CHAPTER 3 - MATERIALS AND METHODS

3.1 Selection of bioindicator species for this study

For this study three species were chosen, the Southern Masked Weaver (*Ploceus velatus*), Red Bishop (*Euplectes orix*) and Red-billed Quelea (*Quelea quelea*). Southern Masked Weavers were used throughout the study from all the sites. Red Bishops and Red-billed Queleas were only used when there were not enough Southern Masked Weavers. Initial statistical analyses showed there were no differences between both, the metal content and biomarker results from the three species. For this reason the data from individuals of the three species were pooled at the different sampling sites.

All are very common seedeaters of the family Ploceidae (weavers and allies), order Passeriformes (Passerines or Songbirds). The Southern Masked Weaver is found throughout southern Africa. The Red Bishop is also found throughout southern Africa, except the dry western areas (Mclean, 1993). The Red-billed Quelea is the most numerous bird species in the world, found throughout sub-Saharan Africa (Perrins, 1990).

The Southern Masked Weaver (also known as the Vittelline or African Masked Weaver) is the most widely distributed southern African weaver. It is not primarily associated with woodland, being more commonly found in open country. It is also common throughout the more arid regions if there is drinking water available. In drier areas local migrations occur, but most ringing recoveries were found within 10 km of the original ringing locality (Harrison *et al.,* 1997).

The Red Bishop is easily recognised by its scarlet and black plumage during the breeding season. They are primarily grassland species, always in close proximity to water. They are also closely associated with agriculture. They may move away during the non-breeding season (Harrison *et al.,* 1997).

The Red-billed Quelea occurs throughout sub-Saharan African outside the forest zone. Access to drinking water limits the distribution of the species in the arid areas. It is a nomadic species and migration is common. Its presence in an area is dependant on rainfall and the condition of the habitat (Harrison *et al.,* 1997).

The three species were selected since fulfilled most of the bioindicator criteria mentioned earlier. These criteria were that they are very common species found throughout very large areas. They inhabit almost all habitat types, including areas inhabited by humans and industrialised areas. They are some of the commonest birds caught during ringing. At some sites they are caught in numbers over the hundred within a few hours. They are easy to handle and easily identified so that even the trainee ringers are able to accurately identify them.

3.2 Site description

Four sites were selected in the province of Gauteng, South Africa (See Figure 3.1). A reference site was chosen in the countryside of the North West Province, South Africa. The Gauteng sites were chosen as they potentially represent a pollution gradient, from the south where more heavy industry occurs to the north. The sites were sampled during the autumn months of February to May in 2002 and 2005. During this time period the birds are either building nests or already breeding (Maclean, 1993). They are thus expected to stay in that specific area.

Rietvlei is located in the Rietvlei Nature Reserve, which lies in the south-eastern parts of the Tshwane Metropolitan Municipality. It is mainly grassland with scattered trees, the majority of them being exotic. There is a large impoundment in the nature reserve that supplies part of the Tshwane Metropolitan Municipality with water. Although the nature reserve contains large mammals and a large number of bird species, the reserve is bordered by residential, industrial and agricultural areas.

