

Polycyclic aromatic hydrocarbons (PAHs) in the aquatic ecosystems of Soweto and Lenasia

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Thesis submitted for the degree *Philosophiae Doctor* in
Environmental Sciences at the Potchefstroom Campus of the
North-West University

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May 2017

Table of contents

List of acronyms and abbreviations	VIII
List of figures	XIV
List of tables	XIX
Acknowledgements	XXII
Abstract	XXIII
Chapter 1: General and thesis introduction	
1.1 General introduction	1
1.2 Hypothesis, aims and objectives	3
1.2.1 Hypothesis	3
1.2.2 Aims and objectives	3
1.3 Study area–Klip River catchment	5
1.3.1 The Klip River catchment	5
1.3.2 Site selection	8
Protea Glen [PG]	11
Lenasia [Le]	11
Fleurhof [FI]	12
Dobsonville [Db]	12
Orlando West [OW]	13
Orlando East [OE]	14
Moroka [Mo]	15
Eldorado Park [EID]	15
Nancefield and Bushkoppies Waste Water Treatment Plant (WWTP) [Nc]	16
1.4 Abiotic and biotic matrices collected	18
1.4.1 Abiotic matrix	18

	Use of sediment as abiotic matrix	18
	Sampling and processing of sediment	18
1.4.2	Biotic matrices	19
	Fish used as biotic matrix for environmental studies	19
	Use of bird eggs as biotic matrix for environmental studies	19
1.4.3	Sampled biota	20
	<i>Clarias gariepinus</i>	20
	Sampling of fish	21
	Hérons and egrets	21
	Ibises	22
	Sampling of bird eggs	23
1.5	References	24
Chapter 2: Polycyclic aromatic hydrocarbons in sediment and biota from Soweto and Lenasia, South Africa		
2.1	Introduction	33
2.1.1	Polycyclic aromatic hydrocarbons (PAHs)	33
2.1.2	Physical and chemical characteristics of polycyclic aromatic hydrocarbons	33
2.1.3	Sources of polycyclic aromatic hydrocarbons	35
2.1.4	Environmental fate	35
2.1.5	Toxicity	36
2.1.6	Sediment toxicity evaluation	36
	Polycyclic aromatic hydrocarbon sediment quality guidelines	36
	Sediment indices	37
	Toxic equivalent quotient calculation	38
2.1.7	Polycyclic aromatic hydrocarbon source diagnostic ratios	39
2.2	Materials and Methods	39
2.2.1	Sample collection	39
2.2.2	Chemical extraction procedure	39
	Sediment extraction	39
	Biota chemical extraction	41

	Fish muscle tissue extraction	41
	Fish bile sample extraction	42
	Wetland bird egg extraction	42
2.2.3	Instrumental analysis	42
	Quantification and quality control	44
2.2.4	Sediment toxicity evaluation	45
	Polycyclic aromatic hydrocarbon sediment quality guidelines	45
	Sediment indices	46
	Toxic equivalent quotient calculation	47
2.2.5	Polycyclic aromatic hydrocarbon source identification and compositions	48
2.3	Results and discussion	49
2.3.1	Chemical analysis results	49
	Sediment chemical analysis results	49
	Fish muscle tissue chemical analysis results	54
	Wetland bird egg chemical analysis results	57
2.3.2	Polycyclic aromatic hydrocarbon compositions and source identification	58
	Polycyclic aromatic hydrocarbon compositions	58
	Polycyclic aromatic hydrocarbon source identification	59
2.3.4	Sediment toxicity evaluation	61
	Polycyclic aromatic hydrocarbon sediment quality guidelines	61
	Sediment assessment indices	64
2.4	Conclusion	69
2.5	References	70

Chapter 3: Quantifying the aryl-hydrocarbon mediated toxicity of polycyclic aromatic hydrocarbons in the sediments of Soweto and Lenasia using the H4IIE-luc reporter gene bioassay

3.1	Introduction	82
3.1.1	Relevance of bioassays	82
3.1.2	Polycyclic aromatic hydrocarbons and cellular responses	83

3.1.3	Polycyclic aromatic hydrocarbons and the H4IIE- <i>luc</i> reporter gene bioassay	84
3.2	Materials and Methods	86
3.2.1	Sample collection	86
3.2.2	Sample extraction	86
3.2.3	Maintenance of H4IIE- <i>luc</i> cell culture	86
3.2.4	H4IIE- <i>luc</i> reporter gene bioassay	86
3.2.5	Calculating bioassay equivalence (BEQs)	87
3.2.6	MTT viability assay	87
3.3	Results and discussion	88
3.4	Conclusion	98
3.5	References	99

Chapter 4: Biomarker responses and fish health assessment of *Clarias gariepinus* from impoundments of Soweto and Lenasia, South Africa

4.1	Introduction	104
4.1.1	Health assessment of aquatic environments	104
4.1.2	Using fish for health assessments	104
4.1.3	Biomarkers and bio-indicators	105
4.1.4	Use of biomarkers and bio-indicators to assess fish health	106
	Biomarker responses	106
	Bio-indicators	109
4.2	Material and methods	111
4.2.1	Sample collection	111
4.2.2	Fish sampling and field necropsy	111
4.2.3	Biomarker responses	112
	Sample preparations	112
	Biomarkers of exposure	113
	Acetylcholinesterase activity assay	113
	Cytochrome P450	113

Biomarkers of Effect	114
Oxidative stress biomarkers	114
Superoxide dismutase activity	114
Catalase activity	114
Biomarkers of oxidative stress damage	115
Malondialdehyde content	115
Protein carbonyl induction	116
Energy allocation biomarkers	116
Cellular energy allocation	116
Available energy (Ea)	117
Energy consumption (Ec)	117
4.2.4 Bio-indicator assessments	118
Health assessment indices calculations	118
4.2.5 Statistical analysis	119
4.3 Results and discussion	120
4.3.1 Biometric information of <i>Clarias gariepinus</i> sampled	120
4.3.2 Biomarker responses	121
Biomarkers of exposure	121
Biomarkers of effect	127
Biomarkers of oxidative stress	127
Biomarkers of oxidative stress damage	129
Cellular energy allocation	130
4.3.3 Bio-indicator assessment	133
Fish health assessment index (FHAi)	133
Organo-somatic indices	138
Fulton's condition factor	138
Hepato-somatic index (HSI)	139

	Spleeno-somatic index (SSI)	140
	Gonado-somatic index	141
4.4	Conclusion	143
4.5	References	145
Chapter 5: Human health risk assessment for matrices of the Klip River of Soweto and Lenasia		
5.1	Introduction	159
5.2	Materials and Methods	161
5.2.1	Site selection	161
5.2.2	Hazard identification	161
5.2.3	Hazard characterisation	162
5.2.4	Exposure assessment	162
5.2.5	Risk characterisation	163
	Calculating non-carcinogenic- and carcinogenic risk	164
	Risk characterisation from exposure to PAHs in water	165
	Risk characterisation from exposure to PAHs in the sediment	166
	Risk characterisation from consuming PAH contaminated fish	167
5.3	Results and discussion	167
5.3.1	Non-carcinogenic risk characterisation (Hazard Index)	169
5.3.2	Carcinogenic risk characterisation	171
	Cancer risk from water exposure	171
	Cancer risk posed by exposures to sediments	173
	Cancer risk from consuming fish from a PAH polluted system	175
	Total cancer risk from the PAH polluted Soweto and Lenasia	175
5.4	Conclusion	178
5.5	References	179
Chapter 6: Statistical integration of results, conclusion, and recommendations		
6.1	Introduction	183
6.2	Data analysis	183
6.2.1	Correlation analysis	183
6.2.2	Multivariate statistics	183

6.3	Polycyclic aromatic hydrocarbons in the aquatic environment of Soweto and Lenasia	184
6.3.1	PAHs in the sediment	184
6.3.2	Relationship between biliary PAHs in <i>Clarias gariepinus</i> and the PAHs in the sediments	186
6.4	Sediment toxicity	188
6.4.1	Sediment toxicity in terms of instrumental data and sediment indices	188
6.4.2	Sediment toxicity in terms of biological responses	189
6.5	Biomarker and bio-indicator responses to polycyclic aromatic hydrocarbons	191
6.5.1	Effects of PAHs on the biomarkers and fish health indices	191
6.6	Conclusion	193
6.7	Recommendations	194
6.8	References	196

List of acronyms and abbreviations

%TOC	Percentage total organic carbon
2,3,7,8-TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin

A

Abs	Absorbance
Acea	Acenaphthene
Acey	Acenaphthylene
AChE	Acetylcholinesterase
Af gut	Gastro-intestinal absorption factor
Af skin	Dermal absorption factor
Ah	Aryl-hydrocarbon
AHH	Aryl-hydrocarbon hydroxylase
AhR	Aryl-hydrocarbon receptor
ANOVA	Analysis of variance
Ant	Anthracene
ARNT	Aryl-hydrocarbon receptor nuclear translator
ASE	Accelerated solvent extraction
AT	Average timing
ATSDR	Agency for Toxic Substances and Disease Registry

B

BaA	Benz(a)anthracene
BaP	Benzo(a)pyrene
BbF	Benzo(b)fluoranthene
BEDS	Biological Effects Database for Sediments
BEQ	Biological equivalents
BgP	Benzo(g,h,i)perylene
BioPy	Biomass combustion
BkF	Benzo(k)fluoranthene
BKME	Bleached kraft pulp and paper mill effluent
BM	Body mass
BSA	Bovine serum albumin

C

CAT	Catalase
CCME	Canadian Council of Ministers of the Environment
CEA	Cellular energy allocation

CF	Condition factor
Chr	Chrysene
CIA	Central Intelligence Agency
CPAH	Carcinogenic polycyclic aromatic hydrocarbon
CR	Cancer risk
CSF	Cancer slope factor
CV	Coefficients of variance
CYP450	Cytochrome P450
D	
D1	Days per week exposed
D2	Weeks per year exposed
D3	Years exposed
DAD	Daily average dose
Db	Dobsonville
DBA	Dibenz(a,h)anthracene
DBMA	7,12-dimethylbenz(a)anthracene
DCM	Dichloromethane
ddH ₂ O	Double distilled water
DDT	Dichlorodiphenyltrichloroethane
df	Film thickness of stationary phase
dl-PCBs	Dioxin-like polychlorinated biphenyls
dm	Dry mass
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
dp	Particle diameter of packing
DR-CALUX	Dioxin response chemically activated luciferase expression
DRE	Dioxin response element
dSPE	Dispersive solid phase extraction
DTPA	Diethylene triamine penta-acetic acid
DWAS	Department of Water Affairs and Sanitation
E	
EA	Environmental Agency
EC	Effective concentration
ECOD	7-ethoxycoumarin-O-deethylase
EDC	Endocrine disrupting chemicals
EDTA	Ethylene diamine tetra acetic acid
EF	Exposure frequency

EID	Eldorado Park
ELISA	Enzyme linked immuno-sorbent assay
enHealth	enHealth Council
ER	Oestrogen receptor
EROD	Ethoxyresorufin-O-deethylase
ERPM	East Rand Proprietary Mines
ETS	Electron transport system
ESI	Electrospray ionisation
EV	Event frequency

F

FAC	Fluorescent aromatic compounds
FAO	Food and Agriculture Organisation of the United Nations
FBS	Foetal bovine serum
FHAI	Fish health assessment index
FI	Fleurhof
Fla	Fluoranthene
Flu	Fluorene
FPF	Fish potency factor

G

GC	Gas chromatograph
GC-TOFMS	Gas chromatograph time-of-flight mass spectrometer
GCxGC-MS-TOF	Gas chromatograph gas chromatograph mass spectrometer time-of-flight
GHB	General homogenising buffer
GPC	Gel permeation chromatography
GSH	Glutathione
GSI	Gonado-somatic index
GST	Glutathione-S-transferase

H

HAH	Halogenated aromatic hydrocarbon
HCH	Hexachlorocyclohexane
HDPE	High density polyethylene
HI	Hazard index
HPAH	High molecular polycyclic aromatic hydrocarbons
HPLC-MS/MS	High pressure liquid chromatography coupled to tandem mass spectrometry
HQ	Hazard quotient
HSI	Hepato-somatic index

HSP Heat shock protein

I

IARC International Agency for Research on Cancer

ID Internal diameter

InP Indeno[1,2,3-cd]pyrene

IR fish Ingestion rate fish

IR sed Ingestion rate sediment

IR water Ingestion rate water

IRIS Integrated Risk Information System

ISQG Interim sediment quality guideline

K

K Fulton's condition factor

Koc Organic carbon-water partition coefficient

Kow Octanol-water partition coefficient

L

Le Lenasia

LE Life expectancy

LOD Limit of detection

LOQ Limit of quantification

LPAH Low molecular polycyclic aromatic hydrocarbons

M

MDA Malondialdehyde

Mo Moroka

MOs Monooxygenases

MTT 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide

MVC Malaria vector control

N

NADPH Nicotinamide adenine dinucleotide phosphate

Nap Naphthalene

Nc Nancefield

nCPAHs Non-carcinogenic polycyclic aromatic hydrocarbons

NMISA National Metrology Institute of South Africa

NTSP National Status and Trends Program

NWU North-West University

O

OCPs organo-chlorinated pesticides

OD	Optical density
OE	Orlando East
OH	Hydroxyl
OW	Orlando West

P

<i>P450</i>	Cytochrome P450 genes
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PC	Protein carbonyl
PCBs	polychlorinated biphenyls
PCDD	polychlorinated dibenzo- <i>p</i> -dioxins
PCDD/Fs	polychlorinated dibenzo furans and dioxins
PCDF	Polychlorinated dibenzofurans
PEC	Probable effects concentration
Pet	Petrogenic
PetPy	Petroleum combustion
PG	Protea Glen
Phe	Phenanthrene
PHAH	Polyhalogenated aromatic hydrocarbon
PMSF	Phenyl methane sulphonyl fluoride
POPs	Persistent organic pollutants
PPB	Potassium phosphate buffer
PSA	Primary secondary amine
Py	Pyrogenic
Pyr	Pyrene

Q

QuEChERS	Quick, Easy, Cheap, Effective Rugged and Safe
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R

REP	Relative potency values
RfD	Reference dose
RLU	Relative light units
ROS	Reactive oxygen species

S

SDS	Sodium dodecyl sulphate
SD	Standard deviation
SEM	Standard error of the mean

SE	Skin exposed
SL	Standard length
SL	Soil loading factor
SOD	Superoxide dismutase
SPE	Solid phase extraction
SQ	Sediment quality
SQG-I	Sediment quality guideline index
SQI	Sediment quality index
SRM	Standard reference material
SSI	Spleeno-somatic index
SSTT	Spiked-sediment toxicity test
StatsSA	Statistics South Africa
T	
TCA	Trichloro-acetic acid
TEC	Threshold effects concentration
TEF	Toxic equivalency factors
TEQ	Toxic equivalency quotient
TEQ _{BaP}	Toxic equivalency quotient in terms of BaP TEFs
TEQ _{FPF}	Toxic equivalency quotient in terms of 2,3,7,8-TCDD calculated using FPFs
TEQ _{TCDD}	Toxic equivalency quotient in terms of 2,3,7,8-TCDD TEFs
Tevent	Event duration
TL	Total length
TMP	1,1,3,3-tetramethoxypropane
U	
UDP-GT	Uridine 5-diphosphate-glucuronosyltransferase
USEPA	United States Environmental Protection Agency
V	
v/v	Volume per volume
W	
WHO	World Health Organisation
WISA	Water Institute of Southern Africa
WMA	Water management area
WRC	Water Research Commission
WWTPs	Waste water treatment plants

List of figures

Chapter 1

- Figure 1.1 Klip River catchment showing the Klip River and its tributary from origin to confluence with the Vaal River
- Figure 1.2 Sampling sites within the greater Soweto and Lenasia area
- Figure 1.3 Protea Glen sampling site: A) Large wetland at Protea Glen; B) Vegetation at sample site
- Figure 1.4 Lenasia sampling site: A) View of the dam showing reed beds; B) Heronry adjacent to the main dam (circled area)
- Figure 1.5 Fleurhof Dam sampling site: A) Thick reed beds lining the eastern shore; B) Construction sites bordering the dam; C) old mine dumps to the western shore
- Figure 1.6 Sampling site in the Dorothy Nyemba Park, Dobsonville: A) Dam where sediment was sampled; B) Dam shore and reed beds showing litter
- Figure 1.7 Orlando West sampling site: A) Klip Spruit opposite to Orlando Stadium, and flowing adjacent to Klipspruit Drive (top background of picture); B) Deep sections and riffles of Klip Spruit
- Figure 1.8 Orlando East sampling site. A) The iconic Orlando Towers and collapsed power station. B) Heavy pollution in dam; C+D) *Clarias gariepinus* carcasses after fish kill
- Figure 1.9 Sediment sampling site at Moroka located under the Chris Hani Road bridge
- Figure 1.10 Sediment sampling site at Eldorado Park, photo taken off the Main road bridge
- Figure 1.11 Sediment sampling site at Nancefield: A) Downstream from the weir; B) Downstream from the collapsed bridge
- Figure 1.12 Fish sampling site at final ponds of the Bushkoppies WWTP
- Figure 1.13 Example of an African sharptooth catfish (*Clarias gariepinus*) male (2.5 kg; 700 mm TL) A) Dorsal view, B) Ventral view C) view of head view showing the eyes, barbels and spinous pectoral fin
- Figure 1.14 Example of herons and egrets of this study: A) a black-headed heron (*Ardea melanocephala*) perched in a tree above a water body; B) a cattle egret (*Bubulcus ibis*) foraging a pond shoreline
- Figure 1.15 Example of ibises: A) a glossy ibis (*Plegadis falcinellus*) foraging in the shoreline; B) a sacred ibis (*Threskiornis aethiopicus*) foraging on bank next to a water body

Chapter 2

- Figure 2.1 Extracted mass chromatogram of the 16 priority phase PAHs in *Clarias gariepinus*
- Figure 2.2 Polycyclic aromatic hydrocarbons metabolite profile, proportional contributions of the 11 OH-PAHs in *Clarias gariepinus* from Soweto and Lenasia 2013: A) Control; B) Orlando; C) Lenasia; D) Fleurhof
- Figure 2.3 Polycyclic aromatic hydrocarbons profile composition at sites from the Soweto and Lenasia study area (2013 and 2014)
- Figure 2.4 Source identification of polycyclic aromatic hydrocarbons in the Soweto and Lenasia sediment of 2013 and 2014
- Figure 2.5 Soweto and Lenasia sediments compared to sediment quality guidelines of MacDonald *et al.*, 2000 and CCME, 2012 in terms PAH levels
- Figure 2.6 Mean toxic equivalent quotient (TEQ) results calculated from literature A) TCDD-TEQs compared to the TEQ guidelines of the CCME (2001); B) BaP-TEQs

Chapter 3

- Figure 3.1 The mechanism of Ah-receptor mediated response in cells (adapted from Hilscherova *et al.*, 2000)
- Figure 3.2 The mechanism of Ah-receptor mediated luciferase reporter gene response of the H4IIE-*luc* bioassay (adapted from Hilscherova *et al.*, 2000)
- Figure 3.3 Luciferase activity (%TCDDmax) for: A) 2,3,7,8-TCDD standard; B) 2013 sediments; C) and 2014 sediments. Bars in A is standard deviation (SD). SD bars were omitted from the other graphs for the sake of simplicity
- Figure 3.4 Relative potencies (REP20) calculated for sediments collected at each site in 2013 and 2014
- Figure 3.5 BEQs compared to the Σ CPAHs for sediments of 2013 and 2014: A) Spearman's correlation graph; B) dual axis line graph showing the relationship between BEQs and Σ CPAHs

Chapter 4

- Figure 4.1 Levels of biological organisation and the order of response to pollutant stress [modified

from Van der Oost *et al.*(2003]

- Figure 4.2 Biomarkers of exposure A) Acetylcholinesterase activity; B) and cytochrome P450 measured in *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014), and control fish. Median values indicated and whiskers set at 5th and 95th percentiles. Bars with common conscripts indicate significant differences (A Tukey's multiple comparison test; B Dunn's multiple comparison test, $p < 0.05$)
- Figure 4.3 Biomarkers of oxidative stress A) superoxide dismutase; B) and catalase activity measured in *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014), and control fish. Median values indicated and whiskers set at 5th and 95th percentiles. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)
- Figure 4.4 Biomarkers of oxidative stress damage: A) malondialdehyde content; B) and protein carbonyl content, measured in *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014), and control fish. Median values indicated and whiskers set at 5th and 95th percentiles. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)
- Figure 4.5 Energy reserves and consumption: A) available carbohydrates; B) total lipids; C) protein content; D) and energy consumption measured in *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014). Median values indicated and whiskers set at 5th and 95th percentiles. Bars with common conscripts indicate significant differences (Tukey's multiple comparison test, $p < 0.05$)
- Figure 4.6 Cellular energetics: A) available energy; B) cellular energy allocation measured in *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014). Median values indicated and whiskers set at 5th and 95th percentiles. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)
- Figure 4.7 Observed abnormalities during necropsy: A) liver enlargement and darker discolouration; B) altered testes containing vesicles [arrows]; C) increase of connective tissue and fusion [arrows]; D) liver discolouration; E) increased fatty deposits in liver
- Figure 4.8 Fish health assessment index values for *Clarias gariepinus* sampled from sites from the greater Soweto and Lenasia area for 2013 and 2014, and control fish. Error bars are SD. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)
- Figure 4.9 Fulton's condition factor for *Clarias gariepinus* sampled from sites from the greater Soweto and Lenasia area for 2013 and 2014, and control fish. Error bars are SEM
- Figure 4.10 The hepato-somatic indices values for *Clarias gariepinus* sampled from sites from the

greater Soweto and Lenasia area for 2013 and 2014, and control fish. Error bars are SEM. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)

- Figure 4.11 The spleeno-somatic index results for *Clarias gariepinus* sampled from sites from the greater Soweto and Lenasia area for 2013 and 2014, and control fish. Error bars are SEM
- Figure 4.12 The gonado-somatic index for A) female and B) male *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014), and control fish. Error bars are SEM. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)

Chapter 5

- Figure 5.1 Hazard index reporting non-carcinogenic risk of polycyclic aromatic hydrocarbons from Soweto and Lenasia for: Water ingestion for adults during A) 2013 and B) 2014, as well as water ingestion hazard risk for children of C) 2013 and D) 2014. The hazard risk for pregnant women ingesting sediments by geophagia during E) 2013 and F) 2014, as well as unintentional ingestion of sediments during G) 2013 and H) 2014. No hazard: $HI < 0.1$; low hazard: $0.1 < HI < 1$; moderate hazard: $1.1 < HI < 10$; high hazard: $HI > 10$, where applicable indicated by a line
- Figure 5.2 Carcinogenic risk of dermal exposure to polycyclic aromatic hydrocarbon contaminated water for A) adults and B) children during 2013, as well as C) adults and D) children during 2014. Unacceptable risk for dermal exposure is greater than 1 in 1 000 000 (1×10^{-6}) (red line)
- Figure 5.3 Carcinogenic risk to ingestion of polycyclic aromatic hydrocarbon contaminated water for A) adults and B) children during 2013, as well as C) adults and D) children of 2014. Unacceptable risk for ingestion is greater than 1 in 10 000 (1×10^{-4}) (red line)
- Figure 5.4 Carcinogenic risk of dermal exposure to polycyclic aromatic hydrocarbon contaminated sediments for A) adults and B) children of 2013, as well as C) adults and D) children of 2014. Unacceptable risk for dermal exposure is greater than 1 in 1 000 000 (1×10^{-6})
- Figure 5.5 Carcinogenic risk to geophagia of polycyclic aromatic hydrocarbon contaminated sediments for pregnant women of Soweto and Lenasia: A) geophagia-, and B) unintentional ingestion during 2013, as well as C) geophagia- and D) unintentional ingestion during 2014. Unacceptable risk for ingestion is greater than 1 in 10 000 (1×10^{-4}) (red line)

- Figure 5.6 Total carcinogenic risk at Soweto and Lenasia from multiple exposures for A) adults and B) children for both survey years (2013 and 2014). Unacceptable risk is greater than 1 in 10 000 (1×10^{-4}) (red line)

Chapter 6

- Figure 6.1 Temporal and spatial representation polycyclic aromatic hydrocarbons in the sediments of Soweto and Lenasia during A) 2013 and B) 2014. Colour scale derived from scale by Baumard *et al.* (1998)
- Figure 6.2 PCA biplot of polycyclic aromatic hydrocarbons in the sediments of Soweto and Lenasia during 2013 and 2014. The ordination explains 92% of the variance in the data with 74.46% by factor 1 and 17.5% by factor 2
- Figure 6.3 RDA triplot of the biliary polycyclic aromatic hydrocarbons in *Clarias gariepinus* and the native polycyclic aromatic hydrocarbons in the sediment from Fleurhof, Lenasia and Orlando, 2013. The ordination explains 57.47% ($p = 0.002$) of the variance, 43.74% by factor 1 and 13.73% by factor 2
- Figure 6.4 Spearman's correlation scatterplot for sediment quality index (SQI) vs sediment quality guideline index (SQG-I) for 2013 and 2014
- Figure 6.5 Spearman correlation scatterplot for: A) the sediment quality guideline index (SQG-I) vs toxic equivalence using the fish potency factors (TEQ_{FPF}); B) the SQG-I vs toxic equivalence using toxic equivalent factors derived by Villeneuve *et al.* (2002) (TEQ_{TCDD}); C) the TEQ_{TCDD} vs TEQ_{FPF}
- Figure 6.6 Spearman correlation scatterplot for: A) the biological equivalence (BEQ) and toxic equivalence (TEQ_{TCDD}); B) BEQ and the concentrations of the carcinogenic polycyclic aromatic hydrocarbons (CPAHs)
- Figure 6.7 Spearman correlation scatterplot for the biological equivalence (BEQ) and the sediment quality guideline index (SQG-I)
- Figure 6.8 Forward selected RDA triplot of polycyclic aromatic hydrocarbons in the sediments of Soweto and Lenasia, biomarker responses and fish health indices of 2013 and 2014. The ordination explains 44.53% of the variance in the data with 36.97% by factor 1 and 7.55% by factor 2

List of tables

Chapter 1

Table 1.1	The potential sources of point- and diffuse pollution in the Klip River (Adapted from Kotze, 2002)
Table 1.2	Selected sites in the greater Soweto and Lenasia co-ordinates, matrices sampled, and physical characteristics (2013 in grey and 2014 in white)

Chapter 2

Table 2.1	Physical and chemical characteristics of the 16 priority PAHs (Table adapted from USEPA, 2008; Neff <i>et al.</i> , 2005; Lee & Vu, 2010; Stogiannidis & Laane, 2015)
Table 2.2	Extraction parameters for the accelerated solvent extraction method
Table 2.3	Mobile phase gradient used in the analysis of hydroxyl-PAHs in fish bile
Table 2.4	Recoveries (%) of polycyclic aromatic hydrocarbons in sampled sediment, <i>Clarias gariepinus</i> and wetland bird eggs, including the limit of detection (LOD) and limit of quantification (LOQ) expressed in µg/kg
Table 2.5	Sediment quality guidelines levels of MacDonald <i>et al.</i> (2000) (µg/kg dm) and of the Canadian council of ministers of the environment (CCME, 2001) (µg/kg 1%TOC)
Table 2.6	Toxic equivalence factors in terms of 2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin (Villeneuve <i>et al.</i> , 2002) and benzo(a)pyrene (Tsai <i>et al.</i> , 2004) as well as fish potency factors (Barron <i>et al.</i> , 2004) used to calculate toxic equivalence quotients
Table 2.7	Diagnostic ratios for source identification of polycyclic aromatic hydrocarbons
Table 2.8	Concentrations (µg/kg) of the PAHs in the sediment from the nine sites in the greater Soweto and Lenasia area for 2013 and 2014
Table 2.9	Concentration of ΣPAHs from literature
Table 2.10	Mean hydroxylated PAH metabolites quantified from <i>Clarias gariepinus</i> from selected sites of 2013 (ng/mL), range in parenthesis
Table 2.11	Levels of naphthalene, acenaphthene and phenanthrene (µg/kg) in wetland bird eggs sampled from Lenasia 2013
Table 2.12	Source identification ratios of PAHs in sediments at sites in Soweto and Lenasia of 2013 and 2014
Table 2.13	Sediment from the sites of Soweto and Lenasia compared to sediment quality guidelines (TEC and PEC) of MacDonald <i>et al.</i> , 2000. Guidelines exceedance indicated by shading
Table 2.14	Sediment from the sites of Soweto and Lenasia compared to sediment quality guidelines of Canada (ISQG and PEL) (CCME, 2012). Guidelines

	exceedance indicated by shading
Table 2.15	SQG-I results for sites from Soweto and Lenasia (2013 & 2014) in terms of the MacDonald <i>et al.</i> (2000) guidelines and the CCME (2012) guidelines. Shading according to index scale
Table 2.16	Sediment quality index (SQI), in terms of PAH contamination, for the sites in the Soweto and Lenasia area for 2013 and 2014. Shading according to index scale
Table 2.17	Toxic equivalent quotient (TEQ _{TCDD}) results, calculated for the sediments of the sites from Soweto and Lenasia (2013 & 2014), compared to the TEQ guidelines of the CCME (2001). Guidelines exceedance indicated by shading
Table 2.18	Toxic equivalent quotient (TEQ _{FPF}) for dioxin-like toxicity towards fish results, calculated for the sediments of the sites from Soweto and Lenasia (2013 & 2014), compared to the TEQ guidelines of the CCME (2001). Guideline exceedance indicated by shading

Chapter 3

Table 3.1	H4IIE-luc reporter gene bioassay results showing %TCDDmax and BEQs (REP20, -50, -80) after exposure to sediment extracts (extrapolated data in italics; the greatest values per column in bold). Viability results were also included as %cell viability
Table 3.2	Bioassay equivalent (BEQ) results, calculated for the sediments collected at the Soweto and Lenasia sites (2013 and 2014) with the H4IIE-luc bioassay, gauged against the TEQ guidelines of the CCME (2001). Shading indicates which guidelines were exceeded

Chapter 4

Table 4.1	Sex ratio, mean mass, mass range, mean total length, and total length range of <i>Clarias gariepinus</i> sampled in Soweto and Lenasia (2013 and 2014). Standard deviation in parenthesis where means are reported
Table 4.2	Summary of the interpretations of biomarker responses (Adapted from van der Oost <i>et al.</i> , 2003 and Wepener <i>et al.</i> , 2011)
Table 4.3	Summary of the relationships of total cytochromes (CYP450), cytochrome 1A isoenzyme, aryl hydrocarbon hydrolase (AHH), ethoxyresorufin-O-deethylase (EROD) and ethoxycoumarin-O-deethylase (ECOD) responses to various pollutants in freshwater fishes reported in literature, as summarised by Van der Oost <i>et al.</i> , 2003

Chapter 5

Table 5.1	Available cancer slope factors and reference doses for polycyclic aromatic hydrocarbons (USEPA, 1999; CIDA, 2015)
Table 5.2	Exposure parameters for human health risk assessment for adults and children (USEPA, 2004; Health Canada, 2004) adapted to represent the

	population in the study area
Table 5.3	Risk characterisation variables for dermal- and oral exposures to soils, water and fish (USEPA, 2004; Health Canada, 2004)
Table 5.4	Extrapolated concentrations (ng/mL) of the polycyclic aromatic hydrocarbons in the water from the nine sites in the greater Soweto and Lenasia area for 2013 and 2014

Chapter 6

Table 6.1	Levels of total polycyclic aromatic hydrocarbons in the sediments (ng/g) of Soweto and Lenasia against the scale by Baumard et al. (1998)
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Acknowledgements

I would like to express my sincere gratitude and thanks to the following people and institutions:

The National Research Foundation (NRF) for the grant-holder linked bursary (Innovation doctoral scholarship)

The Water Research Commission (WRC) for funding the research project (WRC project no. K5/2422)

To my supervisor Prof Rialet Pieters, for all her guidance, patience, support and invaluable contributions throughout my post-graduate career, it is truly appreciated.

