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THE INFLUENCE OF SEASONS ON METAL BIOACCUMULATION AND OXIDATIVE STRESS IN SENTINEL ORGANISMS IN SOUTH AFRICAN URBAN FORESTS

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ABSTRACT

Variations in Al, Fe and Mn concentrations in soil, leaf litter, moss, lichen and pill millipedes, as well as the induced oxidative stress effect of metals on the organisms, between the dry and the wet season within two Afromontane forests in the Western Cape were investigated. The two forests in the closest proximity of the Cape Town city centre and urban pollution sources were chosen to determine the difference of metal contamination between the dry, hot and cold, wet season. Three sampling sites were chosen within each forest at different altitudes on Table Mountain, as well as a control site, where the soil, leaf litter and each organism were randomly collected in both the dry and the wet seasons for metal and biochemical analysis. The metals in the wet season in both of the forests showed higher concentrations, as did the forest closest to the Cape Town city centre. This is indicative of higher levels of pollution due to increased anthropogenic activities in the wet season. Oxidative stress in organisms was apparent in the varying tGSH levels, of which the dry season mostly revealed the higher concentrations. Oxidative lipid damage (measured as MDA) levels were generally increased during the wet season when the metal concentrations were higher as a result of prominent brown haze episodes in winter. Urban forest ecosystems in urban cities may suffer irreparable damage resulting from metal contamination, more so in the colder wet season.

KEY WORDS : Metals, Forests, Season, Soil, Leaf litter, Moss, Lichen, Pill millipedes, Oxidative stress, Brown haze

INTRODUCTION

Forest health is a continual concern, but moreover is their sustained growth and survival, as a result of air pollution, anthropogenic pressure and climate change. It is alarming how forests and wildlife resources are disappearing world-wide. With figures such as seventy million hectares of primary forests lost within a period of twenty years (1995 to 2015) (Mohebalian and Aguilar, 2018) one cannot even fathom the losses in biodiversity, aesthetic view of landscapes, and ecological functions, not to mention the provision of goods and services for human needs. The decline in biodiversity negatively affects rural livelihoods and threatens food, energy and health security of nearby communities (Pudyatmoko *et al.*, 2018). For urban forests that are surrounded by rapidly expanding cities and industrialization, the threat is even more profound (Bytnerowicz *et al.*, 2008) as urban and peri-urban forest vegetation is able to consistently reduce pollution levels by way of the uptake of gaseous pollutants and the adsorption of particulate matter on the surface of leaves (Nowak *et al.*, 2014).

Forest canopies capture aerosols, highly enriched with metals (Shi *et al.*, 2011), which may cause more frequent enrichment of soils via stemflow, throughfall and litter-fall (Mills *et al.*, 2012). Such aerosol depositions can contribute significantly to top soils of forests (Garrison *et al.,* 2003), which can magnify over the years, causing elevated concentrations in soil (Olowoyo *et al.,* 2010). Air pollution has a direct impact on the health of fauna and flora, but is also indirectly affected through contaminated soil and water that they are reliant on (DEADP, 2010). Such soils and plant surfaces become major sinks for these atmospheric pollutants and soil being the basis of the food chain, is the route by which toxic metals are transmitted to humans (Monaci *et al.,* 2000), thereby endangering the health of humans and living organisms (Gurjar *et al.,* 2010).

In urban cities forest ecosystems are subjected to metals in atmospheric pollutants arising from various anthropogenic activities, which is already known to induce reactive oxygen species (ROS) in organisms (Koivula and Eeva, 2010; Sanchez-Virosta *et al.,* 2015) and can cause an overproduction of ROS in these forest ecosystems (Tausz *et al.,* 1998, 2007a, b; Bussotti, 2008). In turn, the organisms in forests can then incur oxidative damage as a result of increased production of ROS in their cells (Gill and Tuteja, 2010; Sharma *et al.,* 2012; Contin *et al.,* 2014).

As a result of anthropogenic activities soils all over the world have deteriorated (Papu-Zamxaka *et al.,* 2010). Soils and their innate, diverse communities are therefore in dire need of protection (Römbke *et al.,* 2005). Studying, the interaction between metals and living organisms, the processes of their biogenic migration and consequently the effects thereof in ecosystems are thus of great significance (Weber and Karczewska, 2004). Biological monitors can provide quantitative information on the atmospheric pollutant load in the environment (Sant'Ovaia *et al.,* 2012). Biological indicators are then used for the detection, deposition, accumulation and distribution of these pollutants in the environment (Markert *et al.,* 2000). Lichens and mosses are well studied biomonitors and indicators of contaminants in the air (Kruger *et al.,* 2019). Their different metal uptake and retention capabilities when used in combination yield even better results (Conti and Cecchetti, 2001; Szczepaniak and Biziuk, 2003), but higher accumulation have been observed in mosses (State *et al.,* 2012; Boltersdorf *et al.,* 2014). Invertebrates, such as millipedes provide important ecosystem services (Lavelle *et al.,* 2006). They are instrumental in soil processes, such as fragmentation, mineralization and redistribution of and decomposition of organic matter as well as influences soil elements and soil aeriation (Smit and Van Aarde, 2001).