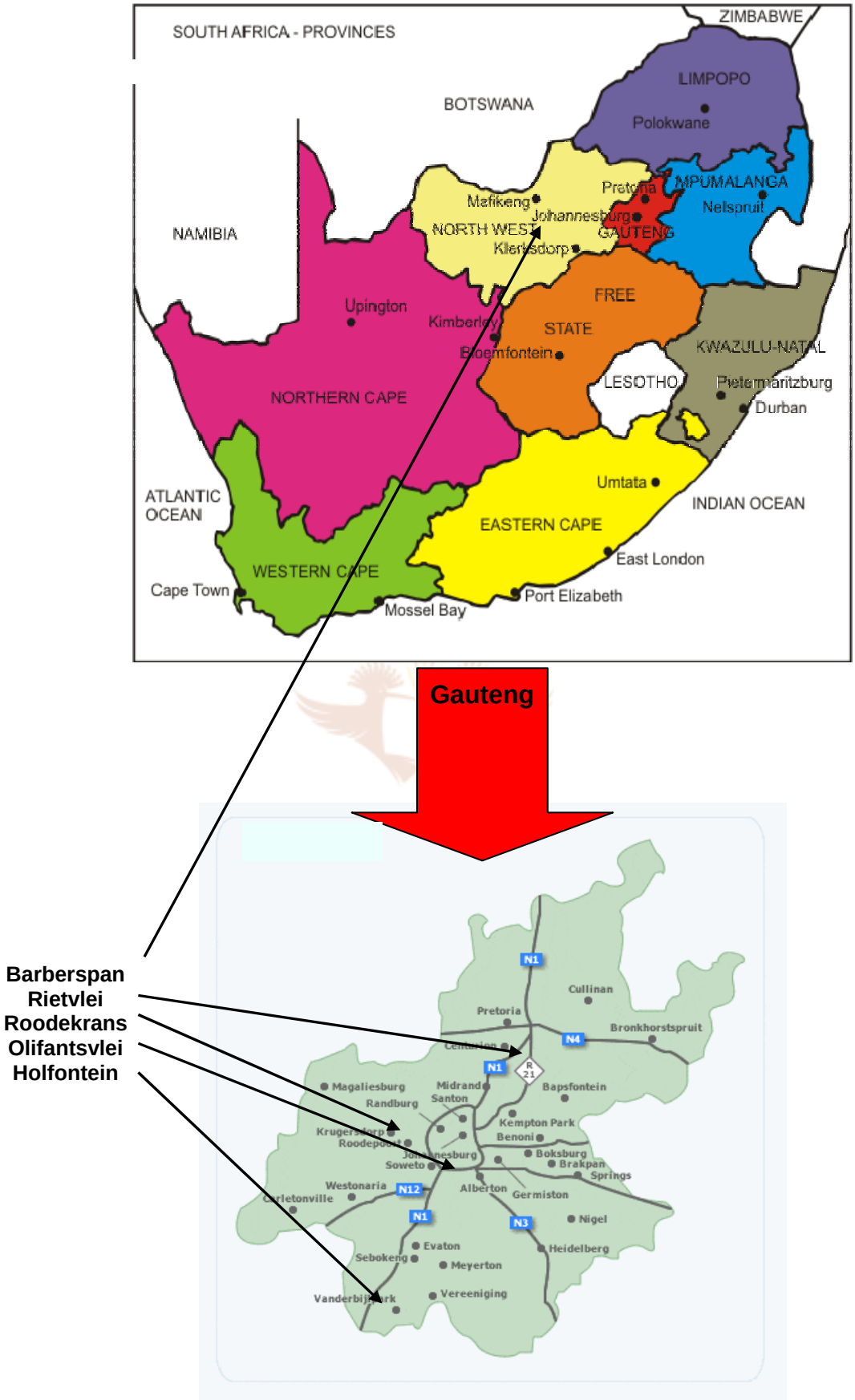

Figure 3.1. The location of the sampling areas

Roodekrans consisted of two locations, the Kloofendal Nature Reserve and the cement quarry at Roodekrans. The Kloofendal Nature Reserve lies within Roodepoort, Gauteng. It is a small nature reserve within a suburban area. It is close to the Alpha Roodekrans quarry at Roodekrans, where sand is excavated from decomposed granite. Birds were sampled from both the quarry and the nature reserve. There are small hills in the area and mainly grassland with trees in the valleys along the streams. Contamination may be due to the excavation occurring in the area. Metals may potentially be deposited in the environment as dust particles from the extensive gold mining activities to the west of the localities.

Olifantsvlei is located in the southern parts of the Johannesburg Metropolitan Municipality. It is a municipal farm where treated water is released into the ecosystem on the farm. It is grassland, with marshy areas and the only trees that do occur are a few exotic species. Sources of contamination might include the treated water that is released into the environment. Sampling at this site could only be undertaken during 2002 as the farm was turned into a residential area by the 2005 survey.