To my co-supervisor, Prof Nico Smit, for his support and guidance, especially for his ability to see a situation from a different angle.

Dr Laura Quinn, the technical supervisor on the project. Thank you for the quality training I received regarding the instrumental analysis component of this project.

Thank you to the following academic staff that were not directly involved in this project, but who's contribution is immensely appreciated: Prof Victor Wepener, Dr Tarryn Botha, Dr Wynand Malherbe, Dr Ruan Gerber and Dr Kyle McHugh.

The Gauteng Department of Agriculture and Rural development, Directorate of Conservation Permit Office for the issue of the sampling permit (CPE2/0097).

Johannesburg Water, for access to the Bushkoppies wastewater treatment plant

Thank you to the following people who assisted during fieldwork and other sampling sessions: Godfrey Sakhele Magodla, Kobus Fourie, Pieter Holtzhausen and Joppie Schrijvershof. Then also thank you to Oom Frans Gigano and Stephen van der Walt at the WRG aquarium for arranging and packing equipment and maintaining the control fish.

A special thank you to Prof Mayumi Ishizuka, Prof Yoshinori Ikenaka, Dr Yared Beyene, and Nesta Bortey-Sam for all their assistance and organisation during my research visit to the Toxicology Laboratory at the Graduate School of Veterinary Medicine Hokkaido University, Japan.

To all my friends at the office—Alewyn Carstens, Nico Wolmarans, Suranie Horn, Tash Vogt, Karin Minnaar, and Anrich Kock—that helped during sampling, in the laboratories, and giving moral support, thank you, your encouragement and support was invaluable.

Then a special thank you to my parents, Etienne and Mauriza Pheiffer, and sister Marnél, for all your love and support. Thank you for encouraging me when the going got tough and for believing in me to accomplish my goals.

The Lord, for His love and granting me the privilege, skills and abilities to explore my interest in the sciences. For His support in this endeavor and giving me the strength and endurance to complete this achievement.

Abstract

In South Africa, our scarce water resources are fully utilised and its quality threatened by pollution and therefore must be protected. One of the pollutant classes of concern is the polycyclic aromatic hydrocarbons (PAHs). PAHs are widely distributed, constantly released into the environment making them persistently present. Sixteen priority congeners have been identified by the USEPA to be monitored and controlled based on their proven harmful effects on humans and wildlife. The main purpose of this thesis was to study the potential exposures of humans and wildlife to the 16 priority PAHs in Soweto and Lenasia—an area known to have high levels of these contaminants. The aims were: to determine the levels of the PAHs in the sediments, fish (*Clarias gariepinus*) and wetland bird eggs from the aquatic ecosystem of the Klip River in the densely populated study area; determine the origins of the PAHs (pyrogenic or petrogenic); finally, to determine the toxicity posed by these PAHs to wildlife and humans. The levels of the PAHs in the matrices were determined by instrumental analysis. The target compounds were extracted for quantification using specific methods for the abiotic matrix (sediment) and the biotic (fish muscle and bird eggs). Sediments were subjected to accelerated solvent extraction, size exclusion chromatography and solid phase clean-up techniques. The biotic samples followed the liquid-liquid extraction based method known as QuEChERS. Biliary metabolites in the fish were isolated by first deconjugation followed by liquid-liquid extraction. The native PAHs were quantified with gas chromatography and time-of-flight mass spectrometry (GC-TOFMS) and the metabolites with high pressure liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). The pollutant profile was calculated by percentage congener contributions and potential origins were determined by diagnostic ratios. The toxicity assessment was a multi endpoint approach: Overall toxicity, sediment quality, and ecological risk were assessed by comparing concentrations to international sediment quality guidelines (SQGs) and calculating sediment quality indices. Investigating specific toxicity via the aryl-hydrocarbon receptor was measured using the H4IIE-*luc* reporter gene bio-assay and compared with toxic equivalence quotients (TEQs). The reaction of fish to environmental stressors was investigated on biochemical level with biomarker assays. These included biomarkers of exposure, oxidative stress, oxidative damage, and cellular energy allocation. Furthermore, individual and community fish health were assessed using various fish health indices. Finally, the potential health risk to the human population dependant on the water bodies was gauged by conducting a theoretical human health risk assessment. The levels of the Σ PAHs in the sediments for both sampling surveys ranged between 274–5 369 ng/g, which were dominated by 3-and 4-ring congeners, mainly from biomass combustion. Evidence of PAHs in the biotic matrices was seen in the form of low levels of low molecular mass PAHs in the eggs, and biliary metabolites (63–1 879 ng/mL) in the fish. Moderate to high toxicity was predicted for benthic organisms, fish, and mammalian systems, based on the instrumentally derived PAH sediment concentrations using international SQGs, sediment indices, and TEQs. These were comparable to the real biological responses of the H4IIE-*luc* reporter gene bioassay proving the usefulness of this bioassay. The clear responses of the biomarkers showed that the fish from the study area were exposed to xenobiotic stressors and there was strong evidence that these were responses to PAHs.

The overall health of fish was visibly affected by environmental stressors (such as pollutant exposure, seasonal changes, and water parameters) and proven to be in poor health. Various routes of human exposure were investigated and the greatest cancer risk was from intentional ingestion and dermal exposure to the sediments. The greatest cancer risk was 227 in 10 000.

Keywords: Soweto and Lenasia, PAHs, sediment, *Clarias gariepinus*, toxicity assessment, H4IIE-*luc* bioassay, biomarker response assays, fish health, human health risk assessment

Chapter 1: General and thesis introduction

1.1 General introduction

South Africa is a water scarce country and is ranked as the 30th driest country in the world. Even though water scarcity is a global challenge, sub-Saharan Africa, especially southern Africa is hardest hit (Cessford *et al.*, 2005; DWAS, 2014). Brand *et al.* (2009) states that South Africa over-utilised its water resources because of the attitude that these resources are inexhaustible. It is believed that many parts of South Africa has reached or are approaching the point where viable freshwater resources are fully utilised (Cessford *et al.*, 2005; DWAS, 2014), placing our ecosystems under immense pressure (Dallas & Day, 2004). Anthropological influences like pollution, misuse and poor management of water resources created environmental problems such as poor water quality and the diminishing of ecosystem health (Brand *et al.*, 2009).

The quantity of our water resources are already under pressure and the decrease in quality escalates the problem. According to the South African National Water Act (Act 36 of 1998) we need to implement monitoring programs to assess aquatic ecosystem health. This, if implemented correctly and efficiently, along with resource management, will promote and support the improvement of aquatic ecosystems.

As a consequence of the above many studies have reported on water quality of South Africa and the effect pollution has on the aquatic environment. These studies focussed on industrial and agricultural pollutants (Schulz & Peall, 2001; Du Preez *et al.*, 2005; Ansara-Ross *et al.*, 2012) and heavy metals (Kotze *et al.*, 1999; Van Aardt & Erdmann, 2004; Jooste *et al.*, 2014). However, there is a paucity in the knowledge of organic pollutants of industrial origins in South African systems, and lately some studies have been conducted to fill this knowledge gap (Nieuwoudt *et al.*, 2009; 2011; Quinn *et al.*, 2009; Barnhoorn *et al.*, 2010; 2015).

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that consist of fused benzene rings containing only hydrogen and carbon atoms (Sims & Overcash, 1983; Angerer *et al.*, 1997; Gehle, 2009). PAHs have relatively high molecular masses: they are solids with low volatility at room temperature and are soluble in many organic solvents, but also relatively insoluble in water (Gehle, 2009). The widespread occurrence of PAHs is largely due to their formation and release in all processes of incomplete combustion of organic materials or high pressure processes (Gehle, 2009): production of cokes and carbon, coal power plants, petroleum processing, furnaces, fireplaces, gas and oil burners, and automobile sources (Angerer *et al.*, 1997; Maliszewska-Kordybach, 1999), all of which are present in South Africa.

Of all the PAHs that exist, the United States Environmental Protection Agency (USEPA) has identified 16 PAH congeners that are classified as priority pollutants due to their adverse effects. This study will focus on these priority congeners, which are: naphthalene, acenaphthene, acenaphthylene, anthracene, phenanthrene, fluorene, fluoranthene, pyrene, benzo(g,h,i)perylene, indeno[1,2,3-cd]

pyrene, benzo(a)anthracene, chrysene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, dibenz(a,h)anthracene (USEPA, 2008) [The last 6 compounds in the list are regarded as human carcinogens (NTP, 2005)].

Studying sediment quality by evaluating the concentration of pollutants can assess the potential toxicity of a system (Chakravarty & Patgiri, 2009). Many studies have investigated the profile and composition within the aquatic environment, by studying the concentrations of these pollutants in the sediment (Fernández *et al.*, 1999; Swartz, 1999; Marvin *et al.*, 2000; Culotta *et al.*, 2006; Nieuwoudt *et al.*, 2009); biota such as molluscs (Wootton *et al.*, 2003; Mirsadeghi *et al.*, 2013; Rodrigues *et al.*, 2013; Sureda *et al.*, 2013), amphibians (Hatch & Burton, 1998; Bryer *et al.*, 2006; Leney *et al.*, 2006), birds (Custer *et al.*, 2001; Triosi *et al.*, 2006;; Pereira *et al.*, 2009), and especially fish (Kayal & Connell, 1995; Vives & Grimalt, 2002; Vives *et al.*, 2004; Reynaud & Deschaux, 2006; Beyer *et al.*, 2010; Harman *et al.*, 2011). It is important to determine the environmental impact these compounds have on the biota, ultimately because it is indicative of possible effects on humans (Väänänen *et al.*, 2005; Cirila *et al.*, 2007; McClean *et al.*, 2007).

The total global anthropogenic release of the 16 USEPA priority PAHs to the atmosphere was estimated to be at 520 000 tons in 2004, of which 18.8% was emitted from Africa (Zhang & Tao, 2009). An appreciable amount of this is expected to return to the surface through deposition and may wind up in the aquatic system. The direct PAH emissions to soil, water and sediment is not known, and there is little data for South African freshwaters (Das *et al.*, 2008; Quinn *et al.*, 2009; Nieuwoudt *et al.*, 2011; Roos *et al.* 2012; Moja *et al.*, 2013; Nekhavhambe *et al.*, 2014). We therefore know that PAHs are present in the South African environment, specifically in the section of the Vaal River catchment running through the Vaal Triangle (Nieuwoudt *et al.*, 2011; Moja *et al.*, 2013). The total concentration of PAHs in the former study ranged between 44 and 39 000 ng/g, dry mass (dm) and the concentration of carcinogenic PAHs ranged between 19 and 19 000 ng/g, dm (Nieuwoudt *et al.*, 2011). The concentrations of native congeners in the water ranged between 23.5 and 110.8 µg/L (Moja *et al.*, 2013). Pyrogenic (burning) processes were the most likely sources, with minimal petrogenic (derived from fuels and oils) contributions (Nieuwoudt *et al.*, 2011; Moja *et al.*, 2013). PAH levels were in the same range as levels reported from other countries.

In the study completed for the Water Research Commission (Project no K5/1561) on POPs in freshwater sites throughout the entire country, the PAHs had the highest levels of all of the organic pollutants analysed for. One of the sites with the highest PAH levels, was within the Soweto and Lenasia urban area, with 5 408 ng/g (Roos *et al.*, 2012). The cumulative probability of developing cancer resulting from exposure to benzo(a)pyrene at this site as a result of exposure to fish contaminated with benzo(a)pyrene was calculated to be between 0.181 and 0.859 in 1 000. [This can be rounded off to 2 in 10 000 and 9 in 10 000]. This is much higher than what is considered as an acceptable risk (approximately 6 in 10 000 versus the acceptable risk of 1 in 100 000 of the WHO (2010)).

1.2 Hypothesis, aims and objectives

The findings of Roos *et al.* (2012) led to the need to investigate the Soweto and Lenasia area in more detail as it showed to be experiencing high PAH exposures and therefore lead to this study. Thus, the main aim of this study was to determine the levels of the 16 priority PAHs in the Klip River that flows through the densely populated urban areas of Soweto and Lenasia, where high levels were previously found to have a more detailed view of PAH pollution and exposure in this particularly populated area of the country.

1.2.1 Hypothesis

Humans and wildlife from Soweto and Lenasia dependant on the Klip River are exposed to the 16 priority PAHs.

1.2.2 Aims and objectives

The hypothesis was investigated along the following aims:

Aim 1: Determine the levels of the 16 priority PAHs in the Klip River that flows through the densely populated urban areas of Soweto and Lenasia where high levels were previously found.

Objectives:

Measure concentrations of 16 PAHs in sediment at 9 sites over a two year period

Measure concentrations of 16 PAHs in fish tissue at 4 sites over a two year period

Measure concentrations of 16 PAHs in wetland bird eggs over a two year period

Aim 2: Investigate the pollutant profile of 16 PAHs in the sediment

Objectives:

Compare site PAH composition percentages by grouping congeners with the same number of cyclic rings to investigate similar pollution profiles between sites.

Calculation of diagnostic ratios to determine origin of the pollution, i.e. pyrogenic vs. petrogenic

Aim 3: Determine the toxicity posed by the PAHs in the study area

Objectives:

Assessing sediment toxicity to benthic organisms, by comparing to international sediment quality guidelines and calculating sediment quality indices

Investigating a very specific form of toxicity: that of the aryl hydrocarbon receptor mediated toxicity, in sediment using the H4IIE-*luc* reporter gene bioassay

Aim 4: Investigating biochemical responses of the fish to the environmental stressors by performing biomarker response assays

Objectives:

Determining the levels biomarkers of exposure (acetylcholinesterase activity and total cytochrome P450s levels), to identify direct effects of xenobiotic stressors on the fish.

Determine the levels of biomarkers of oxidative stress (superoxide dismutase and catalase activity) and oxidative damage (malondialdehyde and protein carbonyl content) to identify potential oxidative stress and associated damage in the fish

Determine the energy budget (available carbohydrates, lipids and proteins and available energy) to indicate if the stressors on the fish affect the cellular energy allocation

Aim 5: Investigating individual and community fish health by applying health indices for fish

Objectives:

Calculating Fulton's condition factor to describe the overall condition of individual fish and the population

Determine the organo-somatic indices for the liver, spleen and gonads, to investigate physiological endpoint deviations within individuals and the population

Applying the fish health assessment index to determine the overall health of individuals and the community

Aim 6: Gauging potential risk to human health by conducting a theoretical human health risk assessment

Objectives:

Determining non carcinogenic risk using the Hazard Index for dermal and ingestion exposure to the various matrices

Calculating cancerous risk from dermal exposures and ingestion of the various matrices

1.3 Study area–Klip River catchment

1.3.1 The Klip River catchment

The Klip River catchment is situated in South Africa's most densely populated province Gauteng, and drains the Witwatersrand region, the southern part of Johannesburg, one of the most developed urban areas in Africa (Kotze, 2002, DWAS 2009). The Klip River catchment is a sub-catchment (along with the Wilge River, Klip River (Free State province), Suikerbosrand-/Bosbok Spruit, Mooi River (North West province), Grootdraai Dam and Vaal River/Vaal Dam/Vaal Barrage catchments) of the Upper Vaal Water Management Area (WMA) (DWAS 2004). The Klip River is the largest tributary of the Vaal River, and together these rivers supply the largest portion of the surface flow of the WMA, downstream of the Vaal Dam (DWAS, 2004). It flows mainly southwards where it joins the Vaal River near Vereeniging.

For the sake of convenience the Klip River catchment was divided into regions based on the Klip River's tributaries and their position within the catchment. The Klip River originates in the south of Roodepoort, northwest of Soweto (Figure 1.1). The river flows south and then turns east along the south of Soweto (Howie & Otto, 1996) (Referred to as Region 1 for this study, Figure 1.1). Here the Klip Spruit joins the Klip River. The Klip Spruit originates north of Soweto, and flows south through the centre of Soweto (Referred to as Region 2, Figure 1.1). The Klip River receives water from three waste water treatment (WWTPs) plants (Olifantsvlei, Bushkoppies and Goudkoppies) that are situated in this area (Figure 1.1). The river continues to flow past the south of Johannesburg towards the east, where the Riet Spruit flows into the Klip River (Region 3, Figure 1.1) and continues towards the confluence with the Vaal River (Region 4, Figure 1.1) near Vereeniging (Howie & Otto, 1996; Kotze, 2002).



Figure 1.1: Klip River catchment showing the Klip River and its tributary from origin to confluence with the Vaal River

Domestic users of the Klip River mainly include rural settlements along the Klip River and its tributaries. The water utility, Rand Water, supplies potable water from the river to various municipalities in the catchment (Howie & Otto, 1996; Kotze, 2002). Industrial use of the water (Regions 1 & 2) is restricted to the middle reaches of the catchment. Main users are processing industries, such as product packaging, roofing and cladding material production, three waste water treatment plants and mining activity (Kotze, 2002). Industrial water is also supplied by Rand Water. Mining (gold, base metals and industrial minerals) is the most important activity in the upper catchment of the Klip River (DWAS, 2012). Agricultural activities such as livestock watering and crop irrigation also use water in the catchment (Kotze, 2002).

Because the Klip River flows through the Witwatersrand region it is considered as one of South Africa's most polluted rivers (McCarthy & Venter, 2006; McCarthy *et al.*, 2007). The mining activities and WWTPs in the catchment act as primary sources of point pollution. The sources of diffuse pollution mainly consist of informal settlements and old mine slime dams/waste dumps (Kotze, 2002). A summary of the potential pollution in the Klip River was compiled by Kotze (2002) (Table 1.1).

Table 1.1: The potential sources of point- and diffuse pollution in the Klip River (Adapted from Kotze, 2002)

Point source pollution			Diffuse pollution	
Klip River upstream from Klip Spruit confluence (Region 1)	Mining activity:	Durban Deep Roodepoort Mine (mine water pumping ceased in 1998)	Mining activity:	Slime dams Rock dumps Old mine waste sites
			Informal settlements:	Kagiso, Durban Deep Roodepoort Mine, Protea Glen, Doornkop West, Soweto, and Moroka
			Municipal:	Leaking sewage systems in informal settlements, mainly Soweto
			Industrial:	Chamdor industrial area
			Waste sites:	Closed solid waste site at Dobsonville
Klip Spruit (Region 2)	Power generation:	Orlando Power Station (ceased operation in 1998, plant collapsed in 2014)	Mining activity:	Slime dams (Central Gold Recovery), Rock dumps, Old mine waste sites
			Informal settlements:	Diepkloof, Power Park, Orlando East, and Pimville
			Municipal:	Leaking sewage systems in Soweto and surrounding suburbs
			Industrial:	Main Reef Road, Industria, Newtown and Selby areas
			Waste sites:	Marie Louise and Robinson Deep solid waste sites (active) and the Meredale solid waste site (closed)
Klip River between Klip Spruit and Riet Spruit confluence (Region 3)	Municipal:	Goudkoppies, Olifantsvlei and Bushkoppies WWTPs	Informal settlements:	Lenasia, Eldorado Park, Eikenhof
			Municipal:	Leaking sewage systems in Eldorado Park
			Industrial:	Nancefield and Olifantsvlei
			Waste sites:	Goudkoppies solid waste site
			Other:	Agricultural run-off
Riet Spruit tributary and Klip River to Vaal River confluence (Region 4)	Mining activity:	East Rand Proprietary Mines (ERPM) gold mine Glen Douglas dolomite mine	Mining activity:	Slime dams (Central Gold Recovery & Ergo Mine), Rock dumps, Old mine waste sites
	Municipal:	Rondebult, Dekema, Vlakplaats and Meyerton WWTPs	Informal settlements:	Central Johannesburg along Main Reef Road in Germiston, Katorus, kwa-Thema and Zonkizizwe
			Municipal:	Leaking sewers in Katorus area
			Industrial:	Village Deep, Alrode and Boksburg, Daleside, Meyerton and ArchelorMittal South Africa. Old Springfield Colliery
			Waste sites:	Henley-on-Klip, Walkerville & Waldrift solid waste sites (active) and Meyindustria solid waste site (closed)
			Other:	Agricultural run-off

1.3.2 Site selection

Of all the compounds investigated by Roos and co-authors (2012) in their WRC study, the PAHs were one of the most abundant. Their report recommended specifically that further investigations were needed at areas where the highest potential risks had been calculated. Soweto and Lenasia was one of these areas, which was also one of the areas with the highest PAH levels (Roos *et al.*, 2012). From the literature on the Klip River catchment—indicating the urbanisation, industrialisation and the pollution sources—as well as the recommendations made by Roos *et al.* (2012), the study area chosen for this project encompassed the greater Soweto and Lenasia area. The chosen study area is formed by Region 1, 2 and part of Region 3 (Figure 1.1). The sampling sites were chosen according to their locations within the study area. They are representative of the drainage area, as they are situated in the upper, middle and lower stretches of the Upper Klip River (from here on only referred to as Klip River) and the Klip Spruit (Figure 1.2).

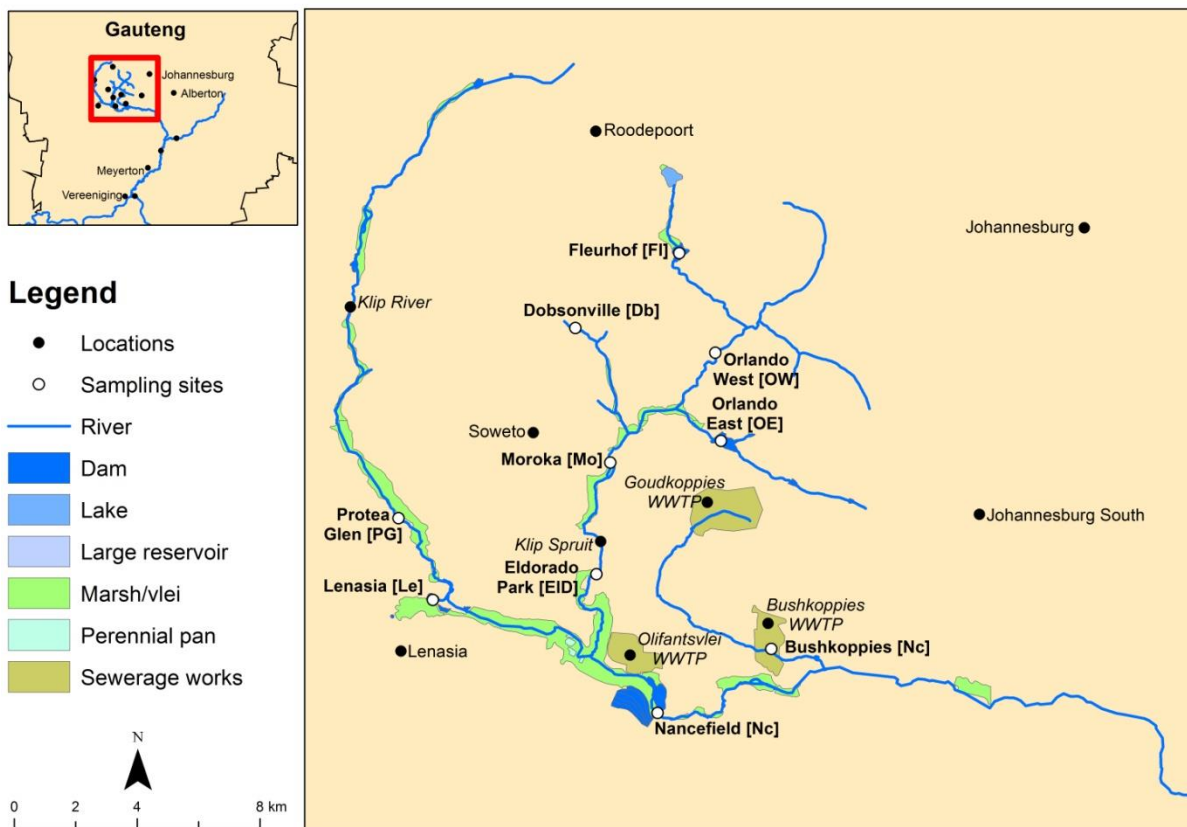


Figure 1.2: Sampling sites within the greater Soweto and Lenasia area

Sediment samples were collected from nine sites within the study area (Table 1.2). Two of these sites formed part of the Klip River upstream of the Klip Spruit confluence. Six sampling sites were located on the Klip Spruit (Region 2) and its smaller tributaries, and one site in region 3 (Figure 1.1). Due to the geographic nature and availability of fish at the sediment sites, fish was sampled only from the sediment sites able to produce fish (Table 1.2). These fish sampling sites were also chosen to represent the different areas within the study area. The Nancefield weir (Nc) (Figure 1.2) was one of these fish sampling sites—representing the farthest downstream area—however after the first

sampling session was unsuccessful an alternative site within the area had to be identified. The closest to the original site where fish could be found was in the Bushkoppies WWTP at the last of the maturation ponds, from where water flows into the Klip River and should be close to the environmental condition (Figure 1.2 & Table 1.2). Potential egg sampling sites were scouted for on foot and after no success aerial reconnaissance was done to locate breeding colonies. After several aerial scouting trips, the only breeding colony within the study area was located in the Lenasia wetland, adjacent to the corresponding fish sampling site (Table 1.2).

Table 1.2: Selected sites in the greater Soweto and Lenasia co-ordinates, matrices sampled, and physical characteristics (2013 in grey and 2014 in white)

Sites	Site codes	Matrices sampled	River/dam characteristics	Flow rate (m/s)	Sediment grain size (%)						%TOC
					>4000 µm Gravel	>2000 µm Very coarse sand	>500 µm Coarse sand	>212 µm Medium sand	>53 µm Fine sand	<53 µm Mud	
Protea Glen 26°15'31.68"S 27°48'45.5"E	PG	Sediment	Deep pool, large rocks. Wetland reeds and riparian shrubs and trees	1	19	8.4	20	38.6	8.7	6.7	3.12
					7.7	12.9	27.9	33.5	10.2	6.1	2.05
Lenasia 26°18'8.33"S 27°50'10.8"E	Le	Sediment, fish and bird eggs	Large dam, forms part of a wetland system. Dam has water grass and weeds	N/A	32.6	4.6	9.1	38.8	5.6	2.6	1.41
					29	12.7	20.1	0	3.9	2	1.84
Fleurhof 26°12'03.49"S 27°54'31.87"E	FI	Sediment and fish	Large dam, weed covered bottom, shore lined with reeds	N/A	0	0.4	18.8	53.2	13.4	7.8	0.42
					3.2	6.7	35.5	38.8	3.5	1.6	1.47
Dobsonville 26°13'22.89"S 27°52'40.35"E	Db	Sediment	Small dam draining into small stream	N/A	2.9	6.9	21.2	43.7	13.7	6.9	1.28
					0	6.4	38.7	68.8	12.9	7.7	1.5
Orlando West 26°13'36.83"S 27°55'26.57"E	OW	Sediment	Relatively fast glides and riffles, followed by deeper glides with large boulders. Riparian zone covered in thick grassland	2.2	1.2	4.1	28.3	38.1	7.4	4.2	0.8
					0.3	8.5	38.4	52.1	8.9	5.1	1.07
Orlando East 26°15'21.63"S 27°55'18.97"E	OE	Sediment and fish	Old power plant reservoir, open areas along the shore line (barren or grass patches), reed beds	N/A	0.5	1.6	37.9	59.6	1.9	0.5	0.53
					0	1.5	10.5	38.1	8.7	3.7	0.71
Moroka 26°15'44.71"S 27°53'17.29"E	Mo	Sediment	Deep fast flowing pools, sandy banks lined with grass and reeds	3.19	0.6	0.5	17.7	0	6	4.9	2.07
					0	0.2	12.2	35	9.8	17.1	0.75
Eldorado Park 26°17'24.27"S 27°53'08.60"E	EID	Sediment	Deep slow stretches linked with faster runs, banks dominated by grass and shrubs	2.68	10.4	14.4	50.9	68.8	3.4	7	2.24
					0	0.3	0.7	53.2	31.1	38.5	1.36
Nancefield 26°19'59.43"S 27°54'11.28"E	Nc	Sediment	Steady flowing, narrow and deep stretch downstream from weir. Rocky banks lined with reeds, veld grass and trees	1.5	8.6	10	22.1	35	13.1	7.6	0.87
					0	3.6	26.3	59.6	10.5	6.5	0.93
26°19'03.13"S 27°56'08.68"E	Nc	Fish	Last ponds of the Bushkoppies WWTP before flowing into the Klip River. Rocky shores lined with trees and veld grass	N/A							

Protea Glen [PG]

The sampling site at Protea Glen is the most western site of the project (Figure 1.3). It is located in the Protea Glen/Naledi residential area. This site is located on the Klip River and represents the most upper part of the Klip (upwards to the origin). Only sediment was collected from this site. The Klip River at PG flows through a large wetland. Sediment was collected where a public road transects this wetland (Figure 1.3A). The river (where sediment was collected) has a slow deep flow (Table 1.3) and the bottom consists of soft mud and large rocks. The riparian zones consisted of mainly wetland reeds, trees and shrubs (Figure 1.3B). Pollution at this site noted included residential waste and litter.