The moss *Hypnum cupressiforme* with its dense carpet is found in the Afromontane forests of Table Mountain, growing on tree trunks, logs, rocks and soil and are known to absorb pollutants, especially metals directly from atmospheric deposition (Sacharová and Suchara, 1998). Lichens accumulate pollutants in the entire surface of their thallus (Aznar *et al.,* 2008; Conti *et al.,* 2011) and is used in the interception of allogeneic atmospheric materials dissolved in wet precipitation, dry depositions, and gaseous emissions (Nash, 2008). *Parmotrema* is a foliose lichen with short and broad, ciliate lobes and are commonly found on Table Mountain on trees, rocks, decaying wood and soil (Crespo *et al.,* 2010), which is also the genus used in this study. Invertebrates such as millipedes are used as bio-indicators to assess soil pollution (Godoy and Fontanetti, 2010; Nogarol and Fontanetti, 2010; Kruger *et al.,* 2019). The bulky, pill shaped pill millipede *Sphaerotherium compressum* (Brandt, 1833) used in this study is a diverse higher taxon of Diplopoda (Hoffman, 1980) and are also commonly found on Table Mountain in the Afromontane forests (SANBI, 2016).

Oxidative stress biomarkers respond to contaminants (Tsangaris *et al.,* 2011), such as metals. The toxic effect of metals in biological systems for example, may lead to the increased production of reactive oxygen species (ROS), affecting various cellular processes, primarily the functioning of the membrane system (Valko *et al.,* 2005). ROS plays a role in regulating cellular signalling via modulating redox status. The impact of pollutants on organisms in field situations can thus be assessed using oxidative stress biomarkers. However, seasonal variations may also change the antioxidant defence systems in animals (Verlecar *et al.,* 2008). Interaction between metals and constituents of the antioxidant defence systems plays a critical part in the ecotoxicological response of an organism to its environment (Regoli *et al.,* 2006). Thus, such studies are imperative in the identification of biomarkers, which can serve as early warning systems for environmental monitoring.

South Africa has typically dry (annual average rainfall of 450 mm) soils (DEAT, 2006) and strong prevailing winds in which dust travels over enormous distances transporting high densities of soil-borne dust (Windfinder, 2014). Metals are blown in this manner to remote mountain areas via long-range atmospheric transport and accumulate in the soil and plants by wet or dry deposition (Wu *et al.,* 2011; Gandois and Probst, 2012), which is the main source of pollution in natural forest areas (Gandois and Probst, 2012). The Cape Peninsula is characterized by a Mediterranean climate with dry summers and winter rainfall (Cowling *et al.,* 1996) and seasonal variations in metal concentrations due to wind and rain have been reported (Keane *et al.,* 2001). The information, thus derived from including seasonal differences in air pollution studies may be of utmost importance (Karar *et al.,* 2006), with regard to metal behavior (Harrison *et al.,* 1997).

The objectives of this study were to compare the dry and wet season with regard to: (a) seasonal fluctuations of the concentrations of the metals Al, Fe and Mn; (b) the induced oxidative stress effect of metals, using two indicators, MDA - an oxidative lipid damage marker and tGSH level - an endogenous, non-enzymatic antioxidant associated with oxidative stress and the redox status in the pill millipede, *Spaerotherium compressum*, the moss, *Hypnumcupressiforme* and the lichen, *Parmotrema sp.* The ultimate aim is to investigate how metal contamination impacts the forest ecosystems in the different seasons.

METHODOLOGY

Study area

Two remnant indigenous Afromontane forest pockets on Table Mountain (Orange Kloof and Newlands), amidst the City of Cape Town, in the Western Cape served as the study areas. Both forests are located on the eastern slopes of the mountain. Orange Kloofis the most intact and oldest indigenous forest on Table Mountain, approximately 22.4 km from the city centre (SA-Venues.com, 2017). Newlands is approximately 8.5 km from the city centre. The climate in Cape Town is Mediterranean with dry warm summers and wet mild winters (Weather Spark, 2015).

Three 20 x 20 m sampling sites within each of the two selected forests (Fernández *et al.*, 2002; Aboal *et al.,* 2006), with the first sampling site at the lowest point at least 300 m away from highways and 100 m from other types of roads or structures. The remaining two sampling sites thereafter were at different altitudes on higher points on the mountain. A control site (site C) in a more remote part of Orange Kloof, the forest furthest away from the CBD of Cape Town was added.

The soil at Orange Kloof forest was characterized at sites 1 and 3 as sandy, and sites 2 and C as loam sandy by means of a 5 fraction analysis. The sandy soils are derived from the parent sandstone, which is sparsely distributed with oxides of Mn and Fe. Newlands forest soil is derived from Table Mountain sandstone and characterized at sites 1 and 3 as loam sandy and site 2 as sandy.

Sampling procedure

The dry season was sampled from January to March 2015, while the wet season was sampled from June to August 2015. Eight replicates, using only five of equal weight or size of the soil, leaf litter and each organism at each of the sampling sites were randomly collected at least 50 cm apart using latex gloves and plastic disposable knives and scoops.

The top 0-5 cm of soil under the leaf litter layer were dug out and the decomposing leaf litter from various Afromontane tree species scraped from the top soil layer. The samples were collected separately in clean 50 ml plastic vials and transported to the laboratory (Køíbek *et al.,* 2010; Stafilov *et al.,* 2010) where the soil was subjected to pH and moisture measurements. pH readings varied between 6 and 7.5. The soil moisture measured during summer was between 3 and 10 % and in winter between 54 and 74 %. The remainder of the soil and leaf litter samples were kept in a freezer before further analysis.

