 mg/kg in soil and 800 mg/kg in leaf litter); Fe (3000 mg/kg in soil and 600 mg/kg in leaf litter) and Mn (24 mg/kg in soil and 95 mg/kg in leaf litter) (Fig. 1). Using soil and leaf litter, collected from their original forest habitat is therefore advisable in order to obtain realistic results. With regards to metal poisoning of the animals and of great significance, is the prospect of detoxification of undesirable elements and, hence the capacity to inactivate, store, and/or to excrete them [46].

Oxidative Stress Indicators

Comparisons of tgsh and Mda Levels in Millipedes between Different Exposure Groups, as Well as Comparisons of tgsh and Mda Levels in Millipedes between Week 0 and Week 6 of Exposure Groups

The mean tGSH and MDA (measured as TBARS) concentrations in millipedes of the exposure groups, as well as the mean tGSH and MDA (measured as TBARS*)* concentrations in millipedes between week 0 and week 6 of the exposure groups are presented in Table 2. Concentrations are expressed in µmol/g.

Induced detoxification mechanisms may have allowed the animals to survive being poisoned by metals [51]. This study highlighted the millipedes' ability to overcome stress factors either by induction of cellular processes or metal detoxification [5].

Both increases and decreases in tGSH levels in millipedes in their respective exposure groups, as well as between week 0 and week 6 were found, indicating

Table 2. The mean tGSH and MDA concentrations (µmol/g) (±SD) in millipedes at the start (week 0) and the end (week 6) of the experimental exposure period for all three exposure groups.

WEEK 0		MILLIPEDES		
		Control	One	Two
tGSH	Mean	^{a}425.43	^{b}201.20	^{b}274.70
	SD	*35.25*	*50.37*	*39.15*
MDA	Mean	^{a}0.60	^{a}0.31	^{a}0.84
	SD	*0.21*	*0.37*	*1.26*
WEEK 6		**MILLIPEDES**		
		Control	One	Two
tGSH	Mean	*^{a}198.17	*^{a}286.53	^{a}291.28
	SD	*99.55*	*8.06*	*26.42*
MDA	Mean	#^{a}0.10	^{a}0.12	#^{a}0.28
	SD	*0.04*	*0.02*	*0.38*

Statistical significant differences (P<0.05) between exposure groups are indicated with different superscripted letters.
An * next to a week 6 mean value indicates a statistical significant difference (P<0.05) between week 0 and week 6 for tGSH. A # next to a week 6 mean value indicates a statistical significant difference between week 0 and week 6 for MDA.
MDA (expressed as µmol TBARS per gram material);
SD = Standard Deviation; N = 6.

that an overproduction of reactive oxygen species (ROS) in these organisms had occurred.

Statistically significant differences on week 0 were found in tGSH (P<0.05) concentrations between the exposure groups control vs group one and control vs group two. The highest tGSH concentration of 425.43±35.25 µmol/g in millipedes were measured in the control group, which was significantly higher than were found in exposure groups one and two (Table 2). The tGSH concentrations found in the millipedes at the start of the exposure period may have arisen from the control soil taken from the collection site at the forest caused by accompanying stress factors in their environment [58]. The highest tGSH concentration measured in the millipedes of the control group (425.43 µmol/g) at the start of the exposure period, was therefore possibly by chance, due to contamination over a long period, as the soil and millipedes came from the same collection site Tables 1 and 2.

Statistically significant differences in tGSH concentrations in millipedes between week 0 and week 6 were found in the control (P = 0.020) and exposure group one (P = 0.044). Millipedes showed the highest concentrations of tGSH in the control group (425.43±35.25) on week 0, as opposed to the significantly (P = 0.020) lower concentration of 198.17±99.55 µmol/g at the end of week 6. (Table 2). This implies that damage to tissues in control millipedes had occurred and could have been due to the chronic exposure to pollutants, such as metals [59] from the site that it was collected from (Table 1). The highest tGSH concentrations at week 0 in the control group millipedes, although not statistically measured seemed to have shown a link with the highest aluminium and iron concentrations, also measured in the millipedes from the control group (Tables 1 and 2).