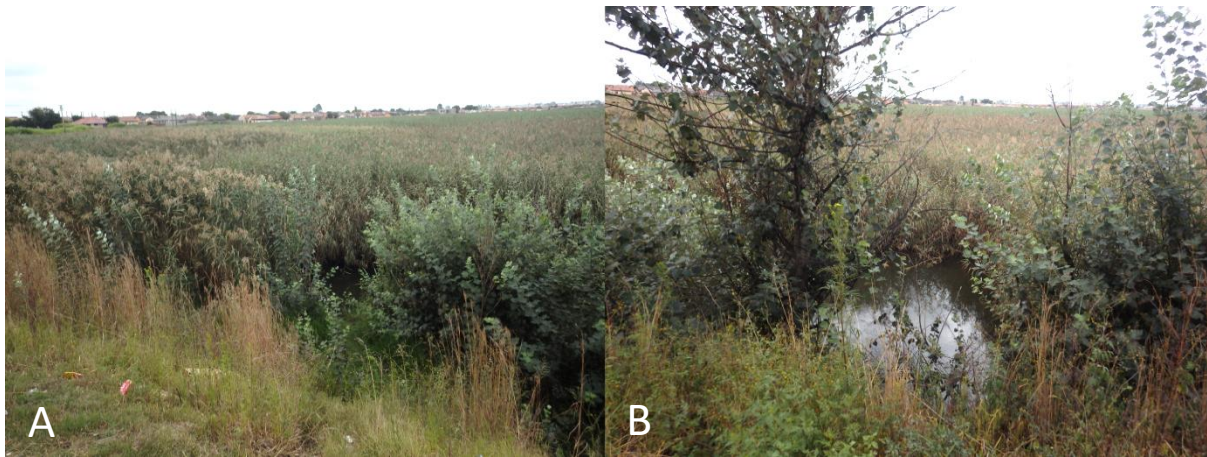


Figure 1.3: Protea Glen sampling site: A) Large wetland at Protea Glen B) Vegetation at sample site

Lenasia [Le]

The Lenasia site, together with Protea Glen forms the western sites. It is downstream from PG (Figure 1.2). At this site sediment, fish, and bird eggs were collected. It was also the only site where eggs were collected. The Lenasia site is located at a dam that forms part of the Lenasia wetland. The Klip River enters this system at the dam and flows through a series of small impoundments and wetland patches before exiting downstream from Lenasia. Fish and sediment were collected from the dam. This dam is a deep impoundment with large patches of water grass and weeds, and most of the shore is covered with reeds (Figure 1.4A). The sediment of the dam has a granular muddy consistency and was filled with small stones. The heronry where the eggs were collected was around a small opening of water in a dense reed bed, which is connected to the main dam (Figure 1.4B). Notable pollution seen at the site included: domestic garbage, construction rubble, condoms (suggests sewage leakage) and evidence of burnt tyres.

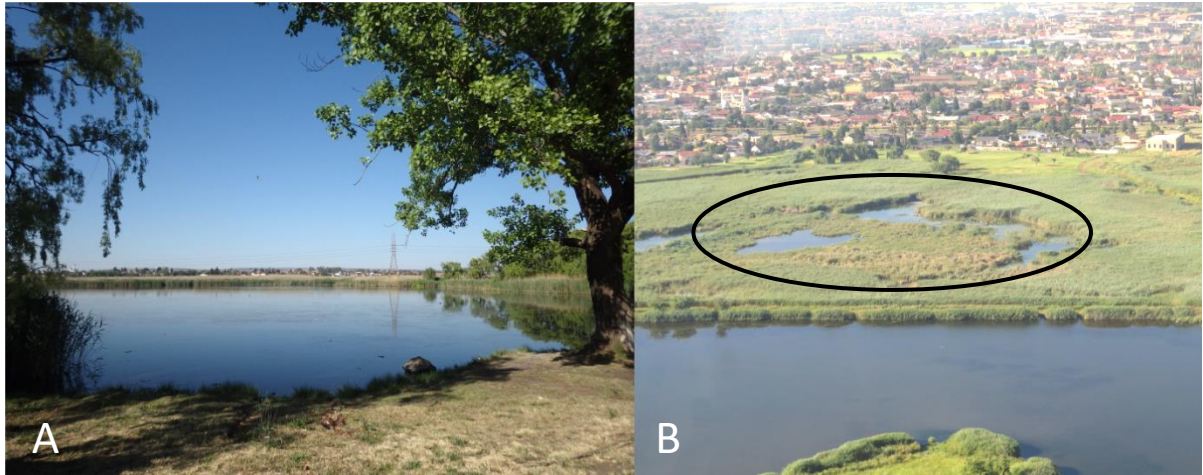


Figure 1.4: Lenasia sampling site: A) View of the dam showing reed beds; B) Heronry adjacent to the main dam (circled area) (Photo B: Henk Bouwman)

Fleurhof [Fl]

Fleurhof Dam is the most northern site of the study area (Figure 1.2), situated in the residential area of Fleurhof, bordering on the industrial areas of Lea Glen, Robertville, and Vogelstruisfontein. The dam is a large impoundment that drains into the Klip Spruit to the south-east. Sediment and fish were collected from this site. The dam had a weed covered bottom and was surrounded by reeds (Figure 1.5A). The sediment collected was sandy. Pollution seen at the site included an informal waste dump and construction rubble. Numerous developments were under construction around the dam (Figure 1.5B), and as the dam is situated lower/downhill of these sites, the run-off from these sites drained into the dam. The western banks of the dam are next to old mine dumps in the Vogelstruisfontein area (Figure 1.5C). Fleurhof Dam is connected to Florida Lake (to the north) by means of a cement channel. The cement was to decrease water seepage into two mine reefs and old mine workings (DWAS, 2010).



Figure 1.5: Fleurhof Dam sampling site: A) Thick reed beds lining the eastern shore; B) Construction sites bordering the dam; C) old mine dumps to the western shore

Dobsonville [Db]

This site is situated to the north west of the Klip Spruit (Figure 1.2) in the residential area of Dobsonville. This site, where only sediment was collected, is in the Dorothy Nyemba Park (maintained

by Johannesburg City Parks). The small dam receives run-off from the surrounding area and the nursery that is located in the park, and then drains into a small stream that flows into the Klip Spruit. The dam is shallow and very small relative to the other sampling dams. The shores of the site were lined with reed beds (Figure 1.6A) and the sediment was firm clay mixed with small rocks. The dam was polluted with municipal garbage littering the edges of the shore and the reed beds (Figure 1.6B).

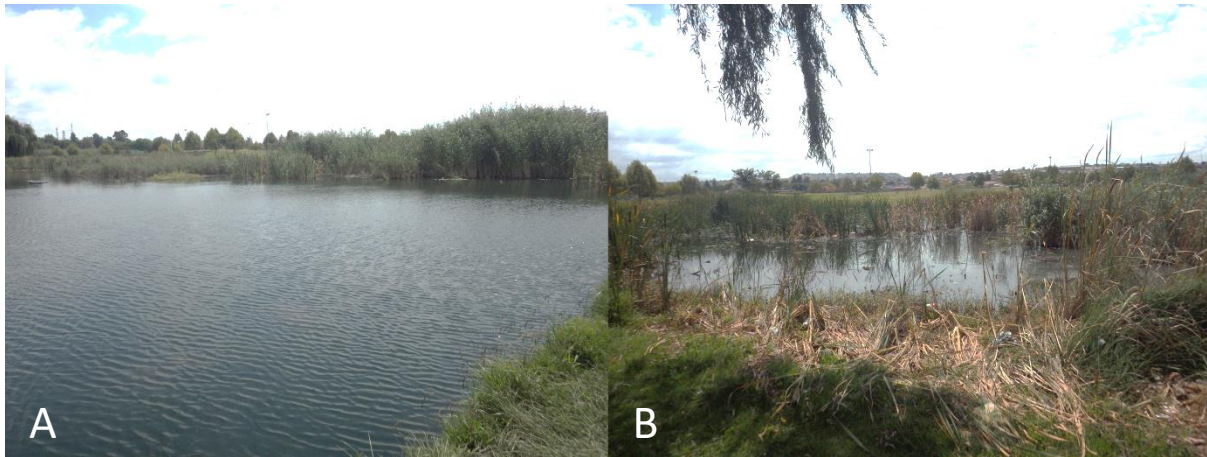


Figure 1.6: Sampling site in the Dorothy Nyemba Park, Dobsonville: A) Dam where sediment was sampled; B) Dam shore and reed beds showing litter

Orlando West [OW]

This is a sediment site directly on the Klip Spruit just below where the stream from Fleurhof drains. It is the most northern sampling site directly on the Klip Spruit (Figure 1.2). It is situated between the residential areas of Orlando East and Meadowlands East. The site is also situated across from Orlando Stadium and flows adjacent to the main road of the area, Klipspruit Valley Drive (Figure 1.7A). On the opposite side of the river is a pre- and primary school. This section of the Klip Spruit flows relatively fast (2.2 m/s) and has deep runs followed by shallower riffles and glides, containing large boulders in the flow (Figure 1.7B). The sediment is soft clay containing sand. The surrounding site is sandy with thick patches of grass lining the banks of the river (Figure 1.7A+B). This site was heavily littered and the water was milky-black with a strong smell of crude oil. A pipe from the residential side of the site pumped water into the river and was possibly a point source of pollution.



Figure 1.7: Orlando West sampling site: A) Klip Spruit opposite to Orlando Stadium, and flowing adjacent to Klipspruit Drive (top background of picture); B) Deep sections and riffles of Klip Spruit

Orlando East [OE]

Orlando East is one of the eastern sites of the project, part of the Klip Spruit tributary (Figure 1.2). This site is located to the south of Orlando East and west of Diepkloof at the iconic Orlando Towers (Figure 1.8A) and is next to Soweto's busy Chris Hani Road. The dam at this site served as part of a spray pond system for the Orlando Power Station together with the cooling towers. This power plant was the first in South Africa to use sewage effluent (from the Klipspruit Sewage Works) as coolant liquid (WISA, 2015). The power plant was decommissioned in 1998. After this the compound was turned into a recreational centre (bungee-jumping at the towers, rowing- and soccer clubs at dam). On 25 June 2014 the main structure of the power station collapsed (Sethusa, 2014). Both sediment and fish were sampled at this site. The shore line of Orlando Dam is lined with reeds. Open areas along the shore are situated at the eastern shore and at the dam wall. The bottom of the dam is mainly sediment with larger rocks and plastic/garbage. The dam is heavily polluted (Figure 1.8B). Pollution included domestic and personal hygiene garbage, litter, construction rubble, and burnt tyres. The stream that flows into the dam receives run-off from informal settlements and is a definite source of pollution for this site (apart from dumping). After the collapse of the power station, a massive fish kill was reported (by locals) (Figure 1.8C+D). The collapse resulted in a high influx of pollutants in the area, the water from the dam turned oily and black. Fish could not be sampled here during the second sampling event.

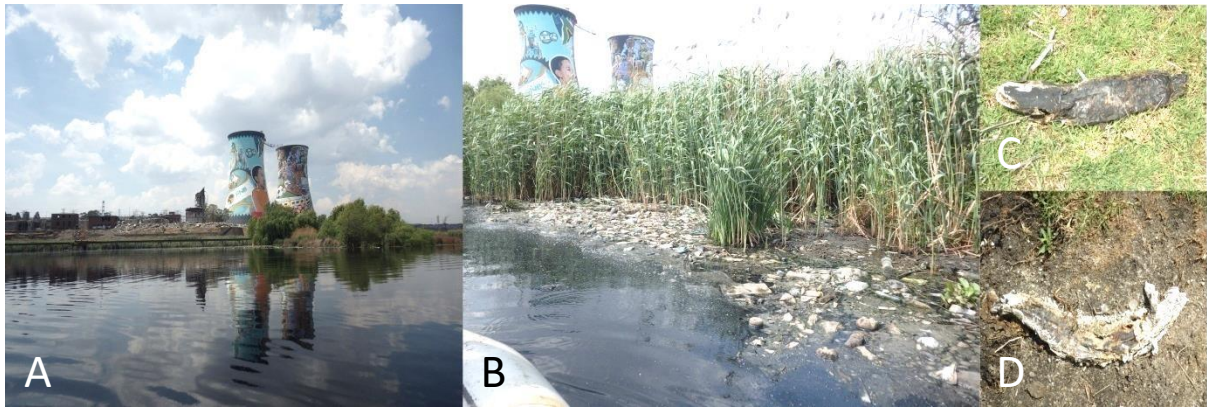


Figure 1.8: Orlando East sampling site: A) The iconic Orlando Towers and collapsed power station; B) Heavy pollution in dam; C+D) *Clarias gariepinus* carcasses after fish kill

Moroka [Mo]

This sediment site is located on the Klip Spruit and represents the central area of the project (Figure 1.2). It is located near the residential areas of Moroka to the west, Molofo South to the north and Pimville to the east. The sampling was done in the river under a bridge next to the Chris Hani Drive and Klipspruit Valley Road intersection. The river at this site is deep and has a flow of 3.19 m/s, before it flows into a wetland farther downstream. The sandy banks of the river are lined with grass and reeds more downstream (Figure 1.9). The sediment sampled was fine and sandy and formed a soft mud. Noted pollution at the site included litter and construction rubble and run-off from urban areas.



Figure 1.9: Sediment sampling site at Moroka located under the Chris Hani Road bridge

Eldorado Park [EID]

Eldorado Park is the most downstream sampling site on the Klip Spruit (Figure 1.2) and is located between Eldorado Park and Klipspruit West. The sampling was done below the bridge of Klipspruit

Valley Road. The vegetation around the site was dominated by grass veld and the banks of the river also had shrubs present. The river flowed at 2.68 m/s and had deep slow stretches and faster runs after formed man-made flow modification (Figure 1.10). The sediment from this site was soft and sandy. The site was polluted with litter and possible run-off from urban and informal settlements.



Figure 1.10: Sediment sampling site at Eldorado Park, photo taken off the Main road bridge

Nancefield and Bushkoppies Waste Water Treatment Plant (WWTP) [Nc]

The sites at Nancefield are the most southern sites of the project (Figure 1.2). This area has two sampling areas because fish could not be found in the weir in the Klip River. Bushkoppies WWTP (operated by Joburg Water) was chosen as a substitute for fish sampling as it is in the same vicinity as the sediment sampling site and it drains into the Klip River just downstream from the weir. The sediment site on the Klip River is situated next to the Golden Highway in the Nancefield area and is downstream from a wetland and the Olifantsvlei WWTP. The river is narrow and deep downstream from the weir (Figure 1.11A) and becomes shallow and fast downstream from the bridge (Figure 1.11B). Here the stream flows at 1.5 m/s and has large boulders in the granular yet muddy sediment. The area where sediment was sampled is directly downstream of a demolished bridge (Figure 1.11B). The rocky bank of the river is lined with reeds (Figure 1.11A), grass and trees (Figure 1.11B). Although litter was not prominent, this site is used by locals for various religious ceremonies and is also a local fishing spot.



Figure 1.11: Sediment sampling site at Nancefield: A) Downstream from the weir; B) Downstream from the collapsed bridge

The fishing site at the Bushkoppies WWTP was strategically chosen as the most downstream sampling site. The complex is located next to the N1, N12 and Golden Highway, in the Nancefield district. To eliminate the potential influence of the plant, only fish was collected from the last set of ponds on the property that drained into the Klip River. These dams were deep and had rocky shores lined with Sweet thorn trees, grass and patches of reeds (Figure 1.12). The visible pollution at this site was minimal as it is a restricted area.



Figure 1.12: Fish sampling site at final ponds of the Bushkoppies WWTP

1.4 Abiotic and biotic matrices collected

1.4.1 Abiotic matrix

Use of sediment as abiotic matrix

The use of sediment quality for evaluating the levels of contaminants is necessary as it assists in assessing potential toxicity in a system (Chakravarty & Patgiri, 2009). Numerous studies have investigated environmental health by studying the concentrations of pollutants in the sediment (Angulo, 1996; Atgin *et al.*, 2000; Meybeck *et al.*, 2004; Nieuwoudt *et al.*, 2009). Organic pollutants in aquatic ecosystems generally exist in low levels in water (Morillo *et al.*, 2009) and accumulate in the sediments (Reid *et al.*, 2000; Zhang *et al.*, 2003). Sediment has a long residence time in the aquatic systems (Saha *et al.*, 2001; Varol, 2011). Due to their variable physical and chemical properties, sediments are important sources for organic and inorganic pollutants (Praveena *et al.*, 2008). Thus because sediments are ideal sinks for PAHs and other pollutants discharged into the environments, they can reflect on the input of point- and non-point sources (Hahladakis *et al.*, 2013; Yohannes *et al.*, 2013). During favourable conditions, sediments play a functional role in the mobilization of contaminants in aquatic systems (Eggleton & Thomas, 2004; Li *et al.*, 2016). In riverine communities, the population is directly and indirectly exposed to sediment and so the pollutants, and are at risk of contamination (Miller *et al.*, 2004). Thus it is important to evaluate the pollution status of the sediment.

Sampling and processing of sediment

Surface sediment samples were collected at the nine identified sites (Figure 1.2) during low flow conditions (June/July) during 2013 and 2014. Three sediment subsamples were collected in a 5 m range at each site using pre-cleaned stainless steel hand shovels. A composite sample was prepared by thoroughly mixing the subsamples in a pre-cleaned stainless steel bowl before storing it in high density polyethylene (HDPE) bottles, pre-cleaned (rinsed thrice with first acetone and then hexane) according to USEPA method 1613 (USEPA, 1994). The samples were protected against microbial- and UV degradation by transporting at 4°C and storing at -20°C in the laboratory. The samples were air dried in the absence of light, ground, and sieved (0.5 mm mesh size) to obtain homogenous samples (Kralik, 1999).

To determine the physical characteristics of the sediments, total organic carbon and grain size was determined. The loss-on-ignition method (Schumacher, 2002) was done on a TruSpec CN analyser to determine the total organic carbon. The different sediment grain sizes was partitioned using a series of sieves (4 000, 2 000, 500, 212, 106, 53 µm) (ISO, 2002).

1.4.2 Biotic matrices

Fish used as biotic matrix for environmental studies

Fish are an ideal biotic matrix of aquatic environment studies, as they are represented in various trophic levels in aquatic food webs (Kidd *et al.*, 2001). Biota act as bio-indicators—which are groups or individual organisms that are used to describe the quality of an ecosystem, depending on their abundance or well-being (Gerhardt, 2002). Disturbances in the lower levels will affect the apex predators, as they feed on prey in lower levels of the food web (Kidd *et al.*, 2001). Fish have been used in numerous studies as biotic matrix, investigating organic pollutants (Vives & Grimalt, 2002; Weber & Goerke, 2003; McHugh *et al.*, 2011; Wepener *et al.*, 2011). The sharptooth catfish (*Clarias gariepinus*) is an opportunistic omnivorous bottom-feeder and have also been found to be intentional detritus feeders, but are known as formidable predators, as well as a hardy and resilient fish, surviving harsh conditions (Skelton, 2001).

Clarias gariepinus was chosen as an indicator species because of its abundance in South Africa, their hardiness and because they are an apex predator. Their position on the food web and its preference for bottom-dwelling in the aquatic systems makes it ideal to study exposure to pollutants, as well as the bio-accumulation and bio-magnification of organic chemical pollutants. These fish are also a valued food source, allowing for investigation in possible transfer of pollutants to humans.

Use of bird eggs as biotic matrix for environmental studies

Birds are also popular biotic matrices for environmental studies and have been used in various studies investigating organic pollution (Custer *et al.*, 2001; Barnhoorn *et al.*, 2009; Pereira *et al.*, 2009; Quinn *et al.*, 2013; Khan *et al.*, 2014). Piscivorous birds represent a different trophic level than fish, and because organic pollutants bio-accumulate and bio-magnify within food webs (Zhou *et al.*, 2006; Antoniadou *et al.*, 2007; Herbert *et al.*, 2011), they can show the trophic transfer between different biotic matrices (fish to birds). The use of bird eggs specifically is considered to be a better matrix than the adult organism itself. Eggs are easy to handle and uses a relatively fast and non-invasive method of collection (Medvedev & Markova, 1995). The eggs are representative of the female parent—as contaminants is transferred from the parent bird to her lipophilic eggs (Van den Steen *et al.*, 2006; Verreault *et al.*, 2006)—reflecting the pollutant body burden of that female parent (Braune, 2007). Wetland birds were chosen as bio-indicators for this study because they are exposed to pollutants from their feeding regimes, their direct habitat selection (aquatic systems) and breeding behaviours. Various types of wetland birds occur in the study area. The only heronry identified in the study area was at Lenasia. Individual target species were not identified beforehand and the species present at the heronry were sampled.

1.4.3 Sampled biota

Clarias gariepinus

Air breathing catfish, from the family Clariidae, form part of one of the world's most abundant fish types (order Siluriformes) (Bruton, 1988; Skelton, 2001). *Clarias gariepinus* is found throughout Africa and parts of Asia and is the most widely spread freshwater species in the world (longitudinally) (Bruton, 1988; Skelton & Teugels, 1992). Its distribution in Africa ranges from as far north as the Nile River and as far south as the Orange- and Umtamvuna systems. In South Africa it has been translocated to the Eastern Cape (Sundays-, Fish- and Keiskamma Rivers) and the Western Cape (Cape Flats and Clanwillam-Olifants River) (Skelton, 2001).

Clarias gariepinus is a dorso-ventrally flattened fish; the body is compressed towards the tail and depressed at the head (Figure 1.13). The dorsal fin extends from behind the head to near the base of the caudal fin. The anal fin extends from the base of the anus to the base of the caudal fin. This fish's fins are mainly of soft rays with exception to the pectoral fins that have a spine with barbs on the outer edge only (Figure 1.13) (Skelton & Teugels, 1992; Skelton, 2001). The eyes are in a super-lateral position and are relatively small (Froese & Pauly, 2015). The head has 4 pairs of long filamentous barbels (Figure 1.13). Both jaws are armed with broad bands of fine sharp teeth. Skin colour varies from black to light brown, often marbled in shades of olive green and grey. The ventral sections of the head and abdomen are white and sometimes have a red flush on the edges of the fins, especially during spawning. *Clarias gariepinus* can attain lengths up to 1.4 m standard length (SL; which is the length from tip of snout to mid-base of caudal fin) (Skelton & Teugels, 1992; Skelton, 2001). The presence of an accessory breathing organ enables *C. gariepinus* to breathe air by gulping air with its mouth. It does this when very active or during dry conditions. *Clarias gariepinus* occur mainly in quiet waters such as lakes and pools but may venture into fast flowing rivers and into rapids. It is tolerant to extreme environmental conditions such as low oxygen concentrations and poor water quality (Froese & Pauly, 2015).

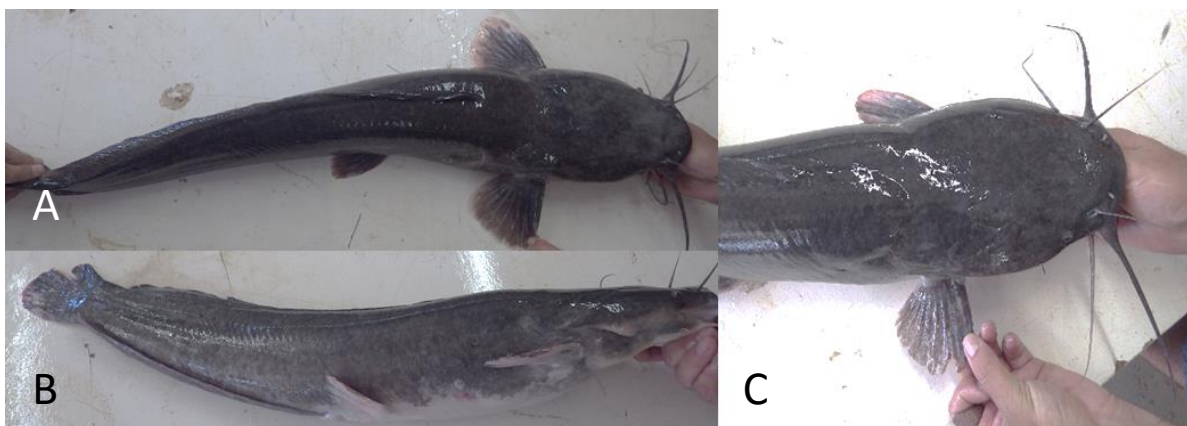


Figure 1.13: Example of an African sharp-toothed catfish (*Clarias gariepinus*) male (2.5 kg; 700 mm TL) A) Dorsal view, B) Ventral view C) view of head view showing the eyes, barbels and spinous pectoral fin

Clarias gariepinus is completely omnivorous, it preys and scavenges on any available organic food source including fish, birds, frogs, small mammals, reptiles, molluscs, crustaceans, seeds, fruit and even plankton. They may hunt in packs, herding and trapping small fish in shallower water. *Clarias gariepinus* breeds in the summer after rain showers when large sexually mature adults migrate to flooded shallow grassy borders of the dam or river (Skelton, 2001).

Sampling of fish

Clarias gariepinus was sampled during the high flow seasons (October) of both 2013 and 2014. Fish were sampled using gill nets (118 and 150 mm) and, rod and line. The gill nets were checked every two hours and caught fish were kept in an aerated container until field analysis was performed. The fish ($n = 20$; 1:1 F:M) used for the control were sampled from various other sites in the Vaal River basin (Boskop Dam, Klerkskraal Dam and Vaal River) and were kept at the Water Research Group aquarium at the NWU (Potchefstroom Campus) for 6 months. Fish were kept in 5000 L recirculating tanks with added aeration. They were kept in aquarium standard water and temperature (CCAC, 2005). The water was replaced bi-weekly.

Herons and egrets

Herons and egrets are non-swimming water birds belonging to the Ardeidae family. These birds are described as tall, slender birds with long legs and -necks (Sinclair *et al.*, 2011). They have dagger-like bills and are mostly aquatic hunters, relying on stealth and occasionally by means of active pursuit (Sinclair *et al.*, 2011). Two species from the family Ardeidae was found at the sampling area: the black-headed heron (*Ardea melanocephala*) (Figure 1.14A) and the cattle egret (*Bubulcus ibis*) (Figure 1.14B).

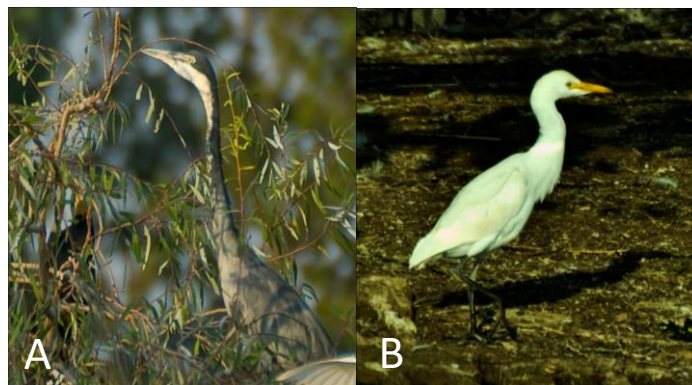


Figure 1.14: Example of herons and egrets of this study: A) a black-headed heron (*Ardea melanocephala*) perched in a tree above a water body; B) a cattle egret (*Bubulcus ibis*) foraging a pond shoreline (Photos: Ig Viljoen)

Ardea melanocephala is common and widespread throughout sub-Saharan Africa. They are found in various habitats such as marshes and floodplains, although they are not depended on them (Hockey *et al.*, 2005; Sinclair *et al.*, 2011). They are solitary hunters, mainly feeding on terrestrial- and aquatic

invertebrates, fish, reptiles, small birds and- mammals (Hockey *et al.*, 2005; Birdlife International, 2015a). Breeding takes place throughout the year with seasonal peaks during the wet months (Hockey *et al.*, 2005; Tarboton, 2011). They breed alongside other species and build their nests to the central, highest points in the heronries, above the smaller species (Hockey *et al.*, 2005; Tarboton, 2011) in large reed beds above water and less often in trees (Tarboton, 2011).

Bubulcus ibis is widely spread throughout South Africa. It is a common resident of open habitats such as grasslands, pastures and agricultural lands (Hockey *et al.*, 2005; Sinclair *et al.*, 2011). They are gregarious birds flocking near watering places before flying to their roosts after sunset. They roost communally with other water birds. The main diet of *B. ibis* consists of insects, fish, amphibians, reptiles and small rodents and nestlings (Hockey *et al.*, 2005; Birdlife International, 2015b). *Bubulcus ibis* often associates itself with larger animals such as livestock and antelope standing on their backs (Hockey *et al.*, 2005; Sinclair *et al.*, 2011). The advantage of this is that they can forage in long grass where visibility is poor as well as prey on the ecto-parasites of the mount (Hockey *et al.*, 2005; Sinclair *et al.*, 2011). *Bubulcus ibis* is a colonial monogamous breeder either nesting in monospecific colonies or mixed species heronries, far outnumbering the other species (Hockey *et al.*, 2005; Tarboton, 2011). Colonies are often over water where they construct their loosely made nests in reeds or overhanging trees from dry twigs, weeds and reed stems (Tarboton, 2011).

Ibises

Ibises are a group of partly aquatic birds belonging to the family Threskiornithidae. Ibises are fairly large, long-legged birds with down curved bills (Hockey *et al.*, 2005; Sinclair *et al.*, 2011) used to probe mud and soft soil for food (Hockey *et al.*, 2005). Two species from the Threskiornithidae family were found breeding at the bird egg sampling site: the glossy ibis (*Plegadis falcinellus*) (Figure 1.15A) and the African sacred ibis (*Threskiornis aethiopicus*) (Figure 1.15B).

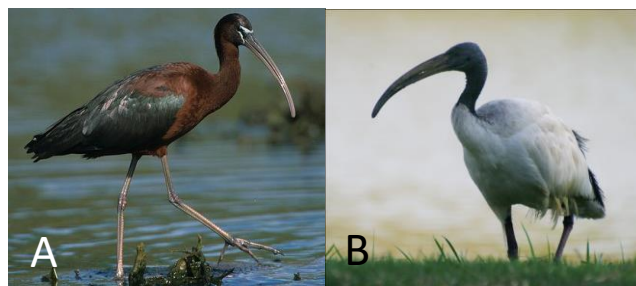


Figure 1.15: Example of ibises: A) a glossy ibis (*Plegadis falcinellus*) foraging in the shoreline; B) a sacred ibis (*Threskiornis aethiopicus*) foraging on bank next to a water body (Photo A: Richard Brooks; Photo B: Ig Viljoen)

Plegadis falcinellus is distributed all over the world. In South Africa their distribution is centred on the central Highveld, and isolated resident populations are found in Kwa-Zulu Natal and the Western Cape (Hockey *et al.*, 2005). This ibis prefers aquatic habitats with muddy substrata such as shallow freshwater water bodies and floodplains, riparian marshes as well as man-made dams, sewage works, irrigated farmland and open grasslands of farms and parks (Hockey *et al.*, 2005; Birdlife

International, 2015c). *Plegadis falcinellus* forages in flocks in shallow water or on soft ground. Their main diet consists of adult and larval insects, molluscs and crustaceans and less frequently small fish, frogs, lizards and small mammals (Hockey *et al.*, 2005). *Plegadis falcinellus* is a monogamous breeder that nests in colonies with other species (Hockey *et al.*, 2005). Its nesting is dispersed rather than clustered within these colonies. They construct their nests in large undisturbed reed beds over water, and sometimes in flooded trees from reeds stems to form a platform (Tarboton, 2011).

Threskiornis aethiopicus is found throughout sub-Saharan Africa and in South Africa throughout the country, especially in high densities in the moist eastern high altitude plateau. The provision of permanent water bodies however has facilitated an increase in numbers in the western part of South Africa (Hockey *et al.*, 2005, Birdlife International, 2015d). The sacred ibis prefers margins of inland freshwater wetlands, specifically the shallows, but also inhabit open grasslands and agricultural lands. *T. aethiopicus* has adapted to the presence of man, by taking advantage of man-made habitats including farm dams, sewage works, abattoirs, refuse tips and farmlands (Hockey *et al.*, 2005).