Whole mosses (*Hypnum cupressiforme)* and lichen (*Parmotrema sp*.) were randomly collected from rocks at a height of 1 to 1.5 m from the ground to prevent any soil contamination (Bargagli and Nimis, 2002). Samples were transported separately in clean plastic bags and cleaned from extraneous material, but not subjected to any washing in order to prevent possible changes in metal contents (Bargagli and Nimis, 2002; Conti, 2002). Samples were kept in a freezer before further analysis.

Similar sizes of the adult millipedes, *Sphaerotherium compressum* were hand collected underneath the litter layer in the soil. They were transported separately in plastic containers in the forest soil in order to prevent unnecessary stress. In the laboratory the millipedes were killed by putting them separately in labelled 10 ml plastic vials in the freezer before further analysis.

Laboratory procedures

Acid digestion

The samples were dried separately in a Memmert oven for 48 hours at 60°C, ground into powder, homogenized with a mortar and pestle and weighed to obtain a weight of approximately 0.2 and 0.3 g per sample. Each millipede was weighed separately and intact on a Precisa XB 220A balance.

The weighed samples and blank were digested in a Grant UBD digester with 10 ml 65% nitric acid at a temperature of 40°C for one hour and 120°C for a period of three hours (Odendaal and Reinecke, 1999). The samples were filtered through Whatman no 6 (90 mm) filter paper and diluted to 20 ml with distilled water using labelled 20 ml volumetric flasks, after which it was filtered through Whatman 0.45 µm cellulose nitrate membrane filter paper using a syringe and Millipore filter holders. Finally, one ml was diluted with 9 ml of distilled water and the prepared samples were stored in centrifuge tubes and taken to the ICP-MS laboratory at the University of Stellenbosch to determine the metal concentrations in the samples.Samples were analysed for three metals (Al, Fe, Mn). The metal concentrations in the samples were determined with an Inductively Coupled Plasma Mass Spectrophotometer (ICP–MS) and calculated using the following formula:

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

Metal concentration is expressed as mg/kg.

Biochemical analysis

Moss *(Hypnum cupressiforme)*, lichen *(Parmotrema sp.)* and adult specimens of the pill millipede *Sphaerotherium compressum* were manually collected in Orange Kloof and Newlands forests at the aforementioned sampling areas during the dry season (January 2015) and the wet season (June 2015).

Samples of moss and lichen were transported to the laboratory in clean plastic bags, on ice in cooler boxes and immediately stored at -80ºC before further analysis. The millipedes were transported in separate plastic containers in the forest soil, leaf litter and decaying wood from their collection sites. Care was taken not to subject them to any additional stress. In the laboratory they were acclimated in tanks for a period of 15 days in the same soil, leaf litter and decaying wood. 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 were adhered to (Godoy and Fontanetti, 2010; Nogarol and Fontanetti, 2010). The millipedes were frozen at -80ºC after the acclimation period, before further analysis. All the samples were freeze dried for 48 hours. The exoskeleton of the pill millipedes was removed prior to being freeze dried.

The samples were prepared for tGSH and lipid peroxidation analysis by using approximately 2 g of the moss, lichen and millipede samples, respectively. Each sample was weighed using a Sartorius 2006 MP Balance and homogenized on ice with ice-cold 10 ml Sodium Phosphate monobasic buffer (7.5) (Sigma Aldridge). Added to the buffer solution was EDTA (Ethylenediamine-tetraacetic acid disodium salt-dihidrate) (99.1%), distilled water and NAOH (Sodium hydroxide) and Triton (0.2%) x 100. The homogenate was stored in eppendorf micro tubes and frozen at -80ºC until further analysis.

Lipid peroxidation

The method of Nair and Turner (1984) was used to determine the concentrations of malondialdehyde (MDA) as a marker of lipid peroxidation (LPO) and was based on the reaction with thiobarbituric acid (TBA). A fresh 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 0.33 ml of the homogenate. A boiling water bath was used to incubate the mixture for 20 min, after which it was cooled. Thereafter 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 method according to Owens and Belcher (1965) was used to measure the total glutathione level at 412 nm using 50, 5-dithio-bis-(2-nitrobenzoic acid) (DTNB). The assay mixture was made up of 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 it was calibrated against the standard curve of GSH.

Statistical analysis of data

T-tests were used to compare the metal concentrations between the dry and wet seasons in forests in terms of soil, leaf litter, moss, lichen and millipedes. Similarly, T tests were used to compare the tGSH and MDA concentrations between seasons in forests in terms of moss, lichen and millipedes.

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 Sigma Plot 13.0 software package.

RESULTS AND DISCUSSION

Seasonal comparisons of metal concentrations (Al, Fe and Mn) in soil, leaf litter and sentinel organisms between the forests: Site C, Orange Kloof and Newlands

The mean metal concentrations in soil, leaf litter and sentinel organisms for each forest for the dry (January 2015) and wet (June 2015) season sampling occasions are presented in Table 1. The metal concentrations for the sites within each forest were pooled to calculate the mean concentrations for each forest.