Holfontein lies just west of Vanderbijlpark and Sasolburg in southern Gauteng. The site was located on a cattle farm close to the industrialised Vaal Triangle area. Air pollution from the steel industries in Vanderbijlpark and petrochemical industries in Sasolburg is a major source of environmental contamination. The area is mainly grassland with trees along the rivers and stream.

Barberspan was chosen as a reference site, away from any major urban areas. The site is situated in the Baberspan Nature Reserve, in the central parts of the North West Province, between the towns of Sannieshof and Delareyville. It is an important bird sanctuary and a declared Ramsar Site. It lies in an area of intensive maize agriculture, but is not close to any other major industries.

For the determination of the EcoQOs, feather samples were collected from the Transvaal Museum in Pretoria. In total 62 samples were collected from the three species. The date of the samples ranged from 1899 to 1959, most being from the period 1900 to 1910. Most of the samples were from the Pretoria region, with some from areas close by. For the number of each species and time of sampling, see Table

3.1. To determine the metal levels for 2002 and 2005, the samples from Barberspan were not used, as this site does not fall in Gauteng.

Table 3.1. Feather sample sizes for the different year groups.

Year Group	*Ploceus velatus*	*Euplectes orix*	*Quelea quelea*	Total Number
1899-1919	18	7	13	38
1920-1939	4	4	8	16
1940-1959	3	2	4	9
2002	39	21	11	71
2005	34	1	9	44

3.3 Sampling protocol

3.3.1 Bird capturing

The birds were caught during ringing sessions by the Witwatersrand Bird Club and BirdLife Gauteng North. The birds were caught using mist nets used by bird ringers. The nets were erected before sunrise in areas close to the roosting sites of the birds. After the birds were removed from the nets they were kept in linen bags in the shade until the sampling took place.

3.3.2 Blood collection and storage

Blood was drawn from the cutaneous ulnar vein (Dein, 1984; Campbell and Dein, 1984). This is also known as the "wing vein" traditionally used in poultry medicine. The blood was drawn with a 1 ml heparinised syringe fitted with a 27-G needle. The blood was stored in polypropylene vials rinsed with heparin. The samples were transported back to the laboratory on ice. Haematological analyses were carried out on the same day as sampling. Following whole blood analysis, the blood samples were centrifuged at 3400 rpm for 10 minutes at 4 °C to separate the blood cells from the plasma. The plasma was pipetted into new vials and stored at -40 °C. The blood cells were stored in Henrikson's Stabalising Buffer at -80°C until further analysis. For haematocrit determination blood was also collected directly from the cutaneous ulnar vein into heparinised haematocrit tubes. The tubes were also transported back to the laboratory on ice and analysed immediately upon return.

3.3.3 Feather collection and storage

Feather samples were collected by plucking several feathers from the breast and belly area of the birds. The feathers were stored at room temperature in metal-free paper envelopes.

3.3.4 Sample sizes

The sample sizes from each site differed. The size of the samples for each site is given in Appendix I.

3.4 Metal Analysis of Feathers

The feather samples were prepared according to the methods of Dauwe *et al.* (2002) and Eens *et al.* (1999). The feathers were washed alternately with MQ water and 1 M acetone. This process was repeated three times to remove all external contamination. The samples were then transferred to pre-weighed polypropylene tubes and dried in an oven for 24 hours at 60ºC.

The feathers were then acidified with a 1:1 mixture of HNO_3 (70%) and H_2O_2 (30%) and left to stand at room temperature for 24 hours. The digestion was completed with the microwave (1000 W) destruction procedure (Blust *et al.,* 1988). The samples were digested for 3 minutes at each of the following power levels: 10%, 20%, 30% and finally 40%. The weight after the digestion was recorded. The samples were then diluted 250 times by mixing 200 µl sample and 50 µl internal standard, and then made up to 50 ml with a 1% nitric acid solution in MQ water. It was then stored in the dark at room temperature until further analysis. The metal content of all the samples were measured using an inductively coupled plasma mass spectrophotometer (X-7 ICP-MS, Thermo Elemental).