They eat a great variety of prey, from insects (aquatic and terrestrial), crustaceans, worms and molluscs. They also feed on vertebrates such as fish, frogs, reptiles and small mammals and nestlings. *Threskiornis aethiopicus* is a monogamous breeder that nests colonially in mixed species heronries (Hockey *et al.*, 2005; Tarboton, 2011). Within these heronries they nest in close proximity of one another, but to the fringes of the colony in discrete groups (Hockey *et al.*, 2005). Sacred ibises will nest in reed beds and trees over water, and often on the ground on isolated islands (Tarboton, 2011).

Sampling of bird eggs

As previously mentioned, the only heronry found in the study area was located in the Lenasia wetland. Water bird eggs (n = 10 per species) were sampled from the nest in the heronries (accessed by boat) during the summer breeding season (November/December) of 2013. The largest egg was collected (assuming it was the first laid) from each nest and marked with a pencil. The eggs were wrapped in pre-cleaned aluminium foil (rinsed thrice with first acetone and then hexane) and also marked on the foil. The eggs were transported in at 4°C and stored at -20°C in the laboratory.

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Chapter 2: Polycyclic aromatic hydrocarbons in sediment and biota from Soweto and Lenasia, South Africa

2.1 Introduction

2.1.1 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds consisting of only fused aromatic rings, without functional groups or heteroatoms (referred to as native PAHs) (Sims & Overcash, 1983; Angerer *et al.*, 1997; Stogiannidis & Laane, 2015) arranged in clustered, angular or linear formations (Table 2.1) (Nadal *et al.*, 2004). They are omnipresent in the environment and according to Neff *et al.* (2005) are major contributors to detrimental effects in aquatic systems. There are 660 native PAHs listed and from these the USEPA (2008) has identified 16 priority PAHs for regulation and priority monitoring in environmental quality (Achten & Hofmann, 2009; Zhang & Tao, 2009). The priority PAHs are naphthalene, acenaphthylene, acenaphthylene, fluorene, phenanthrene, anthracene, pyrene, fluoranthene, chrysene, benz(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(g,h,i)perylene, indeno[1,2,3-cd]pyrene, and dibenz(a,h)anthracene (USEPA, 2008) (Table 2.1).

2.1.2 Physical and chemical characteristics of polycyclic aromatic hydrocarbons

As these compounds are part of a very large group, they often differ from one another in physical and chemical characteristics based on their molecular mass as well as the number of aromatic rings they consist of (CCME, 2008). The PAHs all have a high molecular mass with low volatility and the group is regarded as semi-volatile (Ollivon *et al.*, 1999) (Table 2.1). Polycyclic aromatic hydrocarbons are lipophilic with a high affinity for organic matter (Brenner *et al.*, 2002; Morillo *et al.*, 2007) rather than dissolving in water (Bertilsson & Widefalk, 2002). Although PAHs have high molecular masses, the group of congeners with 2 or 3 rings is referred to as low molecular mass PAHs (LPAHs) (relative to other PAHs), and the high molecular mass PAHs (HPAHs) is the 4–6 ring congeners (USEPA, 2008) (Table 2.1). The LPAHs tend to be more water soluble, but as the number of rings and molecular mass increase the hydrophilicity and mobility decrease (Iqbal *et al.*, 2008). The HPAHs have higher hydrophobicity and lipophilicity as well as increased boiling- and melting points (Haritash & Kaushik, 2009). The high rate of release of these compounds (Boström *et al.*, 2002; Haritash & Kaushik, 2009; Maliszewska-Kordybach *et al.*, 2009) (Table 2.1) and lipophilicity allow them to become resistant to degradation in the environment (as indicated by the K_{ow} values; Table 2.1). Although the PAHs are not classified as persistent organic pollutants, their great volumes of release (Zhang & Tao, 2009) and variety of sources (Maliszewska-Kordybach *et al.*, 2009) allow them to become widespread at high concentrations, and because they degrade slowly under natural conditions—even slower in anoxic and low light conditions (Ahrens & Depree, 2010)—PAHs are occasionally classified as pseudo-persistent.

Table 2.1: Physical and chemical characteristics of the 16 priority PAHs (Table adapted from USEPA, 2008; Neff *et al.*, 2005; Lee & Vu, 2010; Stogiannidis & Laane, 2015)

PAH	Structure	Abbreviation	Rings	Molecular mass	Water solubility (mg/L)	Vapour pressure (Pa)	Boiling point (°C)	LogK _{ow}	LogK _{oc}	Carcinogenicity
Naphthalene		Nap	2	128.17	31	11	218	3.37	3.29	NC
Acenaphthylene		Acey	3	152.2	3.9	9.0×10^{-1}	280	4.1	3.16	NC
Acenaphthene		Acea	3	152.22	3.9	3×10^{-2}	279	3.9	3.94	NC
Fluorene		Flu	3	166.2	1.9	9×10^{-2}	295	4.18	4.13	NC
Phenanthrene		Phe	3	178.2	1.1	2×10^{-2}	340	4.57	4.49	NC
Anthracene		Ant	3	178.2	0.05	1×10^{-3}	342	4.54	4.45	NC
Pyrene		Pyr	4	202.3	0.13	6×10^{-4}	393	5.18	4.83	NC
Fluoranthene		Fla	4	202.26	0.26	1.2×10^{-3}	375	5.22	4.99	WC
Chrysene		Chr	4	228.28	0.002	1.4×10^{-6}	448	5.86	5.61	C
Benz(a)anthracene		BaA	4	228.29	0.009	2.8×10^{-5}	400	5.6	5.57	C
Benzo(b)fluoranthene		BbF	5	252.3	0.0014	6.7×10^{-5}	481	5.8	6.16	C
Benzo(k)fluoranthene		BkF	5	252.3	0.0007	5.2×10^{-8}	480	6	6.18	C
Benzo(a)pyrene		BaP	5	252.3	0.003	7×10^{-7}	496	6	6	SC
Benzo(g,h,i)perylene		BgP	6	276.34	0.00026	1.4×10^{-8}	550	7.1	6.39	NC
Indeno[1,2,3-cd]pyrene		InP	6	276.3	0.00019	1.3×10^{-8}	536	6.6	6.6	C
Dibenz(a,h)anthracene		DBA	6	278.35	0.0005	3.7×10^{-8}	524	6.5	6.59	C

NC = Non-carcinogenic; C = Carcinogenic; WC = Weak Carcinogenic; SC = Strong Carcinogenic

2.1.3 Sources of polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons have both man-made and natural sources (Stogiannidis & Laane, 2015), however their release from anthropogenic activities is one of the most important environmental pollution sources (Van Metre *et al.*, 2000).

The widespread occurrence of PAHs is largely due to their formation and release in all processes of incomplete combustion of organic materials or high pressure processes (Gehle, 2009). This includes the production of coke fuel and carbon, coal power plants, petroleum processing, furnaces, fireplaces, gas and oil burners, and automobile sources and petroleum products (Angerer *et al.*, 1997; Maliszewska-Kordybach, 1999; Yunker *et al.*, 2002). Anthropogenic PAHs originate from two distinct processes namely pyrogenic and petrogenic sources.

Pyrogenic PAHs are formed during the combustion of biomass (coal and petroleum, wood, and grass, and industrial waste) (Liu *et al.*, 2009; Chen & Chen, 2011; He *et al.*, 2014) in oxygen depleted and high temperature conditions (Saber *et al.*, 2006). The HPAHs (4–6 ring PAHs) dominate the pyrogenic PAHs (Neff *et al.*, 2005; Chen & Chen, 2011; Stogiannidis & Laane, 2015). Industrial processes such as power generation (Donahue *et al.*, 2006; Li *et al.*, 2014), coal mining (Pies *et al.*, 2007), metal smelting (Næs & Oug, 1997; Booth & Gribben, 2005) and industrial waste removal (Domeño & Nerín, 2003) are major sources of pyrogenic PAHs. The most abundant PAHs released from pyrogenic sources are fluoranthene, pyrene and to a lesser degree phenanthrene (Page *et al.*, 1999). Carcinogenic PAHs (benzo(a)pyrene, benz(a)anthracene and benzo(b)fluoranthene) with pyrogenic origins are mainly released by motor vehicles (Dickhut *et al.*, 2000; Van Metre *et al.*, 2000; Yadav *et al.*, 2010). Petrogenic sources predominantly consist of 2 and 3 ring PAHs (LPAHs) (Neff *et al.*, 2005; Chen & Chen, 2011; Stogiannidis & Laane, 2015). Petrogenic PAHs are defined as the congeners that originate from petroleum products, including crude oil, petrol and diesel fuels, lubricants and their derivatives (Angerer *et al.*, 1997; Maliszewska-Kordybach, 1999; Yunker *et al.*, 2002; Saber *et al.*, 2006). The PAH profile of different types of petroleum products vary depending on their production process (Stout *et al.*, 2001), for example, fuel with a lighter mass (jet fuel) contain more LPAHs and the heavier fuels, more HPAHs, due to the distillation temperatures and the PAHs' boiling points and vapour pressures (Table 2.1). Apart from spillage and runoff, a major source of petrogenic PAHs is incomplete combustion of fuels. A significant amount of fuel is not ignited during pyrolytic processes (vehicles and combustion engines) (Van Metre *et al.*, 2000; Bucheli *et al.*, 2004)

2.1.4 Environmental fate

After PAHs are released from their various sources they spread into the environment due to their semi-volatility and they can disperse between air, water, soils and sediments (Nadal *et al.*, 2004). The PAHs that finally deposit into the aquatic environment, bind to the sediment (see section 2.1.2). Here they are subjected to various degradation processes: chemical-, photochemical- and biological. These degradation processes include volatilisation, dissolution, emulsification, microbial breakdown, chemical- and photo-oxidation (Page *et al.*, 1996; Brenner *et al.*, 2002; Warren *et al.*, 2003). Once the

degradation processes are active, the physico-chemical properties of the congeners are changed (Kochany & Maguire, 1994; Page *et al.*, 1996). Biological degradation of PAHs seem to be the main pathway of breakdown in sediments and soils (Wilson & Jones, 1993; Lu *et al.*, 2012).

2.1.5 Toxicity

PAH exposure is a danger to wildlife and humans as these compounds are known to be mutagenic and carcinogenic (NTP, 2005). Studies specifically on humans in the workplace where PAH releases are high (industries) or occupations where the products are themselves PAH sources (petroleum- and tar industries) have found that the PAHs enter the body via different exposure pathways and that accumulated PAHs may have detrimental effects (Väänänen *et al.*, 2005; Cirla *et al.*, 2007; McClean *et al.*, 2007). Harmful effects include developmental and reproductive defects, cytotoxicity (i.e. erythrocyte damage), DNA mutation and other health impacts (Zhang & Tao, 2009; Safe *et al.*, 2010; USEPA, 2011). Apart from the before mention effects some of the PAHs are also known carcinogens (Myers *et al.*, 1994; Savinov *et al.*, 2003; Qiao *et al.*, 2006) and according to Elmore & Boorman (2013) PAHs such as benzo(a)pyrene and dibenz(ah)anthracene, are classified as a genotoxic carcinogens—chemicals capable of producing cancer by directly altering cellular genetic material. The International Agency for Research on Cancer (IARC) has classified the carcinogenic PAHs (Table 2.1) into classes based on carcinogenicity: benz(a)anthracene, benzo(a)pyrene and dibenz(ah)anthracene are classified as Group 2A carcinogens (probably carcinogenic to humans), while benzo(b)fluoranthene, benzo(k)fluoranthene, chrysene and indeno(1,2,3-cd)perylene are part of Group 2B carcinogens (possibly carcinogenic to humans) (OEHHA, 2001).

2.1.6 Sediment toxicity evaluation

Polycyclic aromatic hydrocarbon sediment quality guidelines

The assessment of a system's pollution status is achieved by means of various methods and indices based on specific guidelines. Indices are sets of aggregated and measured parameters or indicators (OECD, 2003), that are used to compare results indiscriminately between one another. Quality guidelines are sets of values that act as goals for environmental quality. These quality guidelines often have values that are specific for the purpose of the guideline, i.e. aimed at specific compounds and end points (protection of ecosystems, benthic organisms, and aquatic life, amongst others) (Swartz, 1999; MacDonald *et al.*, 2000).

Currently South Africa does not have any sediment quality guidelines, therefore the sediment quality guidelines used to evaluate the PAH contamination in the present study are those of MacDonald *et al.* (2000) and the Canadian sediment quality guidelines from the Canadian Council of Ministers of the Environment (CCME) (2001). The MacDonald *et al.* (2000) guidelines are consensus based sediment quality guidelines calculated on the PAHs' toxicity to sediment dwelling organisms and based on a three step evaluation. The first step was the matching of biological effects and sediment chemistry from published research. Secondly, each of these sediment samples' measured compounds was

compared to its corresponding existing guidelines, predicting its toxicity. Finally, accuracy of prediction was evaluated by determining if the sediments were toxic to one or more aquatic organisms, using standardised toxicity tests (MacDonald *et al.*, 2000). If a response was significantly different from a reference or control then the concentration in the sediment responsible for the response was regarded as toxic. Simultaneously, if sediments did not cause a significant response in at least one test endpoint the concentrations in the sediments were considered non-toxic. The threshold effects concentrations (TEC; lower values) were set at values where 75% of the sediments were correctly predicted as non-toxic. Similarly, the probable effects concentration (PEC; higher value) was set where 75% of the sediment samples were correctly predicted to be toxic. Thus, the values set out by the guidelines are the TEC, where concentrations below this value is expected not to have harmful effects to sediment dwelling organisms, and the PEC where concentrations above the value are expected to have harmful effects to benthic organisms at a more frequent interval. When the contaminant concentration is between the PEC and TEC guideline harmful effects are expected to occur, depending on the pollutant. These incidence of harmful effects would be greater than that of the TEC and less than the PEC. In the case when a chemical only exceeds the bottom TEC value and falls between the guidelines, interpretation would be that the toxicity toward benthic organisms would increase as the chemical concentration increases (MacDonald *et al.*, 2000).

The Canadian quality guidelines were implemented to protect, sustain and enhance the environment. These guidelines were created using environmental and human health risk protocols, thus are used to protect human and animal health (CCME, 2012). In the case of the Canadian sediment quality guidelines, an interim sediment quality guideline (ISQG; lower value) and a probable effects level (PEL; higher value) for PAHs was created, to evaluate the degree to which adverse effects could occur from exposure to PAH containing sediment and so can be a useful ecotoxicological assessment tool (CCME, 1999; 2012). This set of guidelines was derived on a similar principle as followed by MacDonald *et al.* (2000) that include the combination of both a modification of the National Status and Trends Program (NTSP) approach, as well as the spiked-sediment toxicity test (SSTT). The modified NSTP approach associates filed collected sediment concentrations of each compound measured, with any adverse biological effects observed (compiled in the Biological Effects Database for Sediments, BEDS). The SSTT approach is an independent evaluation of information from toxicity tests completed with spiked-sediments. This approach estimates the concentration of a chemical where adverse effects are not expected.

Sediment indices

The ecological toxicity risk that polluted sediments may pose to benthic organisms can be calculated with the sediment quality guideline index (SQG-I) (Fairey *et al.*, 2001). The quality of the sediment can also be calculated in terms of the target compound or a mixture of compounds by using the sediment quality index (SQI) (Marvin *et al.*, 2003). These tools can then be used to compare toxicity and quality of different sites in a study area in order to determine a status (of wellness) for that ecosystem.

Toxic equivalent quotient calculation

The xenobiotics present in the sediment have the potential to activate specific biochemical pathways in organisms, and through these pathways be toxic to the organism. These may include the inhibition or activation of neurotransmitter enzymes (Ellman *et al.*, 1961; Lionetto *et al.*, 2013), anti-oxidant systems (Lau *et al.*, 2004; Ferreira *et al.*, 2007), and endocrine systems (Jobling & Tyler, 2003; Mills & Chichester, 2005). Another example of such a biochemical pathway is the activation of the aryl-hydrocarbon receptor (AhR), which is a ligand dependant transcription factor that regulates the expression of cytochrome P450 genes, specifically CYP1A1 (Aarts *et al.*, 1995). This enzyme is responsible for the metabolism of the activating xenobiotic. More detail on the mechanism of the transcription of the CYP450s and the AhR will be discussed later (see section 3.1.1). The Ah-receptor only binds to specific compounds—halogenated aromatic hydrocarbons such as the polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs), as well as a selected few PAHs.

Villeneuve *et al.* (2002) derived toxic equivalence factors (TEF) for the PAHs that actively bind to the AhR using the H4IIE-*luc* bioassay. The H4IIE-*luc* bioassay is a rapid and quantitative biological system that functions on AhR binding, thus responses indicate presence of AhR ligands. When compared to the most responsive compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) of known concentration, toxic equivalence can be calculated for the unknown sample (Hilscherova *et al.*, 2000) or for compounds like Villeneuve and co-authors (2002) did for PAHs (see section 3.1.3). Using the TEF values and measured concentrations of the AhR-ligand compounds, the toxic equivalent quotient (TEQ), which can be compared to official guidelines, can be calculated. There are international guidelines available for TEQs and since South Africa does not have TEQ guidelines the Canadian interim sediment quality guidelines (ISQG) for dioxin-like compounds were used in the assessment. This guideline was specifically created for the protection of aquatic life from dioxin-like compounds, using the TEF values for fish (CCME, 2001), and since PAHs use the same mechanism of action, it was used to evaluate PAH toxicity.

Along with the TEF values derived by Villeneuve *et al.* (2002), which are expressed in terms of 2,3,7,8-TCDD, another set of TEF values exists. These were specifically created to express PAH toxicity in terms of the most toxic PAHs, benzo(a)pyrene (Delistraty, 1997; Tsai *et al.*, 2004). There are, however, no guidelines for BaP-equivalency, but it can be used to compare to BaP equivalencies in published literature to the results of the present study.

In conjunction with the TEF values discussed above, a TEQ can be calculated to express a toxic potency specifically toward fish. Barron *et al.* (2004), derived fish potency factors (FPF) from published data on CYP1A induction and AhR binding. Calculating a TEQ using these potency factors can be used to specifically evaluate risk towards fish in a system. TEQs calculated with Villeneuve *et al.* (2002) TEFs represent toxicity towards a broader range of aquatic life. The TEQs calculated using

Barron *et al.* (2004) and Villeneuve *et al.* (2002) factors are comparable, because 2,3,7,8-TCDD was used as the most potent AhR-ligand, and the FPFs were derived accordingly (Barron *et al.*, 2004).

2.1.7 Polycyclic aromatic hydrocarbon source diagnostic ratios

Possible sources of PAHs can be identified by using diagnostic ratios. These ratios are determined by using the concentrations of specific congeners in a sample relative to one another. Low molecular polycyclic aromatic hydrocarbons (LPAHs) congeners with 2 and 3 rings are mostly released by petrogenic sources, while pyrogenic sources are dominated by 4–6 ring congeners (HPAHs). Thus, the ratio between the LPAHs and HPAHs can be used to identify pyrogenic or petrogenic sources (Scolo *et al.*, 2000). The ratio between anthracene and phenanthrene (Ant/Ant+Phe) can also distinguish between pyrogenic and petrogenic sources (Pies *et al.*, 2008; Chen & Chen, 2011). The nature of the pyrogenic source, i.e. identifying whether the combustion fuel was petroleum or biomass (grass, coal, wood), can further be classified using these congener ratios: Fla/Fla+Pyr (Yunker *et al.*, 2002; Liu *et al.*, 2009), BaA/BaA+Chr (Yunker *et al.*, 2002; Raza *et al.*, 2013) and InP/InP+BgP (Yunker *et al.*, 2002; Maliszewska-Kordybach *et al.*, 2009).

2.2 Materials and Methods

2.2.1 Sample collection

Sediment samples were collected from the nine sediment sampling sites proposed for this project (Figure 1.2) during low flow conditions (June/July) in 2013 and 2014. These sites are Protea Glen (PG), Lenasia (Le), Fleurhof (Fl), Moroka (Mo), Eldorado Park (EID), Orlando West (OW), Orlando East (OE), Nancefield (Nc) and Dobsonville (Db). Fish were sampled at the four fish sites in the high flow season (October) in 2013 and 2014 at Lenasia, Fleurhof, Nancefield (Bushkoppies) and Orlando (East). Bird eggs were only sampled at Lenasia (November/December 2013).

2.2.2 Chemical extraction procedure

Sediment extraction

Approximately 5 g prepared sediment (see section 1.4.1) was extracted for chemical analysis. Sediments were spiked before extraction with 450 µL [950 ng/g in toluene] deuterated PAH standard (Nap-D₈, Acey-D₈, Acea-D₁₀, Flu-D₁₀, Phe-D₁₀, Ant-D₁₀, Fla-D₁₀, Pyr-D₁₀, BaA-D₁₂, Chr-D₁₂, BbF-D₁₂, BkF-D₁₂, BaP-D₁₂, InP-D₁₂, BgP-D₁₂ & DBA-D₁₄). The spiked samples were extracted with accelerated solvent extraction (ASE) according to USEPA Method 3545 (USEPA, 1996) using the Dionex ASE 100® instrument (Thermo Fisher Scientific Inc.). The extraction temperature was 100°C and the solvent used was 3:1 dichloromethane (DCM):hexane (v/v). Extraction parameters used are summarised in Table 2.2.

Table 2.2: Extraction parameters for the accelerated solvent extraction method

Parameter	Extraction variable
Solvent (v/v)	3:1 (Dichloromethane:Hexane)
Temperature (°C)	100
Static extraction time (min)	5
Number of static cycles	2
Purge/flush volume (%)	60
Purge/flush time (sec)	90
Pressure	11 721 kPa
Extraction cell size (mL)	100
Filters	Cellulose
Total volume (mL)	±200 mL
Sample size (g)	5–30 g

The sediment extracts were evaporated under a gentle flow of nitrogen gas at 33°C and reconstituted in 2 mL DCM by means of solvent exchange. The samples were further purified by automated gel permeation chromatography (GPC). GPC was used to collect the fraction containing molecules in the size range of the PAHs while removing other interfering compounds including sulphur and lipids from the sample. The GPC system (Waters®) consisted of: two Envirogel™ GPC clean-up columns (19 X 150 mm & 19 X 300 mm), isocratic HPLC pump (1515), autosampler (717plus), dual λ absorbance detector (2487) set at 256 nm, and a fraction collector (model III). This system was controlled by Waters® Breeze™ software. This Waters® setup and method follows Nielsen and Ash (1978) and USEPA method 3640 (USEPA, 1994b). A size-exclusion standard mixture (corn oil [62 500 µg/mL], bis(2-ethylexyl) phthalate [2 500 µg/mL], perylene [50 µg/mL], methoxychlor [500 µg/mL] and sulphur [400 µg/mL]) as well as a PAH standards, were run to determine the retention times of the PAHs as well as known interferences. The PAH fraction in the samples was collected between 15.6 and 20.6 minutes after injection of the sample. This collection period was optimised to exclude high molecular mass lipids as well as sulphur while collecting all 16 of the US EPA priority PAHs. The GPC fraction was evaporated under a gentle stream of nitrogen gas at 33°C and reconstituted in 10 mL hexane.

The final step was solid phase extraction (SPE). Normal phase SPE is a technique used to remove polar compounds from an extract in a non-polar solvent. The highly polar packing material, magnesium silicate, strongly adsorbs polar compounds while non-polar compounds, including PAHs pass through the packing material (USEPA, 1996; 2007). For this project commercially available SPE cartridges Supelco 12 mL 2 g/2 g LC-Si/Florisil® cartridges were used. These cartridges are designed to remove nitrogen compounds from a hydrocarbon sample (Florisil® component) and also to remove polar substances by forming hydrogen bonds (silica gel component) (Zhang, 2007). The method followed was adapted from USEPA methods 3620C and 3630C (USEPA, 1996; 2007): The cartridge was conditioned with 10 mL hexane under gravitational pull. The conditioning solvent was discarded as waste. As soon as the conditioning solvent passed the top of the matrix, but before it ran dry, the 10 mL sample was added and collected in a Turbo-Vap flask positioned under the SPE cartridge. Once the sample passed through the matrix, 24 mL DCM:hexane (1:1, v/v) was added, 6 mL at a time, ensuring the surface of the matrix never ran dry followed by 8 mL DCM. The bioassay samples

were evaporated under nitrogen gas at 33°C and reconstituted in 1 mL hexane. The samples destined for chemical analysis were reconstituted in 1 mL toluene. All samples were stored at -80°C until analysis.

Biota chemical extraction

Polycyclic aromatic hydrocarbons were extracted from fish tissue and wetland bird eggs using a liquid-liquid extraction, coupled with dispersive solid phase extraction (dSPE) known as QuEChERS (**Quick, Easy, Cheap, Effective, Rugged and Safe**). This method was developed as a rapid screening extraction method (Anastassiades & Lehotay, 2003; Lehotay, 2006). It became a useful method for PAH extraction after the trans-ocean oil-drilling rig Deepwater Horizon sank (Yeudakimau *et al.*, 2013) in the Gulf of Mexico in April 2010 and spilled millions of barrels of oil (NOAA, 2016). The advantage of using QuEChERS for fish extraction has been optimised for high lipid matrices, such as fish (Forsberg *et al.*, 2011) and mussel (Madureira *et al.*, 2014). All QuEChERS consumables used in this project was supplied by Agilent Technologies and the method followed was adapted from Anastassiades & Lehotay (2003) and Lehotay (2006).

Fish muscle tissue extraction

Fish sampled in the field or kept in the aquarium (see section 1.4.1) were euthanized by severing the spinal cord. Muscle tissue samples were harvested. For consistency the fillet was collected from the left dorsal side, in line with the left pectoral fin. The samples were wrapped in pre-cleaned aluminium foil (rinsed thrice first with acetone and then hexane) according to USEPA method 1613 (USEPA, 1994a). The samples were transported at 4°C and stored at -20°C in the laboratory. The samples were mechanically homogenised using a cleaned heavy duty bladed homogeniser. Homogenised fish (2 g) was added to the 50 mL centrifuge tubes. Deuterated PAH standards (Nap-D₈, Acey-D₈, Acea-D₁₀, Flu-D₁₀, Phe-D₁₀, Ant-D₁₀, Fla-D₁₀, Pyr-D₁₀, BaA-D₁₂, Chr-D₁₂, BbF-D₁₂, BkF-D₁₂, BaP-D₁₂, InP-D₁₂, BgP-D₁₂ & DBA-D₁₄) were added to the tissue (450 µL [950 ng/g in toluene]) and mixed by vortex. The samples were suspended in 5 mL double deionised water (ddH₂O), after which 10 mL extraction solvent, acetone:hexane (1:1,v/v) was added. Liquid-liquid extraction was performed by vigorously shaking the samples for 2 hours. The QuEChERS extraction salts (anhydrous magnesium sulphate and sodium acetate) were added to the samples and mixed by vortex for a minimum of two minutes. These salts remove the polar fraction and promote partitioning of the analytes to the non-polar organic solvent phase (Lehotay, 2006). After the addition of the salts the samples were spun at 181 g for 5 min at 5°C. The organic phase supernatants were decanted into Turbo-Vap flasks and were evaporated under gentle nitrogen gas flow at 33°C to 1 mL. The flasks were rinsed with extraction solvent and the extract transferred into the 1.5 mL dSPE tube. The dSPE sorbents (primary secondary amine [PSA], C18 and anhydrous magnesium sulphate) removes fatty acids and other interference as well as any remaining water from the extract (Lehotay, 2006). The salts and extracts were mixed by vortex, and centrifuged for 5 min at 181 g (5°C). The supernatants were transferred

into gas chromatography (GC) vials, evaporated under gentle nitrogen gas flow at 33°C, and reconstituted in 1 mL toluene. These samples were stored at -80°C until instrumental analysis.

Fish bile sample extraction

Bile was also sampled during fish necropsy (see section 4.2.2). Bile was either withdrawn from the gall bladder (in the case of large volumes) or the gall bladder was collected as a whole (when there was little bile content) and stored in pre-cleaned 4 mL amber vials, transported at 4°C and stored at -20°C in the laboratory. The bile extraction method was adapted from Guo *et al.* (2013) and Bortey-Sam *et al.* (2016). Thirty microliters bile was decanted into 15 mL centrifuge tubes. Enzymatic deconjugation was done by adding 10 µL β-glucuronidase (bovine liver, Type B-1, 1 240 U/mL), 10 µL aryl-sulfatase (*Patella vulgata* Type V 34 U/mL) and 2 mL sodium acetate buffer. The pH was adjusted to between 5.5 and 5.6 with acetic acid and incubated for 6 hours at 37°C. The sample volume was increased with 2 mL ddH₂O. The internal standard (OHFlu-¹³C₆, OHPhe-D₉ and OHPyr-¹³C₆) was added before liquid-liquid extraction commenced with 5 mL pentane for 30 minutes on a rotary shaker. The samples were subsequently centrifuged at 1 600 g for 10 minutes and the organic phase decanted into an evaporation flask. The pentane extraction was repeated and the second organic phase was added to the first. The collected supernatant was evaporated under a gentle flow of nitrogen gas at 37°C to 50–100 µL. The samples were filtered through a 0.2 µm syringe filter and reconstituted in 500 µL methanol for analysis. Samples were stored at 4°C before instrumental analysis.

Wetland bird egg extraction

The sampled eggs (see section 1.4.3) were homogenised by means of sonication. The extraction of the bird eggs followed the same extraction process as the fish samples using the QuEChERS method. Due to higher lipid levels in the egg samples, an additional GPC clean-up step followed the QuEChERS method to remove these excess fats. On the last step of the QuEChERS method, the supernatant of the dSPE was evaporated under nitrogen gas at 33°C and solvent exchanged into 2 mL DCM. The GPC step was the same as with the sediment extraction: The sample passed through both the Envirogel™ GPC clean-up columns—separating analytes based on molecular sizes—and the PAH containing fraction was collected based on the relative PAH retention times. The collected PAH containing fraction was evaporated under nitrogen gas at 33°C and solvent exchanged into 300 µL toluene and stored at -80°C until instrumental analysis.

2.2.3 Instrumental analysis

The extracted samples were analysed at the National Metrology Institute of South Africa (NMISA). Sediment, fish and egg samples were analysed for the 16 priority PAHs (Table 2.1). The chromatographic system used was a LECO Pegasus IV, gas chromatography coupled to a time of flight-mass-spectrometer (GC-TOFMS) system. Separation was achieved on an Rxi®-PAH (60 m,

0.25 mm ID, 0.1 µm df) priority phase column. The autosampler, GC and TOFMS methods were optimised, in the presence of matrix to achieve optimal separation between the various target analytes and matrix interferences. During analysis compounds were identified based on retention time, elution order and obtained mass spectra (Figure 2.1). Additionally selected sediment analysis were repeated using comprehensive two dimensional chromatography coupled to time of flight mass spectrometry (GCxGC-TOFMS) using a non-polar Rxi®-5SilMS (30 m, 0.25 mm ID, 0.25 µm df) as the primary column and a mid-polar Rxi®-17SilMS (1 m, 0.25 mm ID, 0.25 µm df) as the secondary column. This method was used due to enhanced sensitivity and selectivity for the target PAHs with a smaller molecular mass as chromatographic interferences were more pronounced in the beginning of the chromatogram.