Aluminium concentrations showed statistically significant differences between seasons in soil at Newlands forest (P=0.050); leaf litter at site C (P=<0.001) and Newlands forest (P=0.002); lichen at Orange Kloof forest (P=0.005) and millipedes at site C (P=0.016) and Newlands forest (P=<0.001). All of the Al concentrations were higher in the wet season, except for the millipedes that showed higher concentrations at site C in the dry season (3042.68 ± 3935.36 mg/kg). Moss Al concentrations between seasons, however were not statistically significantly different at any of the forests: site C (P=0.359), Orange Kloof (P=0.934) and Newlands (P=1.000), although the highest Al concentrations in moss were measured at site C in the wet season (3341.43 mg/kg ± 2251.93 mg/kg) (Table 1).

Iron concentrations were statistically significantly different between seasons in soil at site C (P=0.005); leaf litter at all the forests: site C (P=<0.001), Orange Kloof (P=<0.001) and Newlands (P=0.005); lichen at Orange Kloof forest (P=0.014) and millipedes at Newlands forest (P=0.002). Moss Fe concentrations showed no statistically significant differences between seasons at any of the forests: site C (P=0.359), Orange Kloof (P=0.407) and Newlands (P=0.803). Most of the Fe concentrations were higher in the wet season, with the exception of soil, lichen and millipedes at site C and moss at Newlands forest (Table 1).

Manganese concentrations compared between seasons showed statistically significant differences in soil (P=<0.001); leaf litter (P=<0.001) and moss (P=0.005) at site C, as well as in millipedes at site C (P=0.005) and Newlands forests (P=<0.001). Lichen Mn concentrations showed no statistically significant differences between the seasons at any of the forests: site C (P=0.868), Orange Kloof (P=0.081)

Table 1. The mean Al, Fe and Mn concentrations (mg/kg) (± SD) in soil, leaf litter and sentinel organisms in the two forests for the dry and wet season sampling occasions in January and June 2015.

Al						
FOREST	SEASON	SOIL	LEAF LITTER	MOSS	LICHEN	MILLIPEDES
Site C	Dry	13450.31 ± 2851.26	*220.42 ± 175.48	1992.10 ± 661.79	965.81 ± 305.38	*3042.68 ± 3935.36
	Wet	13940.73 ± 3263.24	1021.3 ± 263.66	3341.43 ± 2251.93	768.84 ± 194.82	1072.29 ± 959.60
Orange Kloof	Dry	4122.74 ± 4495.73	265.89 ± 249.21	1127.36 ± 643.69	*671.66 ± 225.57	382.10 ± 220.37
	Wet	10669.29 ± 11070.58	513.1 ± 553.72	1126.37 ± 477.10	1269.4 ± 773.65	411.31 ± 513.85
Newlands	Dry	*5846.29 ± 2575.23	*447.11 ± 367.82	1769.21 ± 1252.20	839.49 ± 646.42	*528.41 ± 548.51
	Wet	8049.87 ± 3272.21	980.0 ± 553.96	1667.44 ± 1121.64	1283.52 ± 983.03	2101.99 ± 1469.16
Fe						
FOREST	SEASON	SOIL	LEAF LITTER	MOSS	LICHEN	MILLIPEDES
Site C	Dry	*8016.54 ± 1592.90	*176.11 ± 124.29	1694.47 ± 436.48	1115.87 ± 417.65	1556.14 ± 2023.16
	Wet	6503.82 ± 1225.01	683.95 ± 96.78	2379.9 ± 1288.61	885.15 ± 448.33	623.14 ± 497.07
Orange Kloof	Dry	1832.03 ± 1012.07	*178.14 ± 129.83	938.87 ± 538.29	*566.54 ± 148.43	325.69 ± 380.81
	Wet	5922.22 ± 6318.44	609.39 ± 433.81	1031.69 ± 486.84	1119.43 ± 676.68	364.2 ± 326.99
Newlands	Dry	9029.98 ± 3251.47	*552.64 ± 530.52	2554.81 ± 2193.75	1068.40 ± 876.27	*922.63 ± 1065.97
	Wet	11856.77 ± 4628.74	1466.95 ± 1117.24	2035.24 ± 1297.96	1660.31 ± 1249.90	2890.29 ± 1813.82
Mn						
FOREST	SEASON	SOIL	LEAF LITTER	MOSS	LICHEN	MILLIPEDES
Site C	Dry	*98.97 ± 23.56	*64.97 ± 14.28	*77.51 ± 21.77	62.98 ± 16.77	*33.28 ± 26.22
	Wet	186.67 ± 47.59	177.24 ± 35.29	150.27 ± 59.78	69.77 ± 39.45	47.1 ± 12.46
Orange Kloof	Dry	255.38 ± 295.89	792.98 ± 874.64	460.76 ± 324.04	445.44 ± 651.45	86.81 ± 69.18
	Wet	204.34 ± 158.31	461.57 ± 445.46	286.57 ± 219.38	138.07 ± 110.84	102.4 ± 93.34
Newlands	Dry	354.15 ± 147.88	605.56 ± 287.19	327.31 ± 196.01	181.61 ± 104.74	*58.31 ± 37.46
	Wet	449.49 ± 148.45	647.35 ± 309.69	403.6 ± 257.68	156.06 ± 209.77	129.07 ± 66.76

Statistical significant differences (P<0.05) between dry and wet seasons are indicated with an asterisk. Comparisons were done separately for soil, leaf litter and sentinel organisms. N=5.

and Newlands (P=0.158). The Mn concentrations were higher in the wet season with the exception of soil at Orange Kloof forest, leaf litter at site C, moss at Orange Kloof and lichen at Orange Kloof and Newlands forests (Table 1).