3.5.Biomarker Analysis

3.5.1 DNA damage

The method of Black *et al.* (1996) was used for the extraction of the DNA from the red blood cells. In relation to the tissue weight, extraction buffer was added and mixed

lightly with a Teflon stirrer. For each 1 mg tissue, 10 µl of DNA extraction buffer (7.3 g NaCl (250 mM), 18.5 g EDTA (100 mM), 6.05 g Tris Base (100 mM) made up to 500 ml with distilled water) was added.

Then 1% of the total mass (= mg tissue + µl DNA extraction buffer) of the 10% sarcosyl solution (1 g Na-laurylsarcosinate dissolved in 10 ml distilled water) was added and mixed lightly. RNAse (2 to 3 µl) was added and the samples were incubated on ice for 10 minutes. The RNAse (10 mg/ml) was dissolved in a Tris/NaCl solution (0.1576 g Tris-HCl (10 mM), 0.0876 g NaCl (15 mM) dissolved in 100 ml distilled water, pH adjusted to 7.5) and boiled for 15 minutes at 100 °C.

To this 5 µl Proteinase K (1 mg/ml) was added and the samples were incubated 30 minutes in the water bath at 55 °C. One volume of Phenol/Chloroform/Iso-amylalcohol (PCI) was added, mixed lightly and then centrifuged for 7 minutes at 3 000 rpm at 4 °C. After this the upper layer was pipetted into a sterile polypropylene vials.

The previous step was repeated, but the samples were only centrifuged for 5 minutes at 3 000 rpm at 4˚C. The samples were again centrifuged for 5 minutes at 3 000 rpm at 4˚C, but one volume of chloroform was added in stead of PCI. The last supernatant was pipetted into a sterile polypropylene vial, as this was the DNA extract. The extract was stored for 1 day at 4 °C

The extracted DNA samples were run on an Agarose gel (0.5%). The electrophoresis system was made level and then the combs were inserted. The gel was made by dissolving 1.5 g agarose in 300 ml (1x) TBE buffer (32.7 g TrisBase, 5.56 g boric acid and 1.84 g EDTA dissolved in 2 L distilled water). The gel was poured into the electrophoresis system and left at room temperature until the gel was set (about 2 hours). After the combs were removed, the samples (5 µl) were loaded onto the gel in duplicate. This was covered with 5 µl of 40% sucrose solution (4 g sucrose dissolved in 10 ml distilled water). λ DNA Hind III digested DNA Molecular Weight Marker II (0.12-23.1 kbp) from Roche was used as DNA marker. DNA marker (5 µl) was loaded onto the gel and covered with 5 µl of 40% sucrose solution. To follow the migration of the samples across the gel, 5 µl bromophenol blue (25 mg bromophenol blue and 4 g

sucrose solution dissolved in 10 ml (1x) TBE buffer) was also loaded and covered with 5 µl 40% sucrose solution.

After the samples were loaded, the gel was covered with buffer (1x TBE buffer). The gel was run for about 3 hours at 75 V and 400 Amp.

The gel was stained using SYBR Green-I nucleic acid stain. 20 µl of Green-I nucleic acid stain was mixed with 200 ml (1x) TBE buffer. The gel was submerged in the stain for 40 minutes in the dark. The gel was then visualised using an ultraviolet transilluminator (UVP) and captured using GrabIt Software version 2.5.

The images were analysed using the OptiQuant software. For each sample the amount of DNA in each class of base pair lengths were determined and expressed in percentage of the total.

3.5.2 Catalase activity

The catalase activity in the plasma samples was determined using the methods of Cohen *et al* (1970). The samples (9.26 µl) were loaded into the wells of a microplate. The same amount of distilled water was used for the blank. To initiate the enzymatic reaction 92.60 µl H_2O_2 was added and mixed thoroughly. This was left to stand for 3 minutes. To stop the reaction 18.52 µl of 6 N H_2SO_4 was added and mixed thoroughly. $KMnO_4$ (129.63 µl) was added and the samples were read at 492 nm within 30 to 60 seconds on an ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc.).