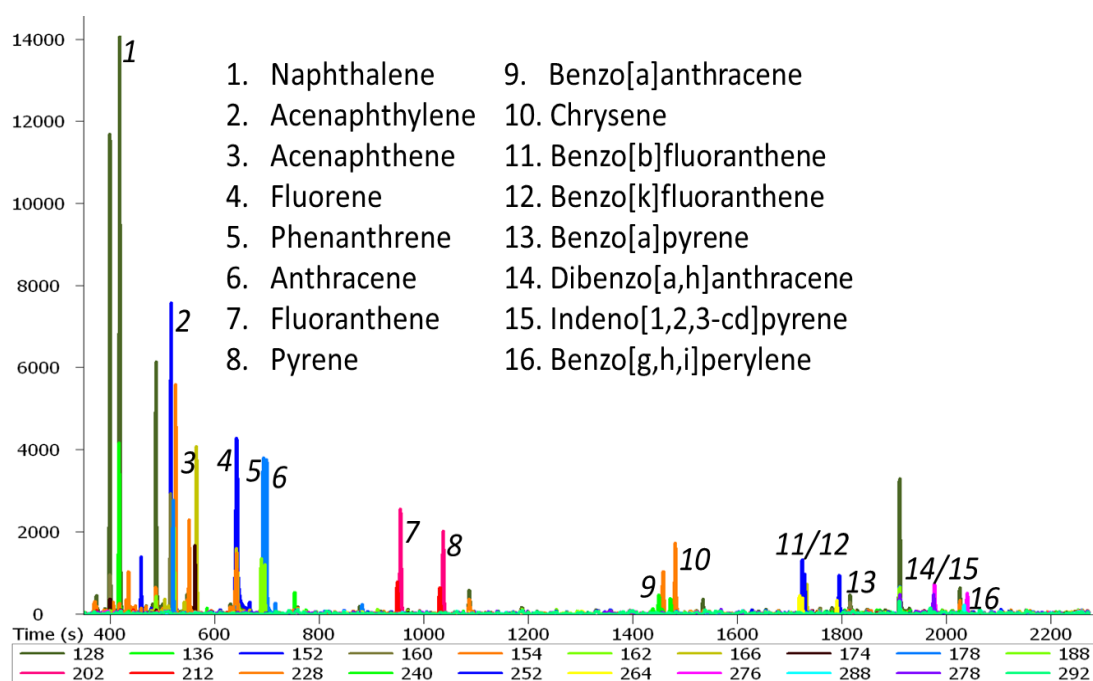


Figure 2.1: Extracted mass chromatogram of the 16 priority phase PAHs in *Clarias gariepinus*

Bile sample analysis was done at the Toxicology Laboratories of the Faculty of Veterinary Medicine at Hokkaido University, Japan. Polycyclic aromatic hydrocarbons metabolites were analysed using a Shimadzu Triple-Quad 8040 HPLC-MS/MS with an ultrafast (UF) lens for 2-OH Nap, 9-OH Flu, 2-,3-OH Flu, 2-OH Phe, 1-,9-OH Phe, 3-OH Phe, 4-OH Phe, 1-OH Pyr, 6-OH Chr, 3-OH Benzo(e)pyrene [BeP] and 9-OH BaP and separated on an Agilent Eclipse PAH column (150 x 2.1 mm, 3.5 µm dp) with gradient mobile phase (Table 2.3) at a flow rate of 250 µL/min. Target analytes were identified based on retention time, elution order and obtained mass spectra in the negative electrospray ionisation (ESI) mode. Only selected bile samples of 2013 were analysed as part of a screening for OH-PAHs in the Soweto and Lenasia fish. Mean recovery with the bile analysis was 116%±10.7.

Table 2.3: Mobile phase gradient used in the analysis of hydroxyl-PAHs in fish bile

Time (min)	Methanol:water (2:3 v/v)	Methanol
0–2	95%	5%
2–20	60%	40%
20–25	60%	40%
25–30	5%	95%
30–35	5%	95%
35–35.01	95%	5%

Quantification and quality control

Ten and eight point matrix-matched calibration curves were constructed for quantification using extracted blank matrix. Matrix matched calibration curves were selected due to the complexity of the samples analysed. Matrix matching eliminates many of the errors and biases associated with matrix effects, and allows for more accurate quantification. For the sediment quantification a ten point non-matrix matched calibration curve (0–5 000 ng/mL) was used. The quantification of the bird eggs and the fish samples used an eight point matrix matched calibration curve (0–700 ng/mL). Calibration curves were constructed for each of the 16 PAHs analytes and the 10 OH-PAH metabolites, each with R^2 values greater than 0.9. Quantification was done using peak areas to mass ratios, as described by the following formula:

$$\text{Mass of native PAH} = \frac{\left(\frac{\left(\frac{\text{Peak area of native PAH}}{\text{Peak area of deuterated PAH}} - a \right)}{m} \times \text{mass of deuterated PAH} \right)}{\text{Sample mass extracted}}$$

Where m is the gradient of calibration curve and a is the y-intercept of calibration curve.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using linear regression analysis of the constructed calibration curves, where S_a was defined as the intercept (Miller & Miller, 2010). The LOD was defined as three times the standard deviation of $S_{Y/X}$ and the LOQ was defined as ten times the standard deviation of $S_{Y/X}$.

Recovery was assessed through the analysis of either gravimetrically spiked blank matrix or where available reference material including NIST SRM 1944 (heavily contaminated New York/ New Jersey Waterway sediment) for soil and NIST SRM 2974a (organics in freeze dried mussel tissue) as a representative of the biological material analysed. The recoveries obtained across all matrices analysed are summarised in Table 2.4. Additionally a matrix blank and solvent blank were analysed with every batch of samples and retention times were confirmed throughout the course of the analysis using SRM 2260a (aromatic hydrocarbons in toluene).

Table 2.4: Recoveries (%) of polycyclic aromatic hydrocarbons in sampled sediment, *Clarias gariepinus* and wetland bird eggs, including the limit of detection (LOD) and limit of quantification (LOQ) expressed in µg/kg

PAH	Recovery in sediment	LOD	LOQ	Recovery in fish	LOD	LOQ	Recovery in bird eggs	LOD	LOQ
Nap	126	3.21	10.69	118	43.89	146.3	55	33.95	113.17
Acey	101	1.69	5.63	84	31.19	103.98	104	13.5	45.01
Acea	103	13.71	45.71	90	42.7	142.33	102	11.86	39.52
Flu	92	10.82	36.05	77	55.35	184.5	11	16.42	54.74
Phe	87	7.17	23.9	80	47.51	158.36	95	14.67	48.9
Ant	85	22.52	75.08	84	71.39	237.96	102	7.06	23.53
Fla	100	1.99	6.64	72	55.93	186.42	99	17.4	57.99
Pyr	106	18.34	61.13	102	65.64	218.8	111	15.3	51
BaA	103	18.84	62.81	78	86.68	288.94	93	25.5	85
Chr	98	3.5	12.48	75	79.68	265.58	96	23.83	79.42
BbF	93	2.09	6.98	87	93.52	311.75	100	29.66	98.85
BkF	97	1.85	6.17	85	92.29	307.63	97	18.91	63.04
BaP	94	8.42	28.06	71	100.52	335.08	95	36.49	121.62
InP	91	1.93	6.43	115	113.66	378.86	100	35.25	117.51
DBA	152	46.03	153.42	62	124.15	413.83	104	37.36	124.54
BgP	65	2.25	7.51	107	116.5	388.34	110	91.29	304.3

2.2.4 Sediment toxicity evaluation

Polycyclic aromatic hydrocarbon sediment quality guidelines

The concentrations of polycyclic aromatic hydrocarbons in the sediment of Soweto and Lenasia were gauged against the guidelines of proposed by MacDonald *et al.* (2000) and the CCME (2001). The congeners were categorised according to their exceedance of the guidelines: below threshold level (lower level), between guidelines (lower and upper level), and above the probable effects level (upper level). MacDonald *et al.* (2000) only derived guideline values for Nap, Fl, Phe, Ant, Fla, Pyr, BaA, Chr, BaP, DBA and ΣPAHs (by addition) are expressed as µg/kg dry mass (dm) (Table 2.5). Similarly, the CCME has guidelines for Nap, Acey, Acea, Flu, Phe, Ant, Fla, Pyr, BaA, Chr, BaP and DBA (expressed as µg/kg 1%TOC) (Table 2.5).

Table 2.5: Sediment quality guidelines levels of MacDonald *et al.* (2000) (µg/kg dm) and of the CCME (2001) (µg/kg 1%TOC)

MacDonald <i>et al.</i> (2000) guidelines (µg/kg dm)			CCME (2001) guidelines (µg/kg 1%TOC)	
PAH	TEC	PEC	ISQG	PEL
Nap	176	561	34.6	391
Acea	-	-	5.87	128
Acey	-	-	6.71	88.9
Flu	77.4	536	21.2	144
Phe	204	1 170	41.9	515
Ant	57.2	845	46.9	245
Fla	423	2 230	111	2 355
Pyr	195	1 520	53	875
BaA	108	1 050	31.7	385
Chr	166	1 290	57.1	862
BaP	150	1 450	31.9	782
DBA	33	-	6.22	135
ΣPAHs	1 610	22 800	-	-

TEC = threshold effects concentration; PEC = probable effects concentration; ISQG = interim sediment quality guidelines; PEL = probable effects levels

Sediment indices

In the present study the sediment quality guideline index (SQG-I) was used to determine the ecological risk the sediment pose to benthic organisms (Fairey *et al.*, 2011). The SQG-I incorporates the more protective guideline (lower) values and the measured concentrations of the target compounds to calculate the index value.

$$SQG-I = \frac{\sum_{i=1}^n C_{PAH}(\text{Sample})/C_{PAH}(\text{Threshold})}{n}$$

The SQG-I is the arithmetic mean of how many times the measured concentration ($C_{PAH}(\text{Sample})$) of individual PAHs at a specific site were higher than the lower guideline levels ($C_{PAH}(\text{Threshold})$) (Fairey *et al.*, 2001).

In addition to the SQG-I, the quality of the sediment can be calculated in terms of the PAH contamination. The sediment quality index (SQI) as described by Marvin *et al.* (2003), incorporates the percentage of PAHs per site that did not meet the lower guidelines and their magnitude of exceedance.

$$SQI = 100 - \frac{\sqrt{F_1^2 + F_3^2}}{\sqrt{2}}$$

The calculation of the index takes into account two elements, namely the scope (F_1) and amplitude (F_3). The scope is the percentage of variables that did not meet the guidelines

$$F_1 = \left(\frac{\text{number of failed variables}}{\text{total variables}} \right) \times 100$$

Amplitude is the magnitude by which the failed variables exceed the guidelines.

$$F_3 = \left(\frac{\text{mdnc}}{0.001\text{mdnc} + 0.01} \right)$$

Where:

mdnc = Mean degree of non-compliance

$$\text{mdnc} = \sum_{i=1}^p \text{non-compliance}_i$$

$$\text{non-compliance}_i = \left(\frac{\text{failed test value}_i}{\text{guideline}_i} \right)$$

Failed test value = amount of samples not meeting guidelines

i = Individual guideline

p = Total amount of guidelines used

Toxic equivalent quotient calculation

The TEQ was calculated using the following equation:

$$\text{TEQ} = \sum (C_i \times \text{TEF}_i)$$

Where:

C_i is the concentration of the polycyclic aromatic hydrocarbon congener

TEF_i is the toxic equivalence of the polycyclic aromatic hydrocarbon congener

The TEF values used for the calculation of the TEQ_{TCDD} , TEQ_{BaP} , as well as the TEQs using the FPFs (TEQ_{FPF}) are listed in Table 2.6.

Table 2.6: Toxic equivalence factors in terms of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Villeneuve *et al.*, 2002) and benzo(a)pyrene (Tsai *et al.*, 2004) as well as fish potency factors (Barron *et al.*, 2004) used to calculate toxic equivalence quotients

PAH	TEF _{TCDD} ^a	TEF _{BaP} ^b	FPF ^c
Nap	-	0.001	-
Acey	-	0.001	-
Acea	-	0.001	-
Flu	-	0.001	-
Phe	-	0.001	-
Ant	-	0.01	-
Fla	-	0.001	2.0×10^{-9}
Pyr	-	0.001	3.9×10^{-7}
BaA	-	0.1	2.0×10^{-3}
Chr	2.3×10^{-6}	0.01	6.6×10^{-5}
BbF	5.1×10^{-6}	0.01	6.6×10^{-4}
BkF	1.4×10^{-4}	0.01	1.3×10^{-3}
BaP	1.6×10^{-6}	1	2.4×10^{-4}
InP	1.5×10^{-5}	0.01	1.9×10^{-3}
BgP	-	0.01	1.0×10^{-5}
DBA	4.6×10^{-6}	1	2.7×10^{-4}

a = Villeneuve *et al.*, 2002

b = Tsai *et al.*, 2004

c = Barron *et al.*, 2004

2.2.5 Polycyclic aromatic hydrocarbon source identification and compositions

The diagnostic ratios used to identify the possible sources of the PAHs (see section 2.1.7) are listed in Table 2.7. The respected sources were determined by the ranges of each of the ratios.

Table 2.7: Diagnostic ratios for source identification of polycyclic aromatic hydrocarbons

	$\frac{\Sigma \text{LPAHs}}{\Sigma \text{HPAHs}}$	$\frac{\text{Ant}}{(\text{Ant}+\text{Phe})}$	$\frac{\text{Fla}}{(\text{Fla}+\text{Pyr})}$	$\frac{\text{BaA}}{(\text{BaA}+\text{Chr})}$	$\frac{\text{InP}}{(\text{InP}+\text{BgP})}$
Petrogenic	>1	<0.1	<0.4	<0.2	<0.2
Pyrogenic	<1	>0.1			
Pyrogenic: petroleum combustion			0.4–0.5	0.2–0.35	0.2–0.5
Pyrogenic: biomass combustion			>0.5	>0.35	>0.5

2.3 Results and discussion

2.3.1 Chemical analysis results

Sediment chemical analysis results

The detailed chemical analytical results of both 2013 and 2014 sediment samples are shown in Table 2.8. Samples that showed poor separation of the smaller molecular PAHs, were subsequently reanalysed using the more sensitive GCxGC-MS-TOF. The samples that were below the limit of detection (LOD) and limit of quantification (LOQ) are reported as half LODs and half LOQs respectively. There was no correlation between the PAH concentrations and either total organic carbon content or the sediment grain size (data not shown).

The summary of the results in Table 2.8 includes:

- individual PAH concentrations
- total PAHs (Σ PAHs),
- low molecular mass PAHs (Σ LPAH: Nap, Acey, Acea, Fl, Phe & Ant)
- high molecular mass PAHs (Σ HPAH: Fla, Pyr, BaA, Chr, BbF, BkF, BaP, InP, BgP & DBA)
- carcinogenic PAHs (Σ CPAH: BaA, Chr, BbF, BkF, BaP, InP & DBA).

Table 2.8: Concentrations (µg/kg) of the PAHs in the sediment from the nine sites in the greater Soweto and Lenasia area for 2013 and 2014

2013 sites	Nap	Acey	Acea	Flu	Phe	Ant	Fla	Pyr	BaA	Chr	BbF	BkF	BaP	InP	DBA	BgP	ΣPAH	ΣLPAH	ΣHPAH	ΣCPAH
Protea Glen	119.8	2.8	77.4	31.1	112.7	22.2	156.3	118.4	69.0	79.2	83.2	40.3	14.0	47.0	23.0	80.7	1 077.2	366.0	711.2	355.8
Lenasia	125.4	2.8	82.8	37.1	56.0	17.9	27.5	30.6	12.9	13.8	13.3	11.6	14.0	3.2	23.0	15.0	487.0	322.0	164.9	91.8
Fleurhof	70.1	0.8	22.9	12.8	99.0	22.7	169.0	128.6	79.0	41.1	45.3	29.0	14.0	26.6	23.0	34.3	818.1	228.3	589.8	257.9
Moroka	62.4	6.4	22.9	46.8	326.5	59.4	900.3	590.1	369.6	214.9	335.5	181.5	30.7	184.9	76.7	275.6	3 684.2	524.4	3 159.8	1 393.8
Eldorado Park	105.7	2.8	63.9	15.6	79.3	15.4	124.2	94.2	56.1	39.7	59.6	42.2	14.0	19.3	23.0	50.6	805.5	282.7	522.8	253.8
Orlando West	34.7	2.8	6.9	15.3	126.3	22.9	233.1	176.6	95.8	58.4	70.5	44.9	14.0	43.4	23.0	56.9	1 025.6	208.8	816.7	350.1
Orlando East	144.2	7.1	100.8	52.5	138.5	27.8	148.1	119.9	55.3	36.0	42.5	27.3	17.3	31.8	23.0	45.4	1 017.5	470.9	546.6	233.2
Nancefield	90.4	0.8	49.6	11.7	32.5	8.0	12.0	9.2	5.2	6.2	3.5	3.9	14.0	3.2	23.0	1.1	274.3	193.0	81.3	59.0
Dobsonville	59.5	0.8	22.9	5.4	29.8	7.0	24.5	9.2	14.3	14.1	16.6	9.5	14.0	10.6	23.0	14.5	275.7	125.4	150.4	102.2
2014 sites	Nap	Acey	Acea	Flu	Phe	Ant	Fla	Pyr	BaA	Chr	BbF	BkF	BaP	InP	DBA	BgP	ΣPAH	ΣLPAH	ΣHPAH	ΣCPAH
Protea Glen	113.1	44.1	22.9	38.7	77.8	15.4	99.7	74.0	42.9	37.3	51.7	24.5	14.0	30.0	23.0	48.7	757.8	312.0	445.7	223.4
Lenasia	240.9	100.8	195.8	280.0	184.0	36.9	239.2	212.1	140.9	89.7	53.4	29.1	14.0	94.8	23.0	155.1	2 089.7	1 038.3	1 051.4	445.0
Fleurhof	160.0	48.8	113.2	39.6	78.9	16.8	85.5	95.5	39.5	25.1	48.9	30.4	14.0	34.0	23.0	49.7	902.8	457.3	445.5	214.9
Moroka	512.7	421.2	470.1	328.3	385.2	92.4	1 086.3	786.6	373.3	241.9	60.0	33.2	44.6	184.9	76.7	272.2	5 369.5	2 209.9	3 159.6	1 014.5
Eldorado Park	255.8	92.0	210.4	52.4	207.5	54.0	479.4	358.7	202.4	127.3	53.8	32.2	28.4	96.1	23.0	139.7	2 412.9	872.0	1 540.9	563.1
Orlando West	122.8	116.9	76.1	119.5	57.6	19.1	103.3	80.5	47.2	30.3	53.8	33.2	14.0	21.2	23.0	28.3	946.7	511.9	434.8	222.8
Orlando East	94.3	0.8	22.9	13.8	42.6	10.3	37.4	30.6	14.6	13.8	50.8	31.1	14.0	9.2	23.0	12.0	421.3	184.8	236.5	156.6
Nancefield	80.6	0.8	22.9	16.5	105.7	23.2	105.3	81.9	46.9	32.0	43.9	32.1	14.0	20.1	23.0	27.5	676.3	249.7	426.6	211.9
Dobsonville	32.3	0.8	6.9	18.0	57.1	12.1	67.4	30.6	28.4	25.0	53.1	28.2	14.0	16.6	23.0	22.2	435.5	127.2	308.4	188.2
½ LOD	1.61	0.85	6.86	5.41	3.59	11.26	1.00	9.17	9.42	1.88	1.05	0.93	4.21	0.97	23.02	1.13				
½ LOQ	5.35	2.82	22.86	18.03	11.95	37.54	3.32	30.57	31.41	6.24	3.49	3.09	14.03	3.22	76.71	3.76				

½ LOD and ½ LOQ values reported in *italics*

In 2013, Moroka sediment had the greatest Σ PAH of 3 684 $\mu\text{g/kg}$, followed by sediments from Protea Glen, 1 077 $\mu\text{g/kg}$. The lowest Σ PAH was in Nancefield and Dobsonville sediments, 274 $\mu\text{g/kg}$ and 275 $\mu\text{g/kg}$ respectively (Table 2.8). Moroka also had the highest sediment Σ PAHs for the 2014 season, 5 369.5 $\mu\text{g/kg}$, which is the highest concentration of both sampling surveys. Eldorado Park and Lenasia also had high Σ PAH concentrations in their sediments (2 412 $\mu\text{g/kg}$ and 2 089 $\mu\text{g/kg}$, respectively) (Table 2.8). The lowest value was in Orlando East sediments (421 $\mu\text{g/kg}$), followed by those of Dobsonville (435 $\mu\text{g/kg}$) (Table 2.8).

Moroka had the highest values of Σ LPAH, Σ HPAH, and Σ CPAH (Table 2.8) in all sediments sampled during 2013 and 2014. A temporal change was noted in the Moroka results between the two sampling events. There was a considerable increase in the Σ LPAH concentration at the site, increasing from 524 $\mu\text{g/kg}$ in 2013 to 2 209 $\mu\text{g/kg}$ in 2014 (Table 2.8). Both Eldorado Park and Lenasia also had high concentrations of low- and high molecular PAHs in the sediments for 2014, where Eldorado Park had predominantly HPAHs (1 540 $\mu\text{g/kg}$, Table 2.8).

As mentioned above, Moroka had the highest concentrations for all the types of PAH groups, including the sum of the carcinogenic PAHs in its sediment. The second highest Σ CPAH for 2013 sediments was at Protea Glen (355 $\mu\text{g/kg}$) (Table 2.8) and the lowest at Nancefield, 59 $\mu\text{g/kg}$ (Table 2.8). The lowest Σ CPAH levels quantified for 2014 sediments was from Dobsonville (188 $\mu\text{g/kg}$) (Table 2.8).

The mean Σ PAHs quantified in the sediments, and their ranges were compared to other South African-, and also African-, European-, North American- and Asian studies (Table 2.9). These ranges and totals are for the 16 priority PAHs only (where available). If authors reported on more than the 16 priority PAHs, new ranges and mean totals were calculated to reflect only the 16 US EPA PAHs if the individual concentrations were available in the paper. In the instances where PAH congeners reported were not for only the 16 priority congeners, the ranges and totals were given as is and it is indicated how many congeners contributed to the totals (e.g. Σ 9PAHs).

The study by Roos *et al.* (2011) in the Soweto and Lenasia section of the Klip River shared many sites with the present study. Therefore the data was used for comparisons in order to determine temporal changes in the study area. The Σ PAHs of Roos *et al.* (2011) were comparable to this study. The highest levels of total PAHs from that study was 5 408 $\mu\text{g/kg}$, measured in the sediment of a site between the Eldorado Park and Nancefield sites of the current study. Overall, the levels measured by Roos *et al.* (2011) were similar to that measured during the present study's 2013 and 2014 seasons (Table 2.9). In a study by Quinn *et al.* (2009), where the authors investigated organic pollutants in the central part of South Africa, which also included the present study area, the mean Σ PAHs was lower, however the range between minimum- and maximum concentrations was larger (Table 2.9). The only results obtained from 2013 that had a higher CPAHs level than that of Roos *et al.* (2011) was for Moroka. The 2014 samples that were comparable to the Roos *et al.* (2011) results were Lenasia, Moroka and Eldorado Park sediments. Nieuwoudt *et al.* (2011) worked in the same area as Quinn *et*

al. (2009), and measured a mean Σ PAH level greater than the aforementioned author's mean Σ PAH (Table 2.9). This indicates that there was a notable temporal increase between the Quinn *et al.* (2009) and Nieuwoudt *et al.* (2011) studies, unlike the similar Σ PAH mean of the present study to Nieuwoudt *et al.* (2011).

The Orange River is considered to be one of the few rivers in South Africa to be less polluted by industry and mining and more by urban development and agriculture (DWAS, 2004; Bucas, 2006) and therefore low PAHs levels can be expected in this system. This was indeed the case in a sample from the Orange River, taken at the agricultural town of Douglas just below the confluence with the Vaal River by Nieuwoudt *et al.* (2011), which had a mean Σ PAH of half that of the lowest Σ PAHs of this study (Table 2.9). The low prevalence of PAHs in the Orange River was further corroborated by findings of Pieters *et al.* (2015) in which the majority of the Orange-Senqu River basin was sampled to screen for the levels and occurrences of PAHs. However, the maximum range of the Orange-Senqu Basin Σ PAH levels was much wider. This is because the basin covers approximately 45% of South Africa, and encompasses industrial and heavy populated areas.

In a study on PAH distribution from sources, Okedeyi *et al.* (2013) investigated PAHs in soils at coal-fired power stations and found high levels of PAHs on site (Table 2.9), which declined over distance away from the source. The Lethabo- and Rooiwal (also known as Kelvin) power stations are 60 and 30 km away from Soweto and Lenasia—close enough for air transported PAHs to end up in aquatic systems.

Other African countries which reported on PAHs in sediments include Egypt and Ethiopia. Lake Maryut, in the Nile Delta, is one of the most polluted lakes in Egypt and is situated close to the highly industrialised and populated city of Alexandria (Barakat *et al.*, 2011). The authors analysed Σ 39PAHs in the sediments, and the mean and range of the 16 priority PAHs has been calculated for the purposes of this study (Table 2.9). The maximum Σ PAHs in Lake Maryut sediments were higher than the sediments from Soweto and Lenasia. However, the mean concentration for Lake Maryut is ten times lower than our study area's (Table 2.9). This is due to outliers in their samples—3 of the 13 samples was above 1 000 ng/g Σ PAHs, one of these measured 6 200 ng/g (Barakat *et al.*, 2011). Mekonnen *et al.* (2015) assessed the distribution of sediment bound PAHs in the Akaki River and in Lakes Awassa and -Ziway in Ethiopia. The Akaki River flows through Addis Ababa and is subjected to domestic waste and untreated industrial effluent (Mekonnen *et al.*, 2015). High levels of organic pollution have been reported in the polluted Lakes Awasa and Ziway (Yohannes *et al.*, 2013). Mekonnen *et al.* (2015) was the first to screen for PAHs. The Σ PAHs quantified in these lake sediments were notably lower than for Soweto and Lenasia (Table 2.9). The Akaki River Σ PAHs were comparable to our levels and similar to Quinn *et al.* (2009)'s results (Table 2.9).

The high altitude lakes of Norway and Austria had levels of the 16 USEPA PAHs within the range of our results (Table 2.9), evidence that PAHs also travel to remote areas via the atmosphere (Fernández *et al.*, 1999). In heavily industrialised countries such as Germany, and to a lesser extent

the Czech Republic, the levels of PAHs in the sediments were immense (Table 2.9). The floodplain soils of the Saar and Mosel Rivers that flow through the coal mining areas of the Rhineland-Palatinate

Table 2.9: Concentration of Σ PAHs from literature

Location	n	Σ 16PAHs ($\mu\text{g/kg}$)		Reference
		Mean	Range	
Klip River, Soweto & Lenasia, RSA	18	1 304	274–5 370	This study
Klip River, Soweto & Lenasia, RSA	12	1 362	215–5 408	Roos <i>et al.</i> , 2011
Multiple rivers, Vaal Triangle, RSA	9	641	44–2 799	Quinn <i>et al.</i> , 2009
South Africa Orange-Senqu River catchment, RSA	62	110	6–870	Pieters <i>et al.</i> , 2015
Klip River, Vereeniging, RSA	1	580		Nieuwoudt <i>et al.</i> , 2011
Riet Spruit, Vanderbijlpark, RSA	1	2 800		Nieuwoudt <i>et al.</i> , 2011
Orange River, Douglas, RSA	1	120		Nieuwoudt <i>et al.</i> , 2011
Lethabo Power station, RSA*	1	18 750		Okedeyi <i>et al.</i> , 2013
Matla Power station, RSA*	1	20 650		Okedeyi <i>et al.</i> , 2013
Rooiwal Power station, RSA*	1	14 440		Okedeyi <i>et al.</i> , 2013
Africa Lake Maryut, Alexandria, Egypt	13	127	39–6 200	Barakat <i>et al.</i> , 2011
Akaki River, Addis Abba, Ethiopia ^a	15	530	0–3 070	Mekonnen <i>et al.</i> , 2015
Lake Awassa, Ethiopia ^a	11	170	24.9–410	Mekonnen <i>et al.</i> , 2015
Lake Ziway, Ethiopia ^a	10	180	15–310	Mekonnen <i>et al.</i> , 2015
Saar- & Mosel Rivers, Germany	11	43 345	20900–81 500	Pies <i>et al.</i> , 2007
Morava- & Drevnice Rivers, Czech	13	18 620	3 500–61 700	Hilscherova <i>et al.</i> , 2001
Europe Tiber River, Rome, Italy ^b	5	215.2	158–272	Patrolecco <i>et al.</i> , 2010
Schwarsee ob Sölden Austria ^c	1	579.7		Fernández <i>et al.</i> 1999
Gossenkölle Austria ^c	1	529.2		Fernández <i>et al.</i> 1999
Øvre Neådalsvatn Norway ^c	1	445.8		Fernández <i>et al.</i> 1999
Arresjøen Norway ^c	1	171.7		Fernández <i>et al.</i> 1999
North America Lower Missouri River, USA	19	421.6	92–2 813	Echols <i>et al.</i> , 2008
Lake Michigan, USA	5	149.4	33–400	Kannan <i>et al.</i> , 2001
Rogue River, Michigan, USA	16	21 717.9	7450–42 800	Kannan <i>et al.</i> , 2001
Detroit River, Michigan, USA	16	12 293.7	17.5–36 200	Kannan <i>et al.</i> , 2001
Hamilton Bay, Lake Ontario, Canada	8	31 287	7200–47 600	Marvin <i>et al.</i> , 2000
Wadamun Lake, Alberta, Canada ^d	1	2 756		Donahue <i>et al.</i> , 2006
Lac Ste. Anne, Alberta, Canada ^e	1	771		Donahue <i>et al.</i> , 2006
Pigeon Lake, Alberta, Canada ^f	1	895		Donahue <i>et al.</i> , 2006
Asia Gomti River, India	10	11 00.9	207.6–3 365.1	Malik <i>et al.</i> , 2004
Yamuna River, India	5	10 150	4500–23 530	Agarwal <i>et al.</i> , 2006
Meiliang Bay, Taihu Lake, China	25	2 563	1207–4 754	Qiao <i>et al.</i> 2006
Yellow River, China	14	1 414	464–2 035	Xu <i>et al.</i> , 2007
Yangtze Delta, China ^g	11	1 318	133–6 338	Chen <i>et al.</i> , 2004

n = number of samples analysed, * Samples from power stations were soil samples

a = Σ 14PAHs (Nap, Acey, Fl, Phe, Ant, Fla, Pyr, BaA, Chr, BbF, BkF, BaP, InP & BgP)

b = Σ 6PAHs (Flu, BbF, BkF, BaP, BgP & InP)

c = Σ 12PAHs (Flu, Phe, Ant, Fla, Pyr, BaA, Chr, BkF, BaP, InP, BgP & DBA)

d = Σ 20PAHs (identity of congeners not reported)

e = Σ 19PAHs (identity of congeners not reported)

f = Σ 10PAHs (identity of congeners not reported)

g = Σ 9PAHs (Flu, Phe, Ant, Fla, Pyr, BaA, Chr, BkF & BaP)

state of Germany had very high levels of Σ PAHs (Pies *et al.*, 2007) (Table 2.9 represents the 11 sites with the highest concentrations), levels that were 33 times greater than the mean measured for this study. Hilscherova and co-authors (2001) sampled the Morava- & Drevnice Rivers from a valley with three industrialised cities in the Czech Republic. The mean Σ PAHs were 14-fold more than the Σ PAHs mean of this study.