A distinct pattern of higher Al, Fe and Mn concentrations in soil, leaf litter and sentinel organisms was observed in the wet season, which may be due to low temperature and temperature inversion. This is a phenomenon that has a major impact on air pollution deposition, especially in winter, as it prevents atmospheric convection from happening, trapping dust and pollutants and keeping it close to the surface. It is further aggravated by anthropogenic activities, such as fossil fuel for heating, as well as higher road traffic, which tend to increase during colder seasons. Dust-borne metals as a direct result further contributes to the metal load, rationalizing the high concentrations of metals found in samples measured during the colder seasons (Norouzi *et al.,* 2017).

The visible smog or brown haze that appears as a result of the inversion and trapped pollution is thus an indication of high concentrations of atmospheric particulate matter (Cheng *et al.,* 2013) that arise from human activities such as vehicle traffic, construction sites, and resuspension processes related to urban surfaces, various industries, the burning of fossil fuels for heating and cooking. Additionally, the geogenic particles contribute to the overall mass concentration of airborne particles and they include minerals transported from regions, which are arid, semi-arid or bare soils within or surrounding cities (Schleicher *et al.,* 2011; Chen *et al.,* 2014), similar to the Cape Flats. The Cape Flats is a large, wind-blown, flat and sandy land, south east of the Cape Town Central Business District (CBD) (SA-Venues, 2021).

Cape Town is situated at the south-western tip of Africa and edged by the Table Mountain range along the west coast, which concurrently with its position, between False Bay and Table Bay, impact air flow within the region. Cape Town's relatively high pollution levels during winter with the brown haze episodes from April to September is as a result of strong temperature inversions and calm conditions during this period, which contains gaseous and particulate pollution from these vehicular emissions (Wicking-Baird *et al.,* 1997), industries and residential biomass burning (City of Cape Town, 2005). Most of the City of Cape Town is covered by this haze and shifts according to the prevailing wind direction (Wicking-Baird *et al.,* 1997). Recently the haze has extended into the summer months as well (City of Cape Town, 2016). The wet season sampling session wasdone during regular spells of the visible brown haze hanging over the city. Similar findings of higher metal concentrations in winter were reported in Paris, France and was due to domestic heating, which escalates in winter in a populated city (Motelay-Massei *et al.,* 2005). Regions such as the Indo-Gangetic Plains in India also reported remarkably high loading of ambient particulates that includes both fine and coarse fractions in winter, which quite often breached the set NAAQS levels (Panda *et al.,* 2016; Sharma *et al.,* 2016a, b).

The higher metal concentrations measured at these forests, surrounded by the City of Cape Town in winter, is therefore an indication of severe haze pollution brought on predominantly by escalating anthropogenic activities, as the fine particle deposition ($PM_{2.5}$) have been found to be elevated in winter (Liu *et al.,* 2016). Similar reports were announced by authors in East Asia, who found Al, Fe and Mn concentrations to double during such brown haze episodes (See *et al.,* 2006). Fine particle deposition is also said to be elevated in winter, as opposed the coarse particles that are generally higher in summer (Liu *et al.,* 2016), which suggests that the higher metal concentrations in winter at these Afromontane forests are predominantly from anthropogenic origin (Handler *et al.,* 2008).

Particulate pollutant concentrations may have been deceased in summer, due to dispersion and dilution through wind turbulence with large mixing height, as well as the sea-breeze (Gupta *et al.,* 2004), which maycause the summer dust to appear less harmful and polluted due to the lower concentrations of toxic metals (Norouzi *et al.,* 2017).

The elevated Al, Fe and Mn concentrations measured in the dry season, especially at site C is a reflection of the dominance of crustal components over anthropogenic elements (Cheng *et al.,* 2005; Xia and Gao, 2011). However, increases in Al and Fe concentrations in the dry season are as a rule associated with their common usage in industrial processes and their subsequent presence in the emissions (Pathak *et al.,* 2015). Similar patterns were noticed with long-range transport during summer in India and a possible mixing of anthropogenic pollutants during transport of dust was affirmed by Chinnam *et al.* (2006).

Other factors that may have contributed to the metal load in the dry season that have been of specific reference to this study in Cape Town are the wild fires that occurred on Table Mountain and the Cape Peninsula during the dry sampling period that are known sources of the metals, Al, Fe and Mn (Karthikeyan *et al.,* 2006a, b). The 'table cloth,' which is a huge cloud that hoovers on top of Table Mountain and drips over the mountainside, creates clouds and rain along the eastern slope (Van der Velden, 2017) and the precipitation thereof contains metals, which are returned to terrestrial or aquatic surfaces and may further have enhanced metal concentrations (Bacardit and Camarero, 2010) in this season.

Higher concentrations of Al, Fe and Mn were measured at the sampling areas more exposed to pollution, such as the Newlands forest, which is the forest closest to forest closest to Cape Town's city centre(8.5 km), as opposed to Orange Kloof forest located further than Newlands forest (22.4 km) from the city centre. Newlands forest, displayed overall higher metal concentrations in the wet season compared to Orange Kloof and site C. The clarification lies in its location closer to the city centre as the level of contaminants are generally higher in samples from forest ecosystems adjacent to industrialized and heavily populated areas (Nakazato *et al.,* 2015), being subjected to various sources of metals due to major anthropogenic activities (São Paulo, 2013). The Newlands Fe concentration was almost twice as high as were found in the Hungarian urban forest study of 6005 mg/kg in the wet season. Their dry season concentration of 4126 mg/kg was also significantly lower than the dry season concentration found in this study (Simon *et al.,* 2016) (Table 1).