The standard was prepared by adding 129.63 µl $KMnO_4$ to a mixture of 101.85 µl 0.01 M phosphate buffer (pH 7) and 18.52 µl 6 N H_2SO_4. The standard was read at 492 nm. For the determination of protein content 225 µl of Bradford's reagent was added to 5 µl of sample in each well of a microplate. This was left to stand for 5 minutes at room temperature. The microplate was then read at 630 nm on an ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc.).

The catalase activity was then calculated using the following equation:

$k = \log (S_0/S_3)$ X 2.3/t

where:
k = first-order reaction rate constant
t = time interval over which the reaction is measured (i.e. 3 minutes)
S_0 = substrate concentration at time zero
S_3 = substrate concentration at 3 minutes

Also:
$S_0 = Abs_{STANDARD} - Abs_{BLANK}$
$S_3 = Abs_{STANDARD} - Abs_{SAMPLE}$

The catalase activity was expressed as µmol H_2O_2/mg prot.min.

3.5.3 Glutathione content

The fluorometric assay for glutathione developed by Cohn and Lyle (1966) was used for the determination of the glutathione content in the plasma samples. The samples (100 µl) were put into the wells of a microplate. To the samples 500 µl EDTA (0.11 g dissolved in 1 L deionised water) and 200 µl H_3PO_4 (25% w/v) was added. The sample was then centrifuged for 10 minutes at 3000 rpm. Some of the supernatant (5 µl) was added to 195 µl phosphate buffer and 10 µl O-pthalaldehyde (0.01 g dissolved in 10 ml absolute methanol). This was mixed thoroughly and read at 460 nm on a fluoroscan (Acent) with the excitation at 355 nm.

3.5.4 Haematology

Whole blood analysis was done with a SYSMEX Micro-Cellcounter CC-120 (Tao Medical Electronics). The blood was first diluted to appropriate volumes and then the total erythrocyte count (TEC), total leucocyte count (TLC), mean corpuscular volume (MCV) and haemoglobin content (HGB) were measured. The haematocrit (HMC) was determined by using the micro haematocrit technique of Korzhuev (1964). The samples were centrifuged at 10,000 rpm for 6 minutes in a Micro-haematocrit

centrifuge (Heraeus-Christ). Afterwards the haematocrit was determined using a HMC ruler. There was no differentiation made between the TEC and the TLC, only the packed cell volume as measured.

The mean corpuscular haemoglobin content (MCHC) and the mean corpuscular haemoglobin (MCH) was calculated with the following formulas (Dein, 1984):

$$MCHC = \frac{HGB}{HMC} \times 100$$

$$MCH = \frac{HGB}{TEC} \times 10$$

3.6 Statistical Analysis

The statistical programme SPSS 12.0.1 was used to do the statistical analysis. The variations in each biomarker and metal concentrations were tested by one-way analysis of variance (ANOVA), considering sites as variables (Zar, 1996). Data were tested for normality and homogeneity of variance using Kolmogorov-Smirnoff and Levene's tests, respectively. When the ANOVA revealed significant differences, post-hoc multiple comparisons between sites and between sampling periods were made using the appropriate Scheffé (parametric) or Dunnette-T3 (non-parametric) test to determine which values differed significantly. When the distribution of the data was not normal the Mann-Whitney U-Test was used to test for significant differences. The significance of results was ascertained at $p < 0.05$. Spearman's rank correlation analyses were used to relate observed distribution to environmental factors and biomarkers (Zar, 1996).

Multivariate analysis based on Bray-Curtis similarity coefficients and group averaged sorting (Bray and Curtis, 1957) was performed on the data using the PRIMER (Plymouth Routines in Marine Environmental Research) program v4.0, (Plymouth Marine Laboratory). Cluster analysis and multi-dimensional scaling (MDS) (Kruskal and Wish, 1978) were performed on the data. For the averaged data the stress value (a

measure of the accuracy of the results) was obtained, to indicate if the ordination is an accurate description of the temporal bioaccumulation patterns (Clarke and Warwick, 1994). The dendrograms and MDS plots resulting from performing cluster analysis on the averaged individual metal concentrations are shown. The graphical presentations were performed using the GraphPad Prism Programme and the data reported as mean +S.E (standard error of the mean).