For the North Americas, Echols *et al.* (2003), Kannan *et al.* (2001) and Marvin *et al.* (2000) reported on PAHs in the sediments of the Lower Missouri-, Rogue-, Detroit Rivers and Lake Michigan, and Hamilton Bay of Lake Ontario, respectively. Similar to the European sites shown in Table 2.9, the sites studied by Kannan *et al.* (2001) had much higher Σ PAH levels in the sediments than found for the present study. Sediments from Lake Michigan and the Lower Missouri River had lower Σ PAH levels than what was found in the present study (Table 2.9). Similarly, Donahue *et al.* (2006), who detected between 10–20 PAHs (Table 2.9), also had lower Σ PAHs quantified in Pigeon Lake and Lac Ste Anne's sediment, in Alberta Canada.

In studies on PAHs in sediment from Asia, the only one that had a higher maximum Σ PAHs came from site in the Yamuna River, in India which was located in the centre of Delhi close to two coal based thermal power stations (Agarwal *et al.*, 2006). Reports for China and India had higher mean Σ PAH levels than Soweto and Lenasia (Table 2.9).

Fish muscle tissue chemical analysis results

No PAHs (in native form) were detected in the fish samples because PAHs are bio-transformed by vertebrates. (Hylland, 2006). Xenobiotics are bio-transformed via two pathways: hydrophilic xenobiotics go through phase I where a polar conjugate is introduced by means of oxidative, reductive and/or hydrolytic processes (Tuvikene, 1995; Rose & Hodgson, 2004; Newman, 2010). Xenobiotics that are already water soluble are directly passed to the phase II pathway which involves the conjugation of xenobiotics or their phase I metabolites. The conjugates that are added to the compounds include acetate, amino acids, glutathione, glucuronic acid, methyl groups and sulphate, to name a few (Tuvikene, 1995; Newman, 2010). Once conjugated the xenobiotic is water soluble and excreted from the organism (Tuvikene, 1995; Newman, 2010).

The main PAH metabolism pathway in fish involves cytochrome P450 (CYP450), monooxygenases, epoxide hydrolase and conjugating enzymes (Tuvikene, 1995, Rose & Hodgson, 2004). The metabolism of PAHs is well studied and the collective process was described in detail by Tuvikene (1995): The subfamily of the CYP450 genes that are activated in fish by PAHs is the CYP1A family. After the PAH has bound to the Ah-receptor (see section 3.1.2), the CYP450s are induced. Specific forms of P4501A1 are induced after exposure to PAHs: aryl hydrocarbon hydroxylase (AHH), ethoxyresorufin-O-deethylase (EROD) and 7-ethoxycoumarin-O-deethylase (ECOD). The release of these enzymes results in the addition of an oxygen atom to the AhR-ligand (like PAHs) and in most cases this oxygen is reduced to a hydroxyl group by monooxygenases. Following these reactions the

metabolites are conjugated by several enzymes and anti-oxidants such as glutathione-S-transferase (GST), uridine 5-diphosphate-glucuronosyltransferase (UDP-GT) and glutathione (GSH). These enzymes complete biotransformation phase II: reducing the toxicity of the compound and making it easier to excrete (Rose & Hodgson, 2004). The deconjugated metabolites or hydroxylated PAHs quantified in the bile samples are presented in Table 2.10.

Table 2.10: Mean hydroxylated PAH metabolites quantified from *Clarias gariepinus* from selected sites of 2013 (ng/mL), range in parenthesis

2018 (ng/mL), range in parenthesis										
	Site									
	Control (n = 10)		Orlando (n = 9)		Lenasia (n = 11)		Fleurhof (n = 10)		DO	Q ₁
OH-PAH	N	Mean	N	Mean	N	Mean	N	Mean		
2-OH Nap	3	5 (5–6)	4	49 (9–89)	0		0		0.09	0.3
9-OH Flu	0		9	90 (21–196)	10	24 (9–49)	10	35 (4.7–96)	0.18	0.6
2-,3-OH Flu	6	131 (55–187)	9	708 (172–1429)	9	220 (87–411)	7	399 (173–783)	0.07	0.25
2-OH Phe	0		6	30 (4–84)	0		2	8.7 (2–16)	0.07	0.23
1-,9-OH Phe	0		0		0		0		0.21	0.71
3-OH Phe	0		0		0		0		0.35	1.16
4-OH Phe	0		6	17 (3–31)	1	3	0		0.04	0.15
1-OH Pyr	3	19 (10–33)	9	86 (26–146)	0		8	69 (32–116)	0.26	0.87
6-OH Chr	0		0		0		0		0.22	0.73
3-OH BeP	0		0		0		0		0.16	0.54
9-OH BaP	0		7	12 (3–34)	0		1	8	0.1	0.32
ΣOH-PAHs*	99.1±80.5		947.4±601.2		201.7±144.3		371.2±340.4			

N = number of fish out of sampled fish (n) with quantifiable levels of respective metabolite

* = Mean of the ΣOH-PAHs per site

There was no significant differences were observed between the sexes. The site with the fish that had the highest mean of total hydroxylated PAHs concentration was Orlando (East), 947.4 ng/mL (Table 2.10), 2-,3-hydroxyfluorene had the highest levels (mean 708 ng/mL, range 172–1 429 ng/mL) contributing to the total OH-PAHs in Orlando fish. The second highest mean ΣOH-PAHs, was in fish from Fleurhof (371.2 ng/mL) followed by Lenasia (201.7 ng/mL). These levels are notably lower than the Orlando results (Table 2.10). As expected, the control fish had the lowest concentrations of OH-PAHs—these fish were kept in a controlled environment and the depuration period would have allowed the majority of the PAHs to be metabolised and excreted. The Orlando fish had the greatest variety of hydroxyl-PAHs in their bile (7 of the 11, Table 2.10). The metabolite profiles indicate relative trends between each other. The 2,3-OH fluorene was invariably the dominant metabolite, contributing between 75% and 89% to the total in the fish from the study area, and 92% in the control fish (Figure 2.2 A–D). The 9-OH phenanthrene and 1-OH pyrene contributed the second most (Figure 2.2 A–D). The 2,3-OH Flu is derived from fluorene that is a main component (but not exclusively) of petroleum sources, along with phenanthrene and petroleum specific alkylated PAHs (Stogiannidis & Laane, 2015). The petrogenic source fingerprinting conducted by Douglas *et al.* (2007) showed that

the 2 and 3-ringed PAHs—such as Flu and Phe—are more abundant in fossil fuels and lubrication oils, than the heavier 4–6 ring PAHs. This indicates that the measured fluorene and phenanthrene metabolites in *C. gariepinus* possibly originated from petrogenic sources.

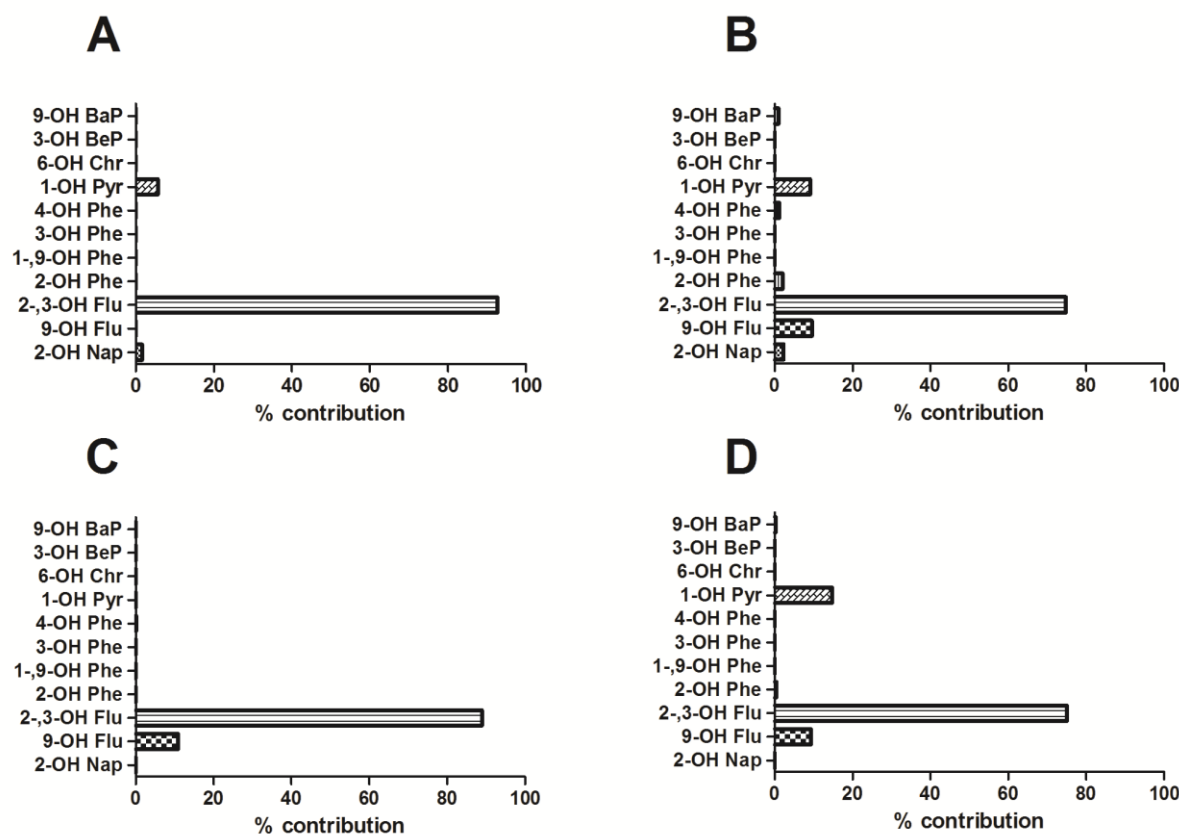


Figure 2.2: Polycyclic aromatic hydrocarbons metabolite profile, proportional contributions of the 11 OH-PAHs in *Clarias gariepinus* from Soweto and Lenasia 2013: A) Control; B) Orlando; C) Lenasia; D) Fleurhof

One of the best general indicators of PAH exposure in fish is considered to be 1-hydroxyl pyrene (Van der Oost *et al.*, 1994; Ruddock *et al.*, 2003) and is the main metabolite of pyrene—one of the most abundant pyrogenic PAHs together with fluoranthene and to a lesser extent, phenanthrene (Page *et al.*, 1999; De Luca *et al.*, 2004). The only phenanthrene metabolite present in the Soweto and Lenasia fish was 1-OH Phe (Figure 2.2). The fact that phenanthrene has a relatively long half-life in soil-like matrices (LeBlanc, 2004) and have a high affinity for sediment particles (Yuan *et al.*, 2001), may be the cause for the lower concentrations in biota (Table 2.10), as these characteristics decrease its bio-availability.

To the author's knowledge the data on biliary PAH metabolites for the fish in this study is the first for South Africa. Only a very few international studies did report on biliary PAH metabolites from fish. The majority of the international PAH metabolite studies in fish bile were on estuarine (Richardson *et al.*, 2001; Ruddock *et al.*, 2003; Jonsson *et al.*, 2004) or marine fishes (Escartin & Porte, 1999a; Aas *et al.*, 2000; Richardson *et al.*, 2001; Kammann, 2007). Thus, the results above will be gauged against

comparable European studies. Ruddock *et al.* (2003) determined the biliary PAHs in the European eel (*Anguilla anguilla*) of the United Kingdom (UK) and although these sites were estuarine, those sites farthest from the mouth were selected to compare with. The levels of 1-OH Pyr were 20 times higher in the most inland site of the Tees River at 3 108 ng/mL [14.24 µM] and 30 times higher (at the most upstream site of the Tyne River at 5 150 ng/mL [23.6 µM]) compared to that measured in *C. gariepinus*. These high levels suggest that the estuarine environments of the UK were far more polluted. Escartin and Porte (1999b), reported on the biliary levels of hydroxylated fluorene, phenanthrene, and pyrene metabolites in brown trout (*Salmo trutta*) from Norwegian and Austrian high altitude lakes. These authors reported a mean of 218.3 ng/mL and 154.2 ng/mL of 1-OH pyr for the Norwegian and Austrian lake fish respectively. These levels are notably higher than those found in the present study. However, the levels of 9-OH Flu in *S. trutta* were lower than that in *C. gariepinus* of South Africa. This trend, along with the high levels of the other Flu-metabolite suggests that the sources of the two areas differ in origins (pyrogenic or petrogenic).

Wetland bird egg chemical analysis results

The chemical analysis results for the wetland bird eggs were similar to that of the fish results. Most of the PAHs were metabolised and only naphthalene, phenanthrene and to a lesser extent acenaphthylene were detected (Table 2.11). The presence of naphthalene and phenanthrene (albeit small) can be attributed to their resistance to metabolism and affinity to accumulate in fish (Liang *et al.*, 2007), which form the main part of piscivorous birds' diets. The slight presence of these PAHs in the egg samples are indicative of the presence of native PAHs in the system and are possibly ubiquitous.

Table 2.11: Levels of naphthalene, acenaphthene and phenanthrene (µg/kg) in wetland bird eggs sampled from Lenasia 2013

N	1	2	3	4	5	6	7	8	9	10	LOD	LOQ
Black headed heron												
Nap	17.0	247.0	17.0	0.0	17.0	17.0					34.0	113.2
Acea	5.9	5.9	0.0	0.0	0.0	0.0					11.9	39.5
Phe	7.3	7.3	7.3	7.3	7.3	7.3					14.7	48.9
ΣPAHs	30.2	260.3	24.3	7.3	24.3	24.3						
Cattle egret												
Nap	17.0	0.0	17.0	17.0	17.0	17.0	17.0	0.0	17.0	17.0	34.0	113.2
Phe	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	14.7	48.9
ΣPAHs	24.3	7.3	24.3	24.3	24.3	24.3	24.3	7.3	24.3	24.3		
Glossy ibis												
Nap	0.0	17.0	0.0	0.0	17.0	0.0	17.0	17.0	17.0	17.0	34.0	113.2
Phe	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	14.7	48.9
ΣPAHs	7.3	24.3	7.3	7.3	24.3	7.3	24.3	24.3	24.3	24.3		
Sacred ibis												
Nap	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	34.0	113.2
Phe	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	14.7	48.9
ΣPAHs	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3		

½ LOD and ½ LOQ values reported in italics

Herbert *et al.* (2011) investigated the 16 priority PAHs in the eggs of two tern and three gull species from northern Alberta, Canada. The PAHs results reported are similar to that of this study—naphthalene and phenanthrene (Table 2.11) were present in most of the samples in low levels, however they also measured Ant, BkF, BaP, DBA, and BgP, also in low concentrations and in a minority of the samples.

2.3.2 Polycyclic aromatic hydrocarbon compositions and source identification

Polycyclic aromatic hydrocarbon sediment compositions

The ratios of the different size classes (based on number of rings) of PAHs measured in the sediments were calculated to determine the composition percentages at each site. These compositions represent the inputs of PAHs at each site from the surrounding area, and therefore references to site names in this section refer to the area, and not so much particular sites. The use of these compositions allows for interpretation of the chemical concentrations and supplements the source identification ratios.

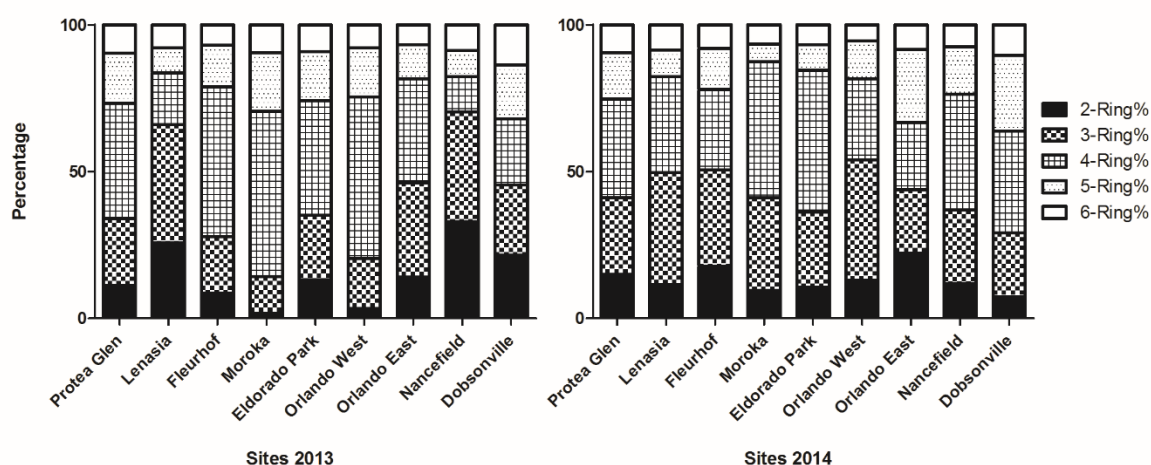


Figure 2.3: Polycyclic aromatic hydrocarbon profile composition at sites from the Soweto and Lenasia study area (2013 and 2014)

The percentage composition by the various congeners in the sediments of the sites between the years varied, except for Protea Glen, which seemed to have received a stable contribution of similar sized PAH molecules between the two years (Figure 2.3). Further investigation revealed that all the sites sampled in 2014 had the most contributions from 3- and 4-ring congeners. Orlando East and Eldorado Park also had the biggest contribution from 3- and 4-ring PAHs during 2013, but the other sites showed a more random size contribution during that year (Figure 2.3). Lenasia, Dobsonville, and Nancefield (2013) had notably more 2- and 3-ring congeners and this corresponds with the source identification for these sites (Figure 2.3, Table 2.12). None of the samples collected in 2013 had high percentages of the 5- and 6-ring congeners; however the sediment sampled in 2014 at Orlando East and Dobsonville had mostly 5-ring congeners (Figure 2.3). Roos *et al.* (2011) reported that the 4-ring

PAHs were the most abundant, followed by the 5-ring PAHs and then the 3- and 6-ring congeners, during their study in the same area.

Polycyclic aromatic hydrocarbon source identification

By calculating the ratios between various PAHs found at a site the original source categories (pyrogenic or petrogenic) can be determined. The results from the source identification are reported in Table 2.12 and Figure 2.4.

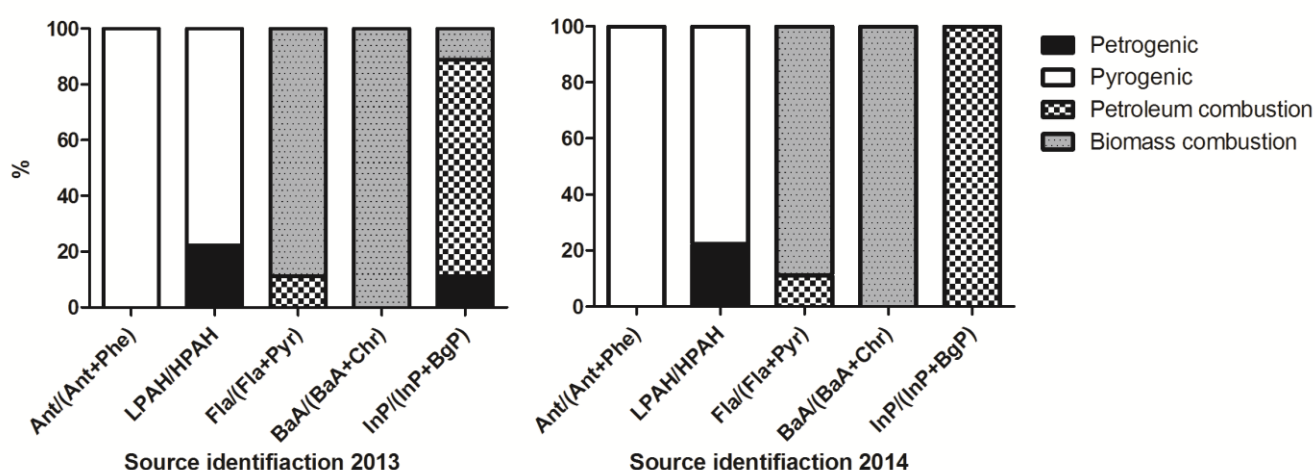


Figure 2.4: Source identification of polycyclic aromatic hydrocarbons in the Soweto and Lenasia sediment of 2013 and 2014

There was little temporal variance between the sources of PAHs in the sediment from 2013 to 2014. The sources were mainly pyrogenic as indicated by the Ant/(Ant+Phe) and LPAH/HPAH ratios (Figure 2.4). More specifically the type of combustion that dominated the formation of PAHs—as indicated by the Fla/(Fla+Pyr), BaA/(BaA+Chr) and InP/(InP+BgP)—is biomass combustion. Petroleum combustion is also present in the urban study area, shown by the InP/(InP+BgP) ratio (Figure 2.4). The only difference between 2013 and 2014 sources were indicated with the InP/(InP+BgP) ratio. This ratio suggests that 11% of the sites had PAHs that originated from petrogenic sources in 2013, and of the remaining 89% pyrogenic sites, 11% of these were due to biomass combustion.

The temporal variation observed for the InP/(InP+BgP) ratio could be attributed to the Lenasia site that seemed to have petrogenic sources for 2013 but indicated a pyrogenic source of petroleum combustion. Although Nancefield stayed in the pyrogenic category for both sampling years, its source nature changed from biomass combustion in 2013 to petroleum combustion in 2014 (Table 2.12). Although the source distribution between petrogenic and pyrogenic showed the same percentages for the LPAH/HPAH ratio between sampling events, the particular sites that were identified with this ratio, differed between the years. In the 2013 sites, Lenasia and Nancefield had the petrogenic sources, but in 2014, the sites were Lenasia and Orlando West (Table 2.12). Similarly, the percentages shown for

the Fla/(Fla+Pyr) ratio were also attributed to different sites: for 2013 Lenasia calculated for petroleum pyrogenic sources and in 2014 it was Fleurhof (Table 2.12). The origins identified by Roos *et al.* (2011) were similar to what was discovered for the present study. It seems that the predominant source of anthropogenic released PAHs in South Africa is pyrogenic, as was discovered when the source ratios were calculated for the other South African studies mentioned in Table 2.5 (data not shown).

Table 2.12: Source identification ratios of PAHs in sediments at sites in Soweto and Lenasia of 2013 and 2014

	Sampling site	Ant/(Ant+Phe)	LPAH/HPAH	Fla/(Fla+Pyr)	BaA/(BaA+Chr)	IcdP/(IcdP+BghiP)
2013	Protea Glen	Py	Py	BioPy	BioPy	PetPy
	Lenasia	Py	Pet	PetPy	BioPy	Pet
	Fleurhof	Py	Py	BioPy	BioPy	PetPy
	Moroka	Py	Py	BioPy	BioPy	PetPy
	Eldorado Park	Py	Py	BioPy	BioPy	PetPy
	Orlando West	Py	Py	BioPy	BioPy	PetPy
	Orlando East	Py	Py	BioPy	BioPy	PetPy
	Nancefield	Py	Pet	BioPy	BioPy	BioPy
	Dobsonville	Py	Py	BioPy	BioPy	PetPy
	Sampling site	Ant/(Ant+Phe)	LPAH/HPAH	Fla/(Fla+Pyr)	BaA/(BaA+Chr)	IcdP/(IcdP+BghiP)
2014	Protea Glen	Py	Py	BioPy	BioPy	PetPy
	Lenasia	Py	Py	BioPy	BioPy	PetPy
	Fleurhof	Py	Pet	PetPy	BioPy	PetPy
	Moroka	Py	Py	BioPy	BioPy	PetPy
	Eldorado Park	Py	Py	BioPy	BioPy	PetPy
	Orlando West	Py	Pet	BioPy	BioPy	PetPy
	Orlando East	Py	Py	BioPy	BioPy	PetPy
	Nancefield	Py	Py	BioPy	BioPy	PetPy
	Dobsonville	Py	Py	BioPy	BioPy	PetPy

Py = pyrogenic, Pet = petrogenic, BioPy = biomass combustion, PetPy = Petroleum combustion

Nieuwoudt *et al.* (2011) suggested that this is mainly from ineffective burning of organic fuels during open burning for domestic heat and cooking, and during incineration of waste. The study by Barakat *et al.* (2011) in Egypt on Lake Maryut reported a source signature of both petro- and pyrogenic. The LPAHs at these sites, according to the Ant/(Ant+Phe) ratio, were of petrogenic origin and the HPAHs evenly divided between biomass- and petroleum combustion (pyrogenic). The PAHs in the Yumana River sediments had similar origins (Agarwal *et al.*, 2006) as the Egyptian lake. The Ant/(Ant+Phe) ratio determined a dominant petrogenic origin for LPAHs present at: the Gomti River, India (Malik *et al.*, 2004); the Yangtze Delta, China (Chen *et al.*, 2004); Lakes Ziway, Awassa and the Akaki River in Ethiopia (Mekonnen *et al.*, 2015). The HPAHs reported on in the publications of Malik *et al.* (2004) and Chen *et al.*, (2004) had their origins very similar to our sites which were dominated by biomass combustion. The Ethiopian sites (Mekonnen *et al.*, 2015) had a mixture of both petrogenic (Lake Ziway) and pyrogenic (biomass combustion—Akaki River and Lake Awassa) sources.

2.3.4 Sediment toxicity evaluation

Polycyclic aromatic hydrocarbon sediment quality guidelines

The sediment quality guidelines were used to determine ecological risk posed by the sediments from the sample sites, based on their chemical concentrations. The results of the application of the PAH sediment quality guidelines on the sediments of Soweto and Lenasia is reported in Figure 2.5 (for the entire study area) and Table 2.13 & 2.14 (for individual sites).

The CCME guidelines regarding PAHs are more protective than that set up by MacDonald *et al.* (2000), and are therefore lower. This is visible in Figure 2.5: When the MacDonald guidelines were used only 11% of the sites had levels exceeding any of the respective guideline levels for 2013 (Figure 2.5A) and in 2014 between 11 and 33% of the sites surmounted the TEC guideline levels (Figure 2.5B). When the CCME guidelines were employed, many more sites had levels exceeding the respective guidelines (Figure 2.5C & 2.5D) and in some instances the individual guidelines were exceeded at all the sites. This was the case for naphthalene (Nap), acenaphthene (Acea), and dibenz(a,h)anthracene (DBA) (Figure 2.5C): In 2014 there were sites with sediment PAH concentrations even surpassing the PEL and not merely the lower ISQG. This was true for acenaphthylene (Acey), acenaphthene (Acea), and fluorene (Flu) (Figure 2.5D).

In 2013, only Moroka sediments exceeded the TEC guideline of MacDonald *et al.* (2000) for all respective PAH congener TEC levels, except for naphthalene (Nap), fluorene (Flu), and benzo(a)pyrene (BaP). The ΣPAHs also exceeded its TEC guideline (Table 2.13). In 2014, Moroka's sediment was beyond the TEC for 9 of the 10 comparative guidelines (Table 2.13). In this year three more sites had levels above the respective TEC guidelines of MacDonald *et al.* (2000) (Table 2.13): Eldorado Park (6 out of 10), Lenasia (4 out of 10) and Orlando West with a single high level. No MacDonald *et al.* (2000) PEC guidelines were exceeded during the entire study.