Aluminium, Fe and Mn are mineral elements, associated with the crustal fraction of PM_{10} and their occurrence in PM_{10} is predominantly as a result of local and regional dust re-suspension, which have been generated by wind, convection, and other natural processes (Shridhar *et al.,* 2010; Pant and Harrison, 2012). The elevated concentrations of these metals found in the forests could therefore be an indication of a higher mineral particle loading in the city due to high dust aerosol levels in the atmosphere resulting from activities such as road expansion and building constructions. Al and Fe concentrations are also generally higher in extensive convective precipitation in mountain regions, which might have brought more dust aerosols in the location (Tripathee *et al.,* 2014). The main sources of particulate matter, however is said to originate from vehicle exhaust, road dust, and industrial production (Dzierzanowski *et al.,* 2011; Gunawardena *et al.,* 2012). These activities, as well as fuel-burning appliances, wild fires, tyre burning and domestic fuel burning, evaporative losses and landfill operations, waste water treatment and fugitive emissions from wind erosion and agriculture are also huge contributors in urbanised cities (Scorgie, 2003) such as Cape Town. Incidentally, the Cape Metropolitan Area with a population count of 3.74 million (Stats SA, 2011) and 1.93 million vehicles that are registered (Wheels 24, 2017) is the most congested city in South Africa (Business Day, 2017).

Site C at Orange Kloof forest which served as the control site, turned out to contain some of the highest Al en Fe concentrations (Table 1). Site specific factors were of special interest atsite. Pathak *et al.* (2015) in India also found that the metals, Al, Fe and Mn showed higher concentrations at some of the sampling sites, and in view of that, suggested anthropogenic addition of metals in soils to be sampling site specific. It was later determined that site C's slightly more exposed location on the mountain seemed to be impacted by natural and anthropogenic pollutants in both seasons transported from the Cape Flats by the South Easter wind (Sen *et al.,* 2017), creating a micro-climate effect at this site (Lawrence and Neff, 2009). During the summer months the strong South Easterly wind blows dust, which exacerbates the problem by elevating the particulate matter levels and further contributes to PM_{10} (DEADP, 2011). Dry season influences that could have contributed to the metal load were dust transporting winds containing the crustal elements Al, Fe and Mn (Handler *et al.,* 2008) as site C is exposed to the Cape Flats.The Al concentrations found in this study in the dry season at site C exceeded concentrations reported in Alcalá de Henares, one of the most densely populated cities in Spain, at an industrial site of 10135.90 mg/kg also in summer. Similarly, the wet season Al concentrations at this site exceeded the wet season concentrations found in the Spanish city of Alcalá de Henare's industrial study site of 11261.40 mg/kg (Peña-Fernández *et al.,* 2015**)** (Table 1).

An important season-specific factor that may have altered metal concentrations between seasons and observed at all three study areas is leaching of metals in the wet season. In soils, decreased metals in the upper layer is often due to high rainfall rates in combination with the lower evaporation rate, which causes leaching of these metals to deep layers. This is especially true in highly porous soils (Madrid *et al.,* 2004, 2007) with a sandy loam texture, such as the soils characterized at Orange Kloof and Newlands forests (Peña- Fernández, 2011). Leaching in soil could also have occurred as a result of post-fire rainfall, where significant decreases in metal concentrations in soil in winter have been reported after the occurrence of wild fires in the summer (Certini, 2005; Zavala *et al.,* 2014), such as were experienced during the dry sampling occasion in January to March 2015 (eNCA, 2015). There were instances when lower concentrations were measured in lichen in winter, which could have been caused by leaching of elements during the wet periods as a result of precipitation. Rainwater can remove particulate that are entrapped onto the lichen surface, which may thus cause lower element content during rainy periods (Adamo *et al.,* 2008). Mn is also easily leached from leaves, which could have resulted in lower concentrations of this metal in leave litter (Avila and Rodrigo, 2004) (Table 1).

Non season specific factors that may have had an influence is biological cycling. Mn concentrations in the surface soil may have been elevated even more (Herndon *et al.,* 2011; Herndon *et al.,* 2015) by vegetation as a result of biological cycling by litter fall, through fall, and uptake (Herndon *et al.,* 2015; Kraepiel *et al.,* 2015). In some tree species the sun leaves are also reported to contain higher Mn concentrations than shade leaves (McCain and Markley, 1989) and may also be considered as an impacting factor, in both increased and deceased concentrations of Mn in leaf litter in both seasons. The initial metal concentrations in leaf litter plays an important role in the accumulation and flow of metals in the decomposing leaf litter (Kaila *et al.,* 2012). Metal accumulation thus occurs mainly from atmospheric depositions and through fall, but also from microbial translocation and immobilization of metals from contaminated soil layers underneath the litter, primarily by fungi (Lomander, 2002; Tyler, 2005) (Table 1).