Enhanced tGSH concentrations in millipedes were noticed after 6 weeks in exposure groups one (286.53 µmol/g) and two (291.18 µmol/g) that have been spiked with the aluminium, iron and manganese cocktail, at the start of the exposure period. These results suggest that these metals were instrumental in the further induction of ROS in these organisms [60]. The highest tGSH concentrations were found in millipedes with the highest aluminium, iron and manganese concentrations from exposure groups one and two. The aluminium and iron concentrations in the millipedes of exposure group one were slightly higher (Table 2), even though the tGSH concentrations in the millipedes showed the highest concentrations in exposure group two. This demonstrates that the endogenous antioxidant is successfully scavenging ROS in an attempt to protect the cells against the induced oxidative stress [61] and simultaneously detoxifying metals [62] at the initial high application of the metals. It thus seems that an overproduction of ROS was caused, which activated the antioxidant defence mechanism in order to protect cells from the induced oxidative stress (Table 2).

MDA content (measured as TBARS) in millipedes showed higher levels at the start of the exposure period in all three groups, without the additional metal application. Significant differences in concentrations in millipedes were noticed between week 0 and week 6 of the exposure group, control (0.6 µmol/g). The exposure group two (0.84 µmol/g) displayed the overall highest MDA concentration (Table 2).

Millipedes showed statistically significant differences between week 0 and week 6 in the exposure group: control (P = 0.018) in terms of mean MDA concentrations. The highest MDA concentration was found in millipedes in exposure group two on week 0 (0.84±1.26), in contrast with the significantly lower concentration (0.28±0.38) at the end of week 6 (Table 2).

The increased MDA content in the millipedes at the start of the exposure period, indicates that they have already been exposed to elevated concentrations of toxic elements [35] over an extended period of time. Pollutant (such as metal) contamination of the millipedes may already have occurred at their collection site, as the metal concentrations measured from this site was already elevated. Lipid peroxidation by means of the generation of free radicals [63] is an indication of oxidative stress [64]. The lowest MDA concentration was found in the control group after week 6 (0.1 µmol/g). Even a low MDA level is an indication that lipid peroxidation (LPO) had occurred. This is due to the fact that MDA, being a decomposition product of polyunsaturated fatty acids, was produced during peroxidation of membrane lipids [65].

The lowest MDA concentrations were measured in millipedes from the control group and the highest MDA content in the millipedes from exposure group two, which is, therefore an indication that the millipedes in the group, exposed to the highest concentrations of the metals, aluminium, iron and manganese (Table 2) experienced induced oxidative stress, associated with peroxidative damage of membrane lipids in the organisms [66]. Metal exposure does induce oxidative stress in invertebrates, which have been reported from laboratory experiments [67] (Table 2).

Induced detoxification mechanisms may have allowed the animals to survive certain acute shocks and avoid being poisoned by metals [51]. This study highlighted the millipedes' ability to overcome stress factors either by induction of cellular processes or metal detoxification [5].

Conclusion

The pill millipedes demonstrated good bioaccumulation abilities. There is, however evidence of tolerance to the metal-containing food in their respective exposure groups, as well as avoidance behavior, through the contaminated substrate that they have ingested during a six-week exposure period. This study showed at which metal concentrations the pill millipedes reacted negatively to contaminated food as it occurs in their natural forest environment, which is already a warning sign. The results of this study is therefore valuable in the management of the health of forests ensuring their continuous survival. It is also recommended that more studies are done in these urban forests to determine concentrations that may cause acute toxicity in millipedes, while simultaneously monitoring metal concentrations over time that may cause chronic toxicity. The millipedes showed the ability to overcome stress factors either by induction of cellular processes or metal detoxification. The higher metal and tGSH concentrations measured in their respective exposure groups indicated that the elevated metal concentrations in the millipedes activated the endogenous antioxidant system to scavenge ROS in an effort to protect cells against the induced oxidative stress and promote detoxification of the metals. The enhanced MDA content in the millipedes at the start of the exposure period, is an indication that these organisms have already been exposed to high concentrations of toxicants, (including metals) over a long period, which may already have occurred at the site, from which the soil, leaf litter and the millipedes were collected. The higher MDA levels measured in millipedes in the group, exposed to the highest metal exposure concentrations also suggests lipid peroxidation possibly by means of the increased generation of free radicals, which suggests that the pill millipedes have experienced induced oxidative stress. These responses suggest that pill millipedes may be utilized as potential biomarkers of exposure to the metals Al, Fe and Mn, which is significant in the conservation of forest ecosystems. More research is, however required to confirm and further explore the use of oxidative stress parameters in invertebrate model systems as an indication of induced environmental metal exposure/toxicity.

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Conflict of Interest

The authors declare no conflict of interest.

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