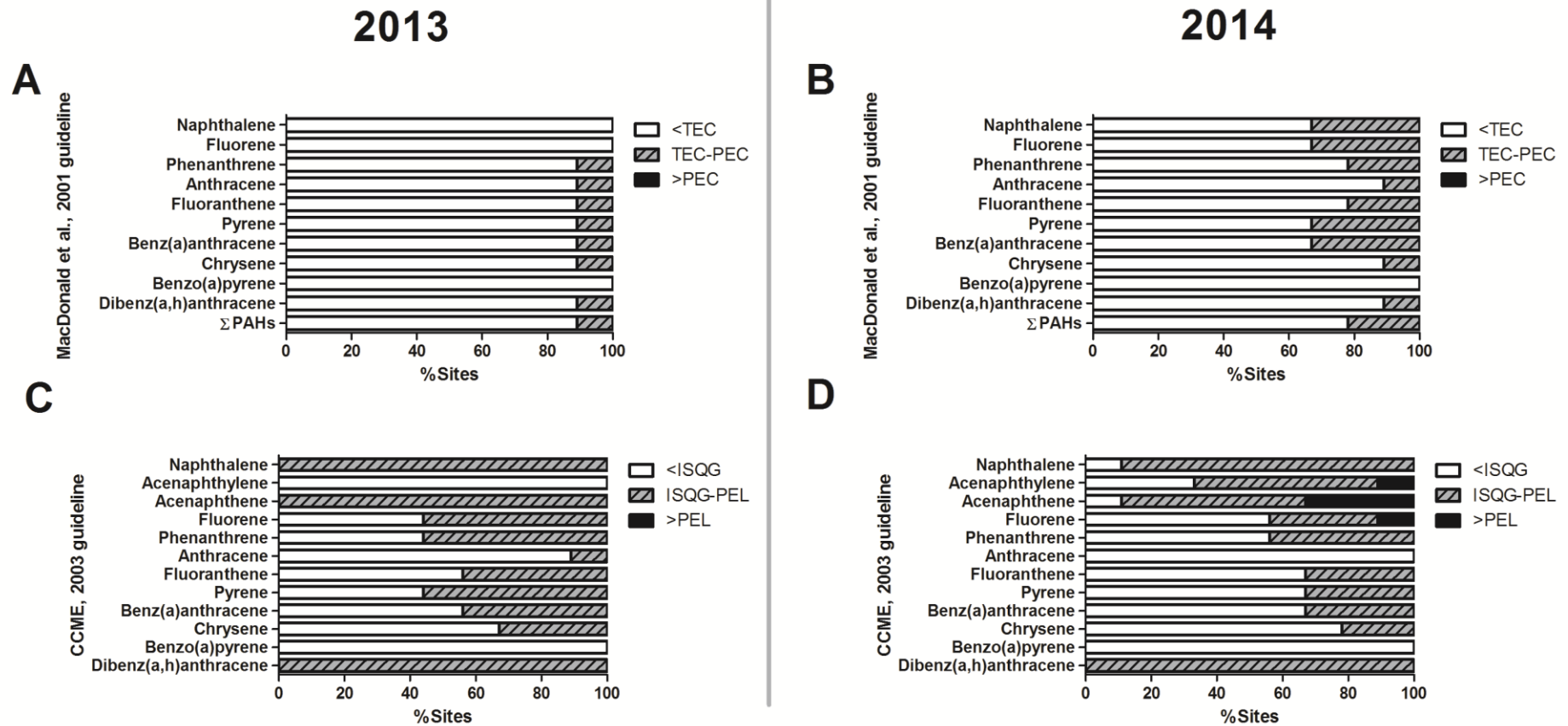


Figure 2.5: Soweto and Lenasia sediments compared to sediment quality guidelines of MacDonald *et al.*, 2000 and CCME, 2012 in terms of PAH levels

Table 2.13: Sediment from the sites of Soweto and Lenasia compared to sediment quality guidelines (TEC and PEC) of MacDonald *et al.*, 2000. Guidelines exceedance indicated by shading

	PAH	TEC	PEC	PG	Le	FI	Mo	EID	OW	OE	Nc	Db
2013	Nap	176	561	119.8	125.4	70.1	62.4	105.7	34.7	144.2	90.4	59.5
	Flu	77.4	536	31.1	37.1	12.8	46.8	15.6	15.3	52.5	11.7	5.4
	Phe	204	1 170	112.7	56.0	99.0	326.5	79.3	126.3	138.5	32.5	29.8
	Ant	57.2	845	22.2	17.9	22.7	59.4	15.4	22.9	27.8	8.0	7.0
	Fla	423	2 230	156.3	27.5	169.0	900.3	124.2	233.1	148.1	12.0	24.5
	Pyr	195	1 520	118.4	30.6	128.6	590.1	94.2	176.6	119.9	9.2	9.2
	BaA	108	1 050	69.0	12.9	79.0	369.6	56.1	95.8	55.3	5.2	14.3
	Chr	166	1 290	79.2	13.8	41.1	214.9	39.7	58.4	36.0	6.2	14.1
	BaP	150	1 450	14.0	14.0	14.0	30.7	14.0	14.0	17.3	14.0	14.0
	DBA	33	-	23.0	23.0	23.0	76.7	23.0	23.0	23.0	23.0	23.0
	Total	1 610	22 800	745.7	358.3	659.3	2 677.4	567.2	800.2	762.5	212.2	200.8
2014	PAH	TEC	PEC	PG	Le	FI	Mo	EID	OW	OE	Nc	Db
	Nap	176	561	113.1	240.9	160.0	512.7	255.8	122.8	94.3	80.6	32.4
	Flu	77.4	536	38.7	280.0	39.6	328.3	52.4	119.5	13.8	16.5	18.0
	Phe	204	1 170	77.8	184.0	78.9	385.2	207.5	57.6	42.6	105.7	57.1
	Ant	57.2	845	15.4	36.9	16.8	92.4	54.0	19.1	10.3	23.2	12.1
	Fla	423	2 230	99.7	239.2	85.5	1 086.3	479.4	103.3	37.4	105.3	67.4
	Pyr	195	1 520	74.0	212.1	95.5	786.6	358.7	80.5	30.6	81.9	30.6
	BaA	108	1 050	42.9	140.9	39.5	373.3	202.4	47.2	14.6	46.9	28.4
	Chr	166	1 290	37.3	89.7	25.1	241.9	127.3	30.3	13.8	32.0	25.0
	BaP	150	1 450	14.0	14.0	14.0	44.6	28.4	14.0	14.0	14.0	14.0
	DBA	33	-	23.0	23.0	23.0	76.7	23.0	23.0	23.0	23.0	23.0
	Total	1 610	22 800	536.0	1 460.7	577.9	3 928.0	1 788.8	617.2	294.4	529.1	307.8

Colour coordination indicates which guidelines were exceeded. TEC = threshold effects concentration; PEC = probable effects concentration

When the more sensitive Canadian guidelines (CCME, 2012) were applied, all the sites of 2013 surpassed three ISQG levels. Moroka was joined by two other sites with multiple congeners higher than the ISQG present in their sediments. Fleurhof had 10 out of 12 levels above the ISQG, followed by Moroka and Orlando West with 9/12. Eldorado Park and Orlando East had 6/12 and 5/12 exceedances respectively (Table 2.14). During the study of Roos *et al.* (2011), only 3 of the 13 sites exceeded the CCME guidelines. The congener that exceeded guidelines by all three these sites was benz(a)anthracene (BaA). Only one site had PAHs other than BaA with levels higher than the ISQG: naphthalene (Nap), phenanthrene (Phe), pyrene (Pyr), and benzo(a)pyrene (BaP) (Roos *et al.*, 2011).

Table 2.14: Sediment from the sites of Soweto and Lenasia compared to sediment quality guidelines of Canada (ISQG and PEL) (CCME, 2012). Guidelines exceedance indicated by shading

	PAH	ISQG	PEL									
				PG	Le	FI	Mo	EID	OW	OE	Nc	Db
2013	Nap	34.6	391	38.4	88.9	167.0	48.8	132.2	65.5	69.7	40.4	68.3
	Acey	5.87	128	0.9	2.0	2.0	5.0	3.5	5.3	3.4	0.4	1.0
	Acea	6.71	88.9	24.8	58.7	54.4	17.9	79.9	12.9	48.7	22.1	26.3
	Flu	21.2	144	10.0	26.3	30.5	36.6	19.5	28.9	25.4	5.2	6.2
	Phe	41.9	515	36.1	39.7	235.7	255.1	99.1	238.3	66.9	14.5	34.3
	Ant	46.9	245	7.1	12.7	54.0	46.4	19.2	43.1	13.4	3.6	8.1
	Fla	111	2 355	50.1	19.5	402.4	703.3	155.2	439.9	71.6	5.3	28.2
	Pyr	53	875	37.9	21.7	306.2	461.0	117.7	333.3	57.9	4.1	10.5
	BaA	31.7	385	22.1	9.2	188.0	288.8	70.1	180.8	26.7	2.4	16.5
	Chr	57.1	862	25.4	9.8	97.8	167.9	49.6	110.1	17.4	2.8	16.2
	BaP	31.9	782	4.5	4.5	4.5	9.8	4.5	4.5	5.5	4.5	4.5
	DBA	6.22	135	7.4	7.4	7.4	24.6	7.4	7.4	7.4	7.4	7.4
2014	PAH	ISQG	PEL	PG	Le	FI	Mo	EID	OW	OE	Nc	Db
	Nap	34.6	391	55.3	117.8	78.2	250.6	125.0	60.0	46.1	39.4	15.8
	Acey	5.87	128	21.6	49.3	23.9	205.9	45.0	57.2	0.4	0.4	0.4
	Acea	6.71	88.9	11.2	95.7	55.3	229.8	102.8	37.2	11.2	11.2	3.4
	Flu	21.2	144	18.9	136.9	19.4	160.5	25.6	58.4	6.8	8.1	8.8
	Phe	41.9	515	38.0	89.9	38.6	188.3	101.4	28.1	20.8	51.7	27.9
	Ant	46.9	245	7.5	18.0	8.2	45.2	26.4	9.3	5.0	11.3	5.9
	Fla	111	2 355	48.7	116.9	41.8	531.1	234.3	50.5	18.3	51.5	32.9
	Pyr	53	875	36.2	103.7	46.7	384.5	175.4	39.3	14.9	40.0	14.9
	BaA	31.7	385	21.0	68.9	19.3	182.5	98.9	23.1	7.1	22.9	13.9
	Chr	57.1	862	18.2	43.9	12.2	118.2	62.2	14.8	6.8	15.6	12.2
	BaP	31.9	782	6.9	7.6	9.5	29.8	26.6	19.8	18.7	10.3	15.1
	DBA	6.22	135	11.3	11.3	11.3	37.5	11.3	11.3	11.3	11.3	11.3

. ISQG = interim sediment quality guidelines; PEL = probable effects levels

An increase in concentration of PAH levels in sediments were noted in 2014. Subsequently, more guidelines were surpassed even to such an extent that some levels were higher than the PEL, e.g. at Moroka, Eldorado Park and Lenasia (Table 2.14). Interestingly, Dobsonville's sediment seemed to have been less polluted in 2014 than in 2013, exceeding only one of the 12 guidelines.

Sediment assessment indices

The potential ecological risk that the sediment of the study area posed to benthic organisms, in terms of PAH exposure is expressed by the SQG-I (Table 2.15). In terms of the MacDonald *et al.* (2000) guideline, Moroka sediment sampled in 2013 and 2014, posed high probability to be toxic to biota. The only other site of 2013 that posed a moderate risk was Orlando East whereas, Lenasia and Eldorado Park scored moderate probability for 2014.

Table 2.15: SQG-I results for sites from Soweto and Lenasia (2013 & 2014) in terms of the MacDonald *et al.* (2000) guidelines and the CCME (2012) guidelines. Shading according to index scale

Site	SQG-I (TEC) MacDonald <i>et al.</i> (2000)	SQG-I (ISQG) CCME (2012)
	2013	
Protea Glen	0.49	0.88
Lenasia	0.30	1.52
Fleurhof	0.43	3.85
Moroka	1.60	4.08
Eldorado Park	0.38	2.67
Orlando West	0.49	3.06
Orlando East	0.52	1.58
Nancefield	0.19	0.54
Dobsonville	0.18	0.86
Site	2014	
	2014	
Protea Glen	0.38	1.09
Lenasia	1.08	3.59
Fleurhof	0.41	1.73
Moroka	2.48	9.70
Eldorado Park	1.06	3.59
Orlando West	0.49	2.10
Orlando East	0.24	0.60
Nancefield	0.37	0.76
Dobsonville	0.24	0.48
Scale		
1.5<	High probability of toxicity to biota	
1.5-0.5	Moderate probability of toxicity to biota	
<0.5	Low probability of toxicity to biota	

Due to the more sensitive nature of the Canadian guidelines when they were applied in the calculations, more sites were deemed to have probable toxic effects (Table 2.15). Applying the Canadian guideline led to the deduction that only Dobsonville sediments (2014) posed no probable toxic risk to benthic organisms. Sediments from Lenasia, Fleurhof, Moroka, Eldorado Park and Orlando West had high probability of being toxic to biota for both years (Table 2.15). Orlando East changed from high to moderate probable toxicity between 2013 and 2014. Dobsonville also seemed to have improved as already mentioned. When the SQG-I was calculated for the sites of Roos *et al.* (2002) (in terms of the MacDonald *et al.*, 2000 guidelines), only one of the 12 sites' sediment showed to have a moderate chance of being toxic to biota. This site was in the same area as where this project collected the Protea Glen sample (SQG-I calculated low probability) (Table 2.15)

From the PAH concentrations in the sediment (Table 2.8), and consequently the sediment quality guidelines, PAH pollution of the sediment increased from 2013 to 2014. It can be expected that the ecological risk would increase concurrently, but that is not the case as seen with the SQG-I (Table 2.15).

The sediment quality index (SQI) (Table 2.16) indicates quality in terms of chemical contamination. Keep in mind that this index incorporates the magnitudes by which the determined levels exceeded the guideline levels. If no guidelines were breached, a SQI value cannot be calculated.

Table 2.16: Sediment quality index (SQI), in terms of PAH contamination, for the sites in the Soweto and Lenasia area for 2013 and 2014. Shading according to index scale

Sites	PEC MacDonald <i>et al.</i> , (2000)		ISQG (CCME, 2012)	
	2013	2014	2013	2014
Protea Glen			47.61	37.33
Lenasia		25.83	30.90	12.75
Fleurhof			4.53	30.79
Moroka	14.10	5.07	8.27	8.22
Eldorado Park		22.48	16.64	8.81
Orlando West		42.41	8.46	27.46
Orlando East			26.06	48.94
Nancefield			50.53	44.74
Dobsonville			37.69	75.61
Scale				
95<Excellent				
80-95 Good				
60-80 Fair				
45-60 Marginal				
0-45 Poor				

The only site with a SQI calculated for the 2013 sediments (in terms of the MacDonald *et al.*, (2000) guidelines) was Moroka. This site scored a poor sediment quality (SQ) (14.1%). The SQI values for 2014 showed four sites to have poor quality sediment: Lenasia, Moroka, Eldorado Park and Orlando West (Table 2.16). All the sites for both years calculated SQI values using the CCME guidelines. All the 2013 samples scored poor sediment quality, except for the sediments of Protea Glen and Nancefield, which were scored as marginal SQ (Table 2.16). Similarly, the SQI scores for 2014 all had poor SQ, except for sediments from Orlando East (50.53%, marginal) and Dobsonville, which had a fair sediment quality of 75.61% (Table 2.16).

SQI scores were calculated for six of the Roos *et al.* (2011) sites. Of these only one was fair, the rest were all in a poor state, achieving a SQI score below 45% (Scale in Table 2.16). The “poor” sites were comparable to the current study sites as they fell within the same urban areas as what we sampled. Lenasia had a poor SQI score, which was the same for Roos *et al.* (2011), but the present study’s Nancefield site was scored as marginal SQ, which was better than the poor SQI of the 3 sites in the area. The only difference in sediment quality between the two studies is at Protea Glen, where for this study a marginal SQ was calculated and for Roos *et al.* (2011) a fair SQI score was assigned.

The toxic equivalent quotient (TEQ_{TCDD}) calculated from the measured polycyclic aromatic hydrocarbons concentrations in sediments are reported in Table 2.17.

Table 2.17: Toxic equivalent quotient (TEQ_{TCDD}) results, calculated for the sediments of the sites from Soweto and Lenasia (2013 & 2014), compared to the TEQ guidelines of the CCME (2001). Guidelines exceedance indicated by shading

	ngTEQ/kg	
	2013	2014
Protea Glen	9.72	6.10
Lenasia	1.73	6.69
Fleurhof	5.54	5.82
Moroka	39.12	9.09
Eldorado Park	7.03	7.24
Orlando West	8.44	6.35
Orlando East	5.22	5.89
Nancefield	0.60	5.26
Dobsonville	2.07	6.16

ISQG	0.85 ngTEQ/kg	PEL	21.5 ngTEQ/kg
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The TEQ_{TCDD} values of all the 2014 sites were higher than the Canadian interim sediment quality guideline (Table 2.17), indicating that the PAHs in the sediment is expected to be harmful to aquatic life. Moroka sediment from 2014 was the only site that exceeded the PEL-TEQ guideline for both sampling seasons (Table 2.17). The only sediments that had a TEQ_{TCDD} value below the guidelines was from Nancefield (2013). These overall results indicate that there are AhR mediated toxic responses to be expected in aquatic organisms.

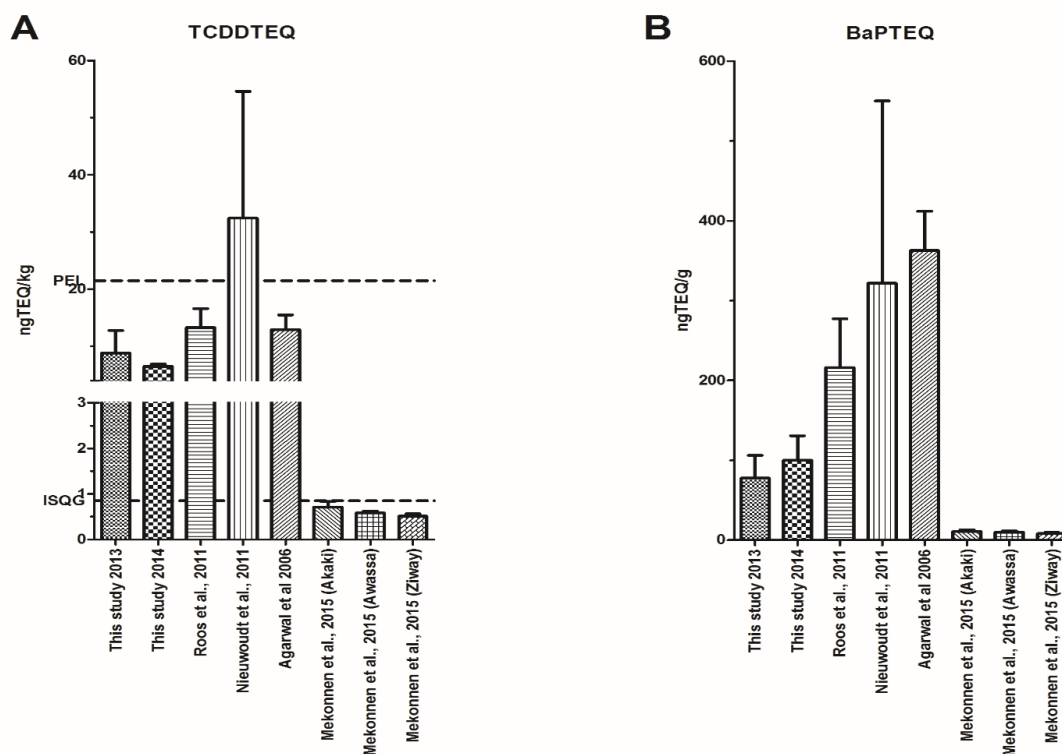


Figure 2.6: Mean toxic equivalent quotient (TEQ) results calculated from literature A) TEQ_{TCDD} compared to the TEQ guidelines of the CCME (2001); B) TEQ_{BaP}

When comparing the mean TEQ_{TCDD}s of this study to those calculated from literature (Table 2.7), most exceeded the CCME (2001) ISQG level, except for the Ethiopian sites—Lake Ziway, Lake Awassa and the Akaki River (Figure 2.5A). The mean TEQ_{TCDD} reported by Nieuwoudt *et al.*, (2011) was the only site to have exceeded the upper PEL level. The Soweto and Lenasia sites for both 2013 and 2014 fell between the ISQG/PEL guidelines. Nieuwoudt *et al.* (2011) had such a high TEQ value, because the measured concentrations of BkF and BaP were high, relative to the remaining PAHs. These congeners have the highest TEF values according to Villeneuve *et al.* (2002) (Table 2.5). A temporal decrease is seen in the mean TEQ_{TCDD} within the sites of this study, but a temporal increase in the TEQ_{BaP} (Figure 2.6B). The Nieuwoudt *et al.* (2011) sites had the highest TEQ_{BaP} followed by the sites in the Yamuna River India (Agarwal *et al.*, 2006). The Roos *et al.* (2011) sites for both the TEQ_{TCDD} and TEQ_{BaP} were higher than the sites from the present study, suggesting a decrease in toxicity in the area over time.

The risk that PAHs in the sediment have toward fish health as shown by the TEQ_{FPF}, using the fish potency factors derived by Barron *et al.* (2004), were also compared to the Canadian interim sediment quality guideline (Table 2.18). All of the sites exceeded the upper CCME (2001) TEQ sediment quality guideline (PEL), showing that the sediments were not only potentially toxic to aquatic organisms (Table 2.17), but also specifically to fish—and potentially higher organisms. Moroka's TEQ_{FPF} value was 30 and 21 times higher, in 2013 and 2014 respectively, than the guideline (Table 2.17). Eldorado Park and Lenasia (both 2014 sediments) also exceeded the guideline with more than 10 fold,

Eldorado Park by 12 times and Lenasia by 11. The remaining sites for both sampling years exceeded the guideline by a range of 1–7 times.

Table 2.18: Toxic equivalent quotient (TEQ_{PPF}) for dioxin-like toxicity towards fish results, calculated for the sediments of the sites from Soweto and Lenasia (2013 & 2014), compared to the TEQ guidelines of the CCME (2001). Guideline exceedance indicated by shading

	ngTEQ/kg	
	2013	2014
Protea Glen	183.3	117.5
Lenasia	36.4	269.8
Fleurhof	123.1	130.7
Moroka	755.1	525.4
Eldorado Park	124.2	294.4
Orlando West	184.1	112.8
Orlando East	126.3	79.2
Nancefield	22.7	107.5
Dobsonville	48.47	93.3

ISQG	0.85 ngTEQ/kg	PEL	21.5 ngTEQ/kg
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2.4 Conclusion

It is clear, from the combined results, that there are sites that were severely affected by the PAHs in the Soweto and Lenasia study area including Lenasia, Moroka, Eldorado Park and Orlando West. The area of most concern is Moroka—the site that had the highest PAH concentrations for both years. Moroka had pyrogenic sources, mainly dominated by 4-ring congeners and was the site that exceeded the most guidelines (both sample sets). Its toxicity assessment indicated that it is likely toxic to benthic biota (from the guideline scores and the SQG-I). Moroka's sediment posed harmful risk to aquatic organisms and specifically to fish. The risk assessment over all indicates that the sediments of the Soweto and Lenasia area may be harmful to the aquatic organisms residing in the area.

Our results were comparable to that of Roos *et al.* (2011) who also studied sites within Soweto and Lenasia. The SQI and SQG-I calculated for sites for this study were better than for Roos *et al.* (2011) where comparable. This means, according to the indices, that the sediment quality has improved and the potential of toxicity to benthos has decreased. The presence of PAHs in the Soweto and Lenasia area (in all matrices investigated) was confirmed by the instrumental analysis. Although the wetland bird eggs did not yield sufficient data, except for some of the LPAHs, the conclusion can be made that the wild birds have often been exposed to PAHs. This was also confirmed with the analysis of the OH-PAH metabolites in the fish bile, indicating exposure to fluorene and pyrene and to an extent phenanthrene.

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Chapter 3: Quantifying the aryl-hydrocarbon mediated toxicity of polycyclic aromatic hydrocarbons in the sediments of Soweto and Lenasia using the H4IIE-*luc* reporter gene bioassay

3.1 Introduction

3.1.1 Relevance of bioassays

Chemicals introduced into the environment occur as complex mixtures. These complex mixtures behave in various manners towards one another and the environment (Hecker & Giesy, 2011). Measuring the levels of these chemicals within an environmental sample is important to determine the level of pollution in that sample (Hilscherova *et al.*, 2001). However, with chemical determination, compounds can only be analysed if applicable analytical methods and standards exist (Garrison *et al.*, 1996). The instrumental analysis of an environmental sample also does not take into account the interactions and synergy of the mixture and provide limited information on their potential biological effects (Hilscherova *et al.*, 2000; Vanderperren *et al.*, 2004). Bioassays address this limitation of instrumental analysis and provide the estimations of the biological effects substances have on living cells and tissues (Hilscherova *et al.*, 2000; Behnisch *et al.*, 2002; Koh *et al.*, 2005). Various types of bioassays exist that investigate different biomarker endpoints. Bioassays were developed to answer the need for rapid and relatively inexpensive methods that detect and estimate relative potencies of complex mixtures (Baston & Denison, 2011) and quantifiably analyse the responses in a biological manner (Behnisch *et al.*, 2002). One of the many types of bioassays is the reporter gene *in vitro* cell bioassays. Cell bioassays offer a rapid and sensitive solution to the limitations of instrumental analysis, with ability to estimate total biological activity of a mixture of chemicals with the same mode of action (Hilscherova *et al.*, 2000).

In vitro cell bioassays are used to assess different modes of toxicity as endpoints such as genotoxicity, endocrine disruption and activation of the aryl-hydrocarbon (Ah) receptor. The DNA-repair-deficient chicken DT40 B-lymphocyte cell line is used to screen and characterise genotoxicity of compounds (Ji *et al.*, 2009). Similarly, the Ames test assesses genotoxic effects like point and frame shift mutations using the Salmonella TA98 and TA100 strains respectively (Mortelmans & Zeiger, 2000). The effect of endocrine disrupting chemicals (EDCs) can be measured with various cell lines, focusing on different sections of the endocrine system. The H295R cell line measures endocrine disrupting activity by modulation of the steroidogenesis pathway (Hecker *et al.*, 2006). Oestrogen activity is quantified using the MVLN oestrogen receptor-mediated luciferase reporter gene bioassay (Demirpence *et al.*, 1993). The androgenic chemical effects are measured similarly by means of the MDA-kb2, androgen receptor-mediated luciferase reporter gene bioassay (Wilson *et al.*, 2002). The Ah-ligand mediated toxic responses are quantified by measuring ethoxyresorufin-O-deethylase (EROD) activity (CYP1A1 activity) using the RTL-W1 cell line (Lee *et al.*, 1993). The H4IIE-*luc* cell line

also measures the CYP1A1 activity as endpoint but quantifies the activity with a receptor-mediated luciferase reporter gene bioassay (Sanderson *et al.*, 1996).

3.1.2 Polycyclic aromatic hydrocarbons and cellular responses

Polycyclic aromatic hydrocarbons (PAHs) are known carcinogens and have adverse effects on human and wildlife health (Balch *et al.*, 1995, Spink *et al.*, 2008, Larsson *et al.*, 2012). Some PAHs are toxic by acting through the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor that mediates many of the biological effects of these compounds (Denison & Heath-Pagiuso, 1998; Baston & Denison, 2011), and a number of PAHs may also interfere with the oestrogen receptor (ER)–mediated signalling (Machala *et al.*, 2001).

The native PAHs that bind to the AhR are: benz(a)anthracene [BaA], chrysene [Chr], benzo(b)fluoranthene [BbF], benzo(k)fluoranthene [BkF], benzo(a)pyrene [BaP], indeno(1,2,3-cd)perylene [InP], and dibenz(ah)anthracene [DBA] (Villeneuve *et al.*, 1999) and are collectively referred to as the carcinogenic PAHs (CPAHs). The AhR-ligands enter the cytoplasm of cells and bind to the AhR complexes—unbound AhRs are complexed with heat shock proteins (HSP) (Denison & Heath-Pagiuso, 1998; Tian *et al.*, 2015) (Figure 3.1). Upon binding, the heat shock proteins dissociate and activates the complex (Hilscherova *et al.*, 2000). The activated complex is translocated into the nucleus, where it rapidly forms a heterodimeric nuclear complex (Safe & Wormke, 2003) with the aryl-hydrocarbon receptor nuclear translator (ARNT) protein (Hilscherova *et al.*, 2000; Safe & Wormke, 2003; Tian *et al.*, 2015) (Figure 3.1). The dimer-complex binds onto the dioxin response element (DRE)—a specific DNA sequence in the CYP1A1 promoter (Denison & Heath-Pagiuso, 1998; Hilscherova *et al.*, 2000; Safe & Wormke, 2003; Denison *et al.*, 2004). Attachment to the DRE leads to the transcription of the adjacent responsive genes (Hilscherova *et al.*, 2000), resulting in the upregulating or induction of proteins responsible for detoxification (Baird *et al.*, 2005) (Figure 3.1). Cytochrome enzymes metabolise the PAHs by addition of an oxygen atom and in most cases this oxygen is reduced to a hydroxyl group (Tuvikene, 1995), and further metabolism can result in epoxide-metabolites. The PAH-epoxide-metabolites are capable of binding to DNA during this stage of detoxification (Baird *et al.*, 2005) causing mutagenesis. The reactive metabolites are conjugated by several enzymes and anti-oxidants: glutathion-S-transferase (GST), uridine 5-diphosphate-glucuronosyltransferase (UDP-GT) and glutathione (GSH) (Tuvikene, 1995; Baird *et al.*, 2005). These enzymes complete biotransformation phase II—reducing the toxicity of the compound and making it easier to excrete.

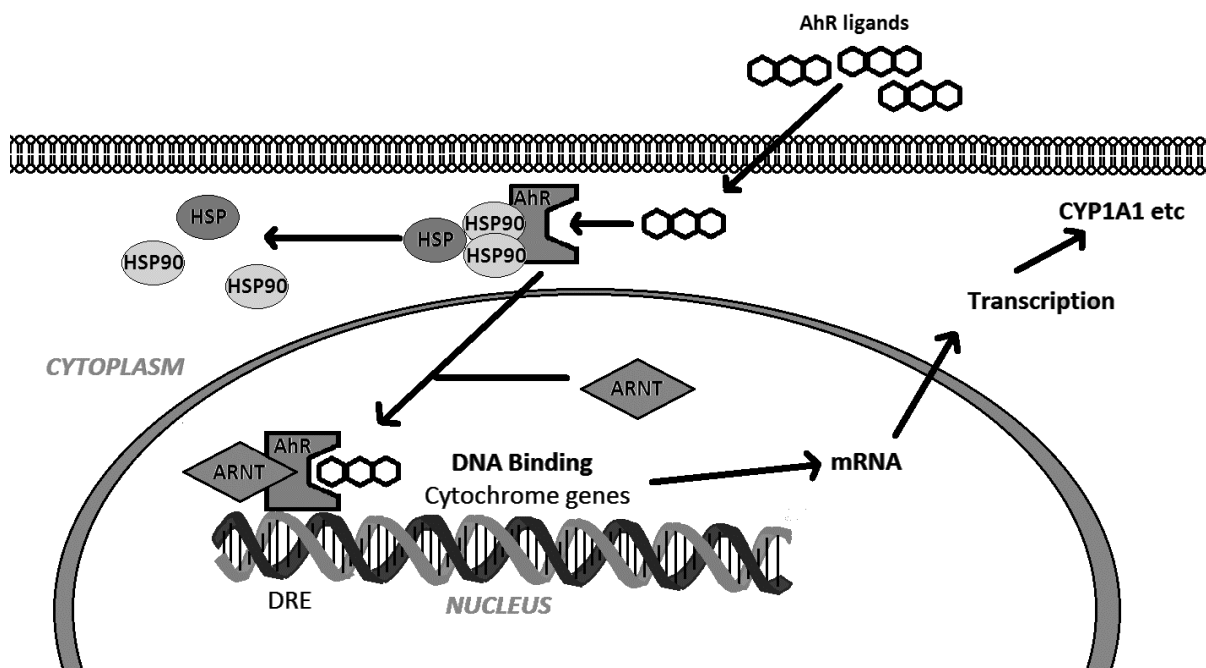


Figure 3.1: The mechanism of Ah-receptor mediated response in cells (adapted from Hilscherova *et al.*, 2000)

The activation of the AhR has been reported to exhibit anti-oestrogenic cross-talk with the oestrogen receptor (Chen *et al.*, 2001), blocking the receptor (ER) (Safe, 2001). This cross-talk mechanism between the AhR-ER α is complex, but involves the inhibition of oestradiol responsive genes by DRE structures that bind to the AhR complex and so disrupting the oestrogen action through multiple mechanisms (Navas & Segner, 2000; Safe *et al.*, 2000), which may lead to detrimental effects. Thus the ability of PAHs to bind to DNA is not their only role in carcinogenesis, but can include disruption in hormone systems (Baird *et al.*, 2005).

3.1.3 Polycyclic aromatic hydrocarbons and the H4IIE-*luc* reporter gene bioassay

Dioxin-like toxicity (AhR mediated toxicity) of PAHs was specifically investigated for the sediment sampled in Soweto and Lenasia. The AhR mediated responses of PAHs can be quantified with the H4IIE-*luc* reporter gene bioassay. The H4IIE-*luc* bioassay results represent the total amount of bio-activity due to AhR-ligands present in the environmental sample as a result of gene activation. The H4IIE-*luc* reporter gene bioassay consists of rat hepatoma cells that had been stably transfected with a firefly luciferase reporter gene. The bioassay indirectly measures cytochrome P450 induction, as mentioned above, which is an endpoint in the AhR mediated response (Hilscherova *et al.*, 2000; Denison *et al.*, 2004). The luciferase gene was inserted downstream of the cytochrome genes and the DRE in the H4IIE-*luc* cells. In the presence of luciferin (substrate for luciferase), light is produced (Figure 3.2). The amount of light that is released is directly proportional to the amount of AhR agonists present in the sample (Hilscherova *et al.*, 2000).