The metal concentrations in the pill millipedes were observed to be higher at site C and Newlands forests, where the highest concentrations of the metals, Al, Fe and Mn in soil and leaf litter were measured, suggesting that these specific pill millipedes are good accumulators of metals. Unfortunately, literature relevant to this study, using pill millipedes is to the best of my knowledge scarce to non-existent. However, Da Silva Souza *et al.* (2014) reported that higher metal concentrations in millipedes are caused by consumption of polluted litter in a polluted soil. Many influencing factors that determine the metal concentrations in invertebrates, such as physiological conditions, feeding habits, breeding, sex, development stage, season (Jelaska *et al.,* 2007; Butovsky, 2011) and excretion ability of the bodies (Avgin and Luff, 2000).

In moss, emission sources largely determine the bioaccumulation of metals in mosses within study regions (Schroder *et al.,* 2008; Holy *et al.,* 2009). Several other factors could have influencedthe metal accumulation in mosses, such as the physicochemical characteristics of the pollutants and the moss binding surfaces (Gonzalez and Pokrovsky, 2014), physiological processes, such as moss growth (Boquete *et al.,* 2014) sampling site characteristics, such environmental conditions, elevation, gradient and level of exposure and canopy structure (Fernández *et al.,* 2015).

Comparisons of tGSH and MDA levels in moss, lichen and millipedes between the forests: Site C, Orange Kloof and Newlands

The mean tGSH and MDA (measured as TBARS) concentrations in moss, lichen and millipedes for each forest for the dry (January 2015) and wet (June 2015) season sampling occasions are presented in Table 2. The tGSH and MDA concentrations for the sites within each forest were pooled to calculate the mean concentrations for each forest. Concentrations are expressed in µmol/g.

Metals play a significant role in the chemical, biological, biochemical, metabolic, catabolic and enzymatic reactions in living tissues (Hashmi *et al.,* 2007) and for that reason multiple biomarkers were used in this ecotoxicological research (Lam, 2009). The purpose was to thoroughly assess exposure of the forest organisms to contaminants and eventually determine the impact on living organisms (Cazenave *et al.,* 2009), which will contribute to the conservation of forests.

In this study, statistically significant differences were found between the dry and wet seasons in terms of mean tGSH levels in moss at Newlands forest (P=0.032), as well as millipedes at site C (P=0.049) and Newlands forest (P=<0.001). Comparisons of mean tGSH levels in lichen between seasons were too low to be detected.The tGSH levels measured were higher in the dry season with the overall highest mean tGSH levels found in moss at

Newlands forest (79.78 ± 54.65 µmol/g) and millipedes at site C (542.81 ± 142.90 µmol/g).

Living organisms develop an antioxidant defense system to balance the reactive oxygen species (ROS) that form during natural cell processes (Valavanidis *et al.,* 2006). Non-enzymatic antioxidants with low molecular weights, such as glutathione is able to reduce oxidative stress by scavenging ROS (Choudhury and Panda, 2004; 2005; Singh *et al.,* 2006). The higher tGSH levels in moss and millipedes in the dry season is an indication of the cellular antioxidant defense system attempting to prevent the effects of peroxidation (Ezemonye and Ikpesu, 2011; Paulino *et al.,* 2012) and the lower tGSH levels, in the wet season on the other hand signifies damage to the tissues, possibly resulting from long term exposure to pollution (Loguercio *et al.,* 1996; Barbaro *et al.,* 1999). It is noteworthy that the metal concentrations were higher in the wet season (Table 1), which may have caused damage to the tissues of the organisms. Both the rise and fall in tGSH levels, nonetheless points to an over production of ROS in cells (Tausz *et al.,* 1998, 2007a, b; Bussotti, 2008).

The dry season metal concentrations were not much lower than the wet season concentrations at site C (Table 1), yet the tGSH concentration in moss was lower in this season (Table 2). The lower tGSH concentration in moss, thus indicates damage to the moss tissues, probably from exposure to these metals (Loguercio *et al.,* 1996; Barbaro *et al.,* 1999), which seems that the antioxidant system was more efficient in its function as protector in the wet season where moss was in its preferred environment.

All three study areas displayed depleted tGSH levels in lichen in summer and winter where the Al, Fe and Mn concentrations measured in lichen in this study were comparable with urban and industrial concentrations found in other studies (Sorbo *et al.,* 2008; Malaspina *et al.,* 2014). One may further suggest that the metals at such concentrations were able to cause excessive ROS production and subsequently induced oxidative stress, causing lipid peroxidation (Jing *et al.,* 2009; Sen *et al.,* 2014). Metals are known to generate ROS, which may have led to the depletion of antioxidants in cells such as tGSH (Koivula and Eeva, 2010; Sanchez-Virosta *et al.,* 2015). The depleted tGSH levels in the lichen tissue is, therefore an indication of damaged tissue that normally occurs when the organisms are exposed to toxicants over a long period of time (Loguercio *et al.,* 1996; Barbaro *et al.,* 1999) (Tables 1, 2).