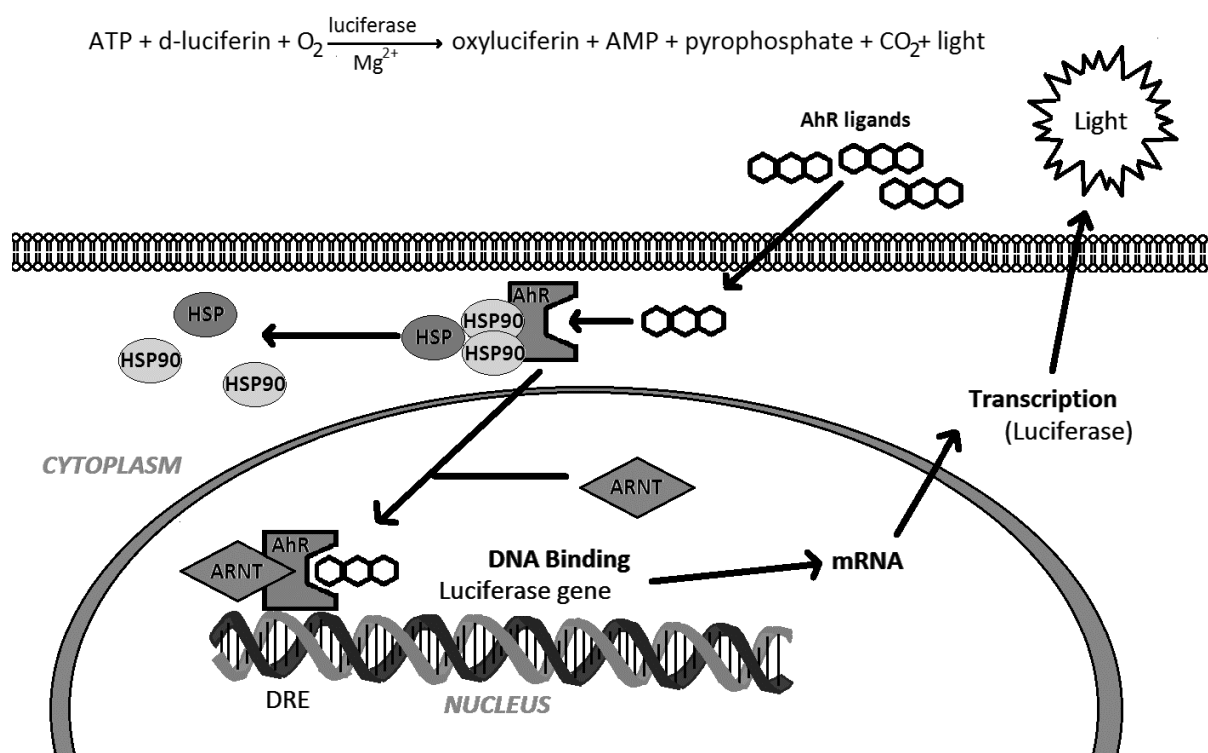


Figure 3.2: The mechanism of Ah-receptor mediated luciferase reporter gene response of the H4IIE-*luc* bioassay (adapted from Hilscherova *et al.*, 2000)

The toxicity of the sample is quantified in terms of the reference compound, 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (2,3,7,8-TCDD). This quantification is based on the assumption that the investigated sample is a diluted form of the reference material, or a mixture of chemicals behaving like the reference compound, 2,3,7,8-TCDD, which is the most toxic congener of the AhR binding compounds (Yoo *et al.*, 2006). The results are given as relative potency values (REP) or TCDD-equivalence.

The results obtained from using this reporter gene bioassay 1) establish whether there are AhR agonists present in a sample and 2) quantify the toxicity of that sample relative to TCDD. The chemical data obtained from instrumental analysis identify the possible AhR agonists and concentrations of occurrence. The bioassay and chemical analysis complement each other: the relative toxicity quotient (TEQ) can be calculated with the chemical data and compared to the biological toxicity equivalent which is the REP or TCDD-equivalence from the assay. These equivalents can be used to assess the risk the compounds pose to humans and the environment (Yao *et al.*, 2002) and can be compared to environmental guidelines, such as international sediment quality guidelines.

3.2 Materials and Methods

3.2.1 Sample collection

Composite sediment samples were collected from the nine sediment sampling sites proposed for this project (Figure 1.2). These sites are Protea Glen (PG), Lenasia (Le), Fleurhof (Fl), Moroka (Mo), Eldorado Park (EID), Orlando West (OW), Orlando East (OE), Nancefield (Nc) and Dobsonville (Db).

3.2.2 Sample extraction

Processed sediment samples (see section 1.4.1), were extracted chemically using the same methods as for instrumental analysis: accelerated solvent extraction, gel permeation chromatography and solid phase extraction (see section 2.2.2). However, no deuterated standards were added prior to extraction because these standards would also bind to the AhR and would elicit a response from the cells indistinguishable from that of the AhR ligands extracted from the sediment producing false positive responses. The final extract was reconstituted into 1 mL hexane.

3.2.3 Maintenance of H4IIE-*luc* cell culture

During routine maintenance of the cell culture, aseptic conditions were followed. The H4IIE-*luc* cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) with L-glutamine and 10% glucose, and without phenol red and sodium bicarbonate. The DMEM was supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich) and 0.04 M sodium bicarbonate. The cells were kept in tissue culture dishes in an incubator at 37°C in humidified air (5% CO₂: 95% air). The cells were rinsed with phosphate buffered saline (PBS) (Sigma-Aldrich) and treated with 1.5 mL trypsin (Highveld Biological) to passage them (Aarts *et al.*, 1995).

3.2.4 H4IIE-*luc* reporter gene bioassay

The method of the luminescence bioassay is a modified version of that described by Tillitt *et al.* (1991). The interior 60 wells of flat bottom 96 well microtiter plates were seeded with the 250 µL H4IIE-*luc* cells at a density of 80 000 cells/mL. The external 36 wells were filled with 250 µL phosphate buffered saline (PBS), to create a homogenous micro environment across each cell containing well. The plates were incubated at 37°C in humidified air (5% CO₂: 95% air) for 24 hours before dosing. A series of three times diluted sample were dosed in triplicate at a volume of 2.5 µL per well, to generate a dose-response curve (Whyte *et al.*, 2004). Along with the environmental samples, controls were also dosed at 2.5 µL/well: a series of four times diluted 2,3,7,8-TCDD [160, 40, 10, 2.5, 0.625, 0.157 nM] (positive control); a 3 well solvent control (hexane); and a blank control, containing only cells and culture media (Hilscherova *et al.*, 2003). After 72 hr incubation the cells were microscopically inspected for viability and confluency. The culture media was removed and the cells were washed with Mg²⁺ and Ca²⁺ supplemented PBS. The ion supplemented PBS ensures that

magnesium and calcium ions were present in excess, which are limiting factors during the light forming reaction of the bioassay (Hilscherova *et al.*, 2000). The cell membranes were lysed using a lysis buffer for mammalian cell cultures (CellLytic™, Sigma-Aldrich). The plates were frozen at -80°C for 30 minutes, to ensure that the cells have ruptured. The luminescence was recorded in a plate reader (Berthold multi-mode micro plate reader, model-LB941). The plate reader automatically injected 100 µl luciferase activating reagent (LAR) (20 mM tricine (Sigma-Aldrich), 1.07 mM $\text{Mg}(\text{CO}_3)_2 \cdot \text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ (Sigma-Aldrich), 2.67 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma-Aldrich), 0.1 mM EDTA-disodium salt (Sigma-Aldrich), 33.3 mM dithiothreitol (Sigma Aldrich), 270 µM coenzyme A (Sigma-Aldrich), 530 µM ATP (Sigma-Aldrich) and 470 µM beetle luciferin (Malford)) (Villeneuve *et al.*, 1999). The digestion of luciferin by luciferase produced light, measured in relative light units (RLUs).

3.2.5 Calculating bioassay equivalence (BEQs)

Dose-response curves were prepared for the samples as well as the positive control by plotting the logarithm of the concentration (in the case of the control) or logarithm of the volume (in the case of the sample) on the x-axis and the %TCDDmax on the y-axis. The %TCDDmax was calculated by expressing the luminescence of each sample dilution as a percentage of the maximum luminescence generated by the positive control (2,3,7,8-TCDD) (Sanderson & Giesy, 1998). The relative effects potencies (REP) for the samples were calculated by dividing the effects concentration (EC20, EC50, EC80) of the positive control by the EC20-80 of the sample (Finney, 1971; Villeneuve *et al.*, 1999) (The unit of these REPs is mass TCDD-equivalents/volume extract). Reporting all three REPs is necessary as it cannot be assumed that the complete mixture of the environmental samples will respond the same as TCDD (Villeneuve *et al.*, 2000). The REP values were back calculated to represent the TCDD-eq in terms of the mass sediment extracted (Koh *et al.*, 2005). The TCDD-eq calculated from bioassay results are commonly known as bioassay equivalents (BEQ) (Baston & Denison, 2011). The limit of detection (LOD) for the H4IIE-*luc* bioassay was calculated by determining the mean EC₀ for the TCDD response curves. The utmost intercept with 95% confidence was calculated and used as the LOD, back calculated to a ngTCDD/g value (Villeneuve *et al.*, 1999; Thomsen *et al.*, 2003).

3.2.6 MTT viability assay

A viability test was performed parallel to the luminescence bioassay and dosed with the same series of samples and controls as in the bioassay. The 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) viability assay was used to prevent false negative results in the luminescence bioassay, where low or below LOD responses in the H4IIE-*luc* bioassay might not necessarily be due to the absence of AhR agonists, but rather from cytotoxicity. The MTT assay mechanism involves the metabolism of yellow MTT solution by the living cells into blue formazan crystals. The viability of the cells was determined by spectrophotometric quantification of formazan formation (Vistica *et al.*, 1991).

The MTT plates were seeded, dosed, and incubated in the same manner as the luminescence plates. On the fifth day, the MTT plates were rinsed with PBS, but did not receive lysis buffer. The negative control cells were killed with 100 μ L MeOH before all the wells received 100 μ L MTT solution and incubated for 30 min at 37°C, 5% CO₂. The cells were inspected, to determine if the formazan crystals had formed. The excess MTT solution was discarded and the formed crystals dissolved with 200 μ L dimethyl sulfoxide (DMSO) (Sigma-Aldrich). After a waiting period of 15 minutes at room temperature, the optical density (OD) at 560 nm was measured (Berthold multi-mode micro plate reader, model-LB941) (Vistica *et al.*, 1991).

Viability was calculated by expressing the OD of the wells that received samples were expressed as a percentage of the OD from the control wells, representing 100% viable cells. Statistical differences between the control cells (100% viability) and the exposed cells were tested using single-tailed Mann-Whitney U tests where p-values lower than 0.05 was considered significant.

3.3 Results and discussion

The H4IIE-*luc* reporter gene bioassay results of the low flow season of 2013 and 2014 are presented and discussed below. Luciferase induction for both years was reproducible with coefficients of variance (CV) less than 11%. The limit of detection for the sediments was 14.8 ng/g TCDDeq/g (95% confidence).

The response elicited by sediment extracts was reported as the maximum response on the dose-response curve (%TCDDmax) relative to TCDD as the standard (Figure 3.3A). The concentrations of the biological equivalents (BEQ) were quantified by comparing the dose-response relationship of the sediment extract (known mass extracted) to the TCDD standard curve and the BEQs were quantified as REP20, 50 and 80 (Table 3.1).

The cell viability reported in Table 3.1 is for the raw extracts only. The 2013 samples that were cytotoxic to the cells were Protea Glen, Moroka, Eldorado Park, Orlando East and -West, and Nancefield (Table 3.1). Cytotoxicity was again seen in the 2014 sample for Moroka, Eldorado Park and Orlando East. The Lenasia site registered cytotoxicity only for the 2014 sample (Table 3.1). Evidence of cytotoxicity is also noticeable in the steep decrease at the far right on the dose-response curves (Figure 3.3B and C). A number of the raw extracts' dilutions were also cytotoxic [specifically, Orlando West 2013 and Moroka 2013 in Figure 3.3B and Eldorado Park 2014 (Figure 3.3.C)]. Thus the maximum elicited responses seen on the dose-responses (Figure 3.3) for the extracts that were cytotoxic are from the diluted samples (see Table 3.1). This indicates that the extracts contained either AhR-ligands that were cytotoxic at high concentrations and/or non-AhR binding compounds that killed the cells. The consequence of this is that the maximum elicited response might very well have been higher than what was reported here. Schirmer *et al.* (1998) revealed that the 2- and 3-ring PAHs were directly cytotoxic to a rainbow trout gill epithelial cell line, RTgill-W1, specifically Nap, Acey, Acea, Flu and Phe and others demonstrated that the toxicity of some PAHs are additive (Beach &

Harmon, 1992; Muñoz & Tarazona, 1993; Schirmer *et al.*, 1998). Therefore, there is a real likelihood that the observed cytotoxicity could have been due to the PAH contents of the extracts even though the concentrations of individual PAH congeners in the sediment (Table 2.8) were not as high as those Schirmer *et al.* (1998) observed cytotoxicity for. It is the additive effect that could have contributed to the cytotoxicity observed in our bioassays.

The maximum luciferase activity of viable cells per site was given as %TCDDmax (Table 3.1) and were included in the table to give an indication of the approximate reactivity, but since the mass of the extracted sediment had not been brought into consideration yet (as for the REP values) the %TCDDmax cannot be used to compare between sites and over time. The 2013 sediment bioassay had a %TCDDmax range of 29.7% and 106.2%. The range for 2014's %TCDDmax was lower than 2013 and varied between 30.8% and 76.1%. Lenasia was also the only site to have a value higher than 50% in 2014 (Table 3.1).

As mentioned earlier in this chapter, it is important to report all three REP values (Table 3.1), as environmental samples do not always respond similar to the reference compound. However, REP50 values were not measured for all the sites, some were extrapolated values, and therefore only the REP20s will be used for comparison between sites (Figure 3.4).

The highest REP20 value was calculated for the Moroka sediments (221 pgBEQ/g) (Table 3.1), followed by Eldorado Park and Orlando East—all greater than 100 pgTCDD-eq/g (Table 3.1). Sediments from Orlando West and Nancefield had responses below the limit of quantification. Moroka was once again the site with the highest REP20 for 2014, followed by Eldorado Park and Lenasia (Table 3.1). Protea Glen was below the limit of quantification and all the remaining sites calculated a REP20 below 100 pgTCDD-eq/g.

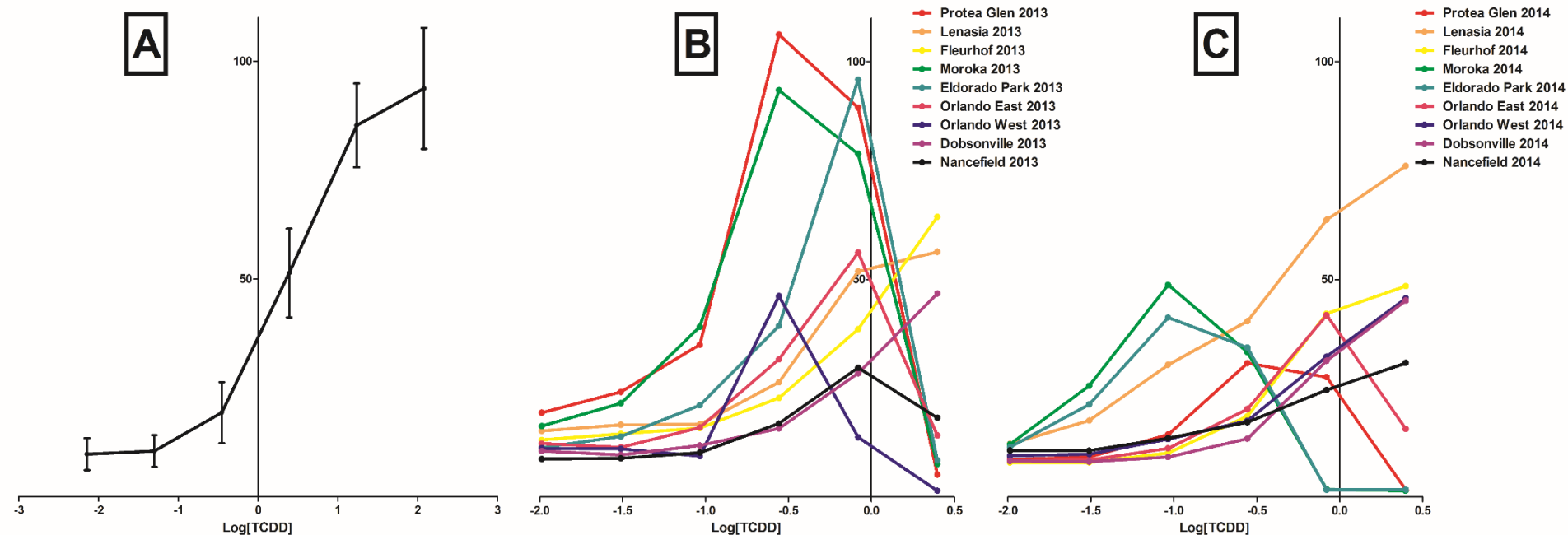


Figure 3.3: Luciferase activity (%TCDDmax) for: A) 2,3,7,8-TCDD standard; B) 2013 sediments; C) and 2014 sediments. Bars in A is standard deviation (SD). SD bars were omitted from the other graphs for the sake of simplicity

Table 3.1: H4IIE-*luc* reporter gene bioassay results showing %TCDDmax and BEQs (REP20, -50, -80) after exposure to sediment extracts (extrapolated data in italics; the greatest values per column in bold). Viability results were also included as %cell viability

	2013 Sediment					2014 Sediment				
	%Cell viability	%TCDDMAX	REP20 (pgBEQ/g)	REP50 (pgBEQ/g)	REP80 (pgBEQ/g)	%Cell Viability	%TCDDMAX	REP20 (pgBEQ/g)	REP50 (pgBEQ/g)	REP80 (pgBEQ/g)
Protea Glen	0%*	106.20	91.6 ± 12.4	273.2 ± 76.4	838.5 ± 397.6	0%*	30.8	<LOQ		
Lenasia	108%	56.35	25.4 ± 2.2	30.0 ± 10.6	37.1 ± 22.1	84%	76.1	352.1 ± 75.9	290.2 ± 12.7	245.8 ± 45.3
Fleurhof	147%	64.39	51.7 ± 14.4	48.5 ± 17.0	50.0 ± 53.9	116%	48.5	55.9 ± 8.1	62.4 ± 21.5	80.0 ± 65.1
Moroka	0%*	93.44	221.1 ± 24.2	642.7 ± 236.3	1410.7 ± 434.4	0%*	48.8	816.8 ± 11.0	972.1 ± 301.6	1271.7 ± 657.1
Eldorado Park	7%*	95.85	137.2 ± 18.3	331.6 ± 13.6	745.7 ± 90.2	5%*	41.2	679.1 ± 28.6	446.7 ± 174.3	360.7 ± 167.3
Orlando East	25%*	56.17	121.7 ± 5.9	128.0 ± 29.0	138.1 ± 57.7	60%*	41.8	78.7 ± 19.5	58.2 ± 42.4	49.2 ± 56.1
Orlando West	0%*	46.15	<LOQ			76%	45.6	45.6 ± 6.4	32.1 ± 16.4	28.8 ± 25.7
Dobsonville	90%	46.78	31.9 ± 0.8	25.3 ± 20.8	28.7 ± 42.4	82%	45.1	34.9 ± 2.5	33.2 ± 6.0	31.9 ± 9.2
Nancefield	16%*	29.72	<LOQ			112%	30.8	6.7 ± 1.4	1.2 ± 0.9	0.2 ± 0.2

*Cytotoxicity of the raw extract, which is significantly different to the control (p<0.05)

It is immediately apparent that the BEQs measured for 2014 sediments were greater than 2013 (Figure 3.4). Moroka 2014 and Eldorado Park's BEQ are 4 times greater than the previous year's (Figure 3.4). The only site that had a notable temporal decrease was Protea Glen, from 91.6 pgBEQ/g to below the limit of detection. Protea Glen was also the only site from the 2014 sampling that was below the LOQ (Table 3.1). In addition to Moroka and Eldorado Park, Lenasia showed a great increase over the two sampling years (Figure 3.4). Orlando West and Nancefield also had temporal increases from below the LOQ to quantifiable BEQs, even if they were low (Figure 3.4). The definite increase in 2014 BEQs suggests that there must have been an increase in AhR-ligands between the sampling events. The extraction method followed to isolate the PAHs works very effective because there is a strong correlation between the BEQs and the concentration of the CPAHs (Table 2.8) of each site (Spearman's correlation $r = 0.78$, $p = 0.0002$) (Figure 3.5A). Thus, the luciferase activity seen in the bioassays could be mainly attributed—but not exclusively—to PAHs. Other compounds such as PCBs could have been co-extracted and isolated with the PAHs based on their size during the GPC step (see section 2.2.2), and dl-PCBs definitely bind to the AhR (Hilscherova *et al.*, 2000).

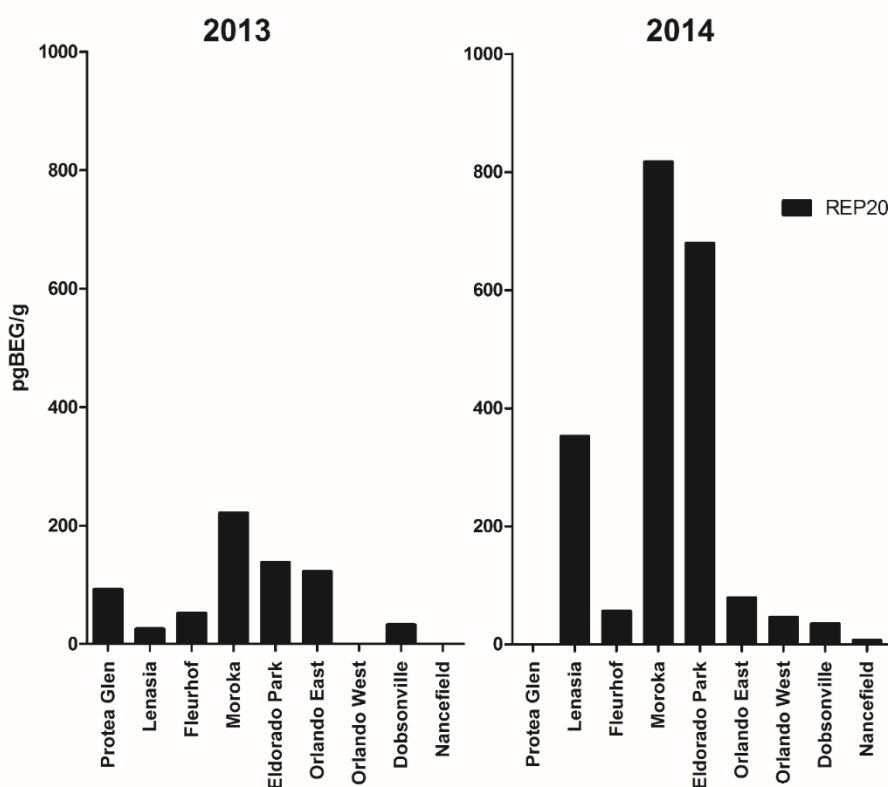


Figure 3.4: Relative potencies (REP20) calculated for sediments collected at each site in 2013 and 2014

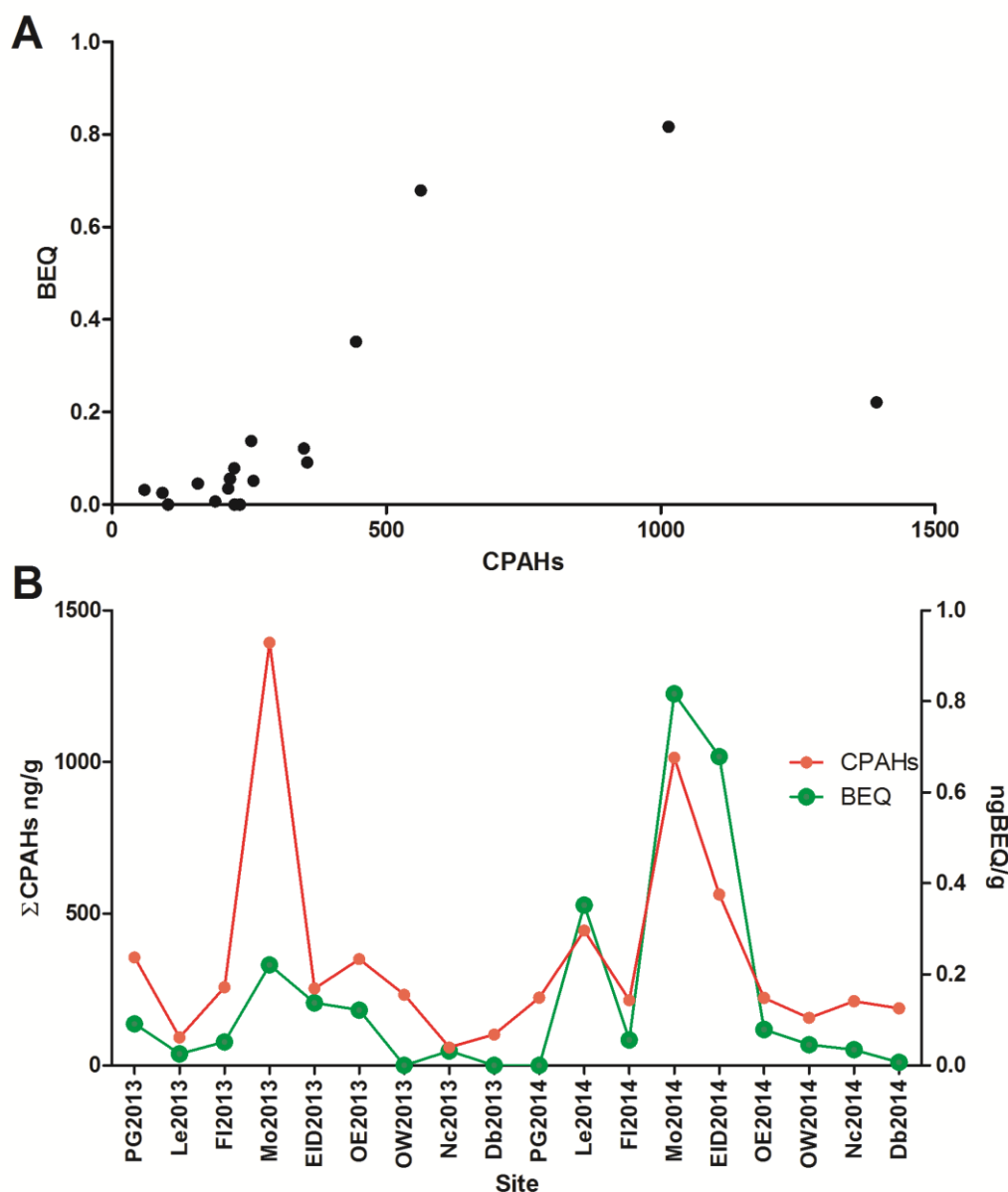


Figure 3.5: BEQs compared to the Σ CPAHs for sediments of 2013 and 2014: A) Spearman's correlation graph; B) dual axis line graph showing the relationship between BEQs and Σ CPAHs

One clear example of the presence of compounds other than PAHs present in the extract is that of the Moroka 2013 sample (Figure 3.5B). The highest levels of Σ CPAHs were measured in this sample (Table 2.8), but its BEQ was not the highest (Figure 3.5). It may be that compounds with a higher affinity for the AhR but less potent than the PAHs prevented many PAHs from binding to the AhR (Brown *et al.*, 1994; Petrulis & Bunce, 2000), causing only a small volume of luminescence. This binding to the AhR may even lead to an inhibitory effect. In the case of Lenasia, Moroka, and Eldorado Park sediments of 2014, the BEQs were greater than the Σ CPAHs (Figure 3.5). It is likely that other AhR-ligands were present at these sites and contributed to a higher BEQ along with the CPAHs.

The toxicity of the sediments has already been gauged against international sediment quality guidelines (see section 2.2.4). The TEQs calculated in chapter 2 were based on instrumental analysis, and therefore reflected the toxicity of the measured PAHs relative to 2,3,7,8-TCDD. The BEQs calculated with the H4IIE-*luc* bioassay are also in terms of 2,3,7,8-TCDD toxicity, but reflect biological reactions to the entire extract contents and is not related to chemical quantification of the separate congeners. The BEQs were also compared to the Canadian interim sediment quality guidelines (ISQG) for dioxin-like compounds, reported in Table 3.2. Only Dobsonville 2014 had the same toxicity assessment results between the TEQs and BEQs (Table 2.17 & 3.2). All the other sites were well above the upper probable effect level (PEL) guideline (Table 3.2). Moroka and Eldorado Park were the two sites that had the greatest BEQs, and subsequently exceeded the guidelines the farthest. Moroka's 2013 BEQ was 260 times higher than the lower guideline, and its 2014 BEQ 960 fold the ISQG (Table 3.2). The upper PEL was exceeded by a factor of 10 and 30 over the two consecutive years respectively. Similarly Eldorado Park surpassed the lower ISQG by 160 and 800 times, and the PEL threshold six and 30 times, for 2013 and 2014. This is of concern as it shows that there is a definite risk to benthic organisms in the aquatic systems of Soweto/Lenasia, exposed to the sediments.

Table 3.2: Bioassay equivalent (BEQ) results, calculated for the sediments collected at the Soweto and Lenasia sites (2013 and 2014) with the H4IIE-*luc* bioassay, gauged against the TEQ guidelines of the CCME (2001). Shading indicates which guidelines were exceeded

	pgTEQ/g	
	2013	2014
Protea Glen	91.6	LOQ
Lenasia	25.4	352.1
Fleurhof	51.7	55.9
Moroka	221.1	816.8
Eldorado Park	137.2	679.1
Orlando West	121.7	78.7
Orlando East	LOQ	45.6
Nancefield	31.9	34.9
Dobsonville	LOQ	6.7
ISQG	0.85 pgTEQ/g	PEL
		21.5 pgTEQ/g

Roos *et al.* (2011) sampled in the same location as the Nancefield site (Figure 1.2), for two consecutive years. The BEQs reported decreased between the two surveys, from 161.24 pgBEQ/g to 86 pgBEQ/g (Roos *et al.*, 2011). The higher BEQ is comparable to the levels of this study (Table 3.1). It is important to note that the Roos *et al.* (2011) study analysed a different fraction of the extract. These authors treated their extracts with sulphuric acid, before running the bioassay. This step would have destroyed most AhR-ligands (non-persistent) including