Oxidative stress causes modifications to important bio-molecules, such as proteins, lipids

Table 2. The mean tGSH levels and MDA concentrations (µmol/g) (± SD) in moss, lichen and millipedes for forests for the dry and wet season sampling occasions in January and June 2015.

tGSH				
FOREST	**SEASON**	**MOSS**	**LICHEN**	**MILLIPEDES**
Site C (N=5)	**Dry**	**14.36 ± 11.44**	**ND**	***542.81 ± 142.90**
	Wet	*51.01 ± 72.25*	*ND*	*385.19 ± 143.67*
Orange Kloof (N=15)	**Dry**	**51.97 ± 65.53**	**ND**	**452.67 ± 123.96**
	Wet	*26.03 ± 11.23*	*ND*	*332.30 ± 127.83*
Newlands (N=15)	**Dry**	***79.78 ± 54.65**	**ND**	***536.74 ± 140.77**
	Wet	*29.64 ± 33.02*	*ND*	*201.85 ± 127.50*
MDA				
FOREST	**SEASON**	**MOSS**	**LICHEN**	**MILLIPEDES**
Site C (N=5)	**Dry**	***0.49 ± 0.26**	***0.10 ± 0.03**	**0.58 ± 0.28**
	Wet	*0.44 ± 0.34*	*2.2 ± 2.03*	*1.4 ± 1.26*
Orange Kloof (N=15)	**Dry**	**0.56 ± 0.34**	***0.03± 0.35**	**1.35 ± 1.78**
	Wet	*0.70 ± 0.55*	*1.20 ± 0.84*	*2.13 ± 2.15*
Newlands (N=15)	**Dry**	***0.1 ± 0.10**	***0.30 ± 0.37**	**0.67 ± 0.52**
	Wet	*0.30 ± 0.26*	*1.11 ± 0.97*	*1.12 ± 0.86*

Statistical significant differences (P<0.05) between dry and wet seasons are indicated with an asterisk. Comparisons were done separately for moss, lichen and millipedes. MDA expressed as µmol TBARS per gram material; ND=Not Detected; SD=Standard Deviation.

and genetic material (Sies and Cadenas, 1985). Membrane lipid peroxidation in the organisms can be estimated by measuring the content of malondialdehyde (MDA), which serves as an indicator of induced oxidative stress (Sujetovien and Galinyt, 2016).

The following forests showed statistically significant differences between seasons in mean MDA concentrations in moss: Site C (P=0.049) and Newlands forest (P=0.027) and lichen: site C (P=<0.001), Orange Kloof (P=0.022) and Newlands (P=0.042). With the exception of moss at site C, the higher MDA concentrations were measured in the wet season. The overall highest in moss at Orange Kloof forest (0.70 ± 0.55 µmol/g); lichen at site C (2.2 ± 2.03 µmol/g) and millipedes at Orange Kloof forest (2.13 ± 2.15 µmol/g) (Table 2).

The higher MDA concentrations in any given season signifies exposure of the organisms to toxic element concentrations that are extremely high (Baèkor *et al.,* 2010; Pisani *et al.,* 2011). Metals, as such are recognized toxicants to cause lipid peroxidation by producing free radicals (Choudhury and Panda, 2005).

With the exception of site C, higher Al and Fe concentrations, as well as higher MDA content were measured in lichen in winter (Tables 1, 2). Aluminium is said to induce MDA accumulation and increase MDA content in lichen (Unal *et al.,* 2010). In this study, metal contamination may therefore have contributed largely to the peroxidation of lipids through the induction of ROS (Choudhury and Panda, 2005).

Except for Al at site C, the Al, Fe and Mn concentrations were slightly higher in the wet season (Table 1), as were the MDA concentrations in the wet season (Table 2). Exposure to metals may causes induction of oxidative stress in invertebrates, which is based on an increase in lipid peroxidation products (MDA) in the invertebrates (Geret *et al.,* 2002) and may have been the case in this study.

The tGSH levels in the organisms showed higher results in both seasons at all three study areas, as opposed to the lower MDA content measured, also in both seasons, indicating tGSH successfully, protecting the sentinel organisms against oxidative stress by scavenging ROS (Choudhury and Panda, 2004, 2005; Singh *et al.,* 2006), as well as sufficiently detoxifying metals (Sanita di Toppi *et al.,* 2008). Organisms are well equipped with antioxidant defense systems to eliminate ROS and protect cells and tissues from impairment and dysfunction (Sugiyama, 1994). By activating the cellular antioxidant defense system, it is thus possible for the organisms to control the increased levels of ROS in tissues (Gomes *et al.,* 2014).

CONCLUSION

Low temperature, temperature inversions and brown haze played a significant role in the higher Al, Fe and Mn concentrations measured in the wet season in both the forests. Site specific factors on the other hand were justification for the elevated metal concentrations at site C and Newlands forest. This is indicative offine particle deposition ($PM_{2.5}$) and haze pollution due to a general increase in anthropogenic activities in the colder season and more so at the forest in closer proximity of an urban city. Metal load contributions indicate crustal origin, road fugitive and construction dust, as well as traffic emissions.

The dry season revealed the higher tGSH levels in moss and millipedes, due to both natural and anthropogenic influences, which activated their defense mechanism. MDA levels in moss and millipedes were mostly higher in the wet season when the metal concentrations were higher, as a result of the prominent brown haze episodes in winter. In addition, moss seemed to be more tolerant to environmental stressors than lichen. Pill millipedes showed good potential as bioindicators and biomonitors of metal pollution and their response to oxidative stress can be seen as good biomarkers of exposure to the metals, Al, Fe and Mn. This information is especially valuable in light of the fact that pill millipede studies in terms of metal contamination and oxidative stress are virtually non-existent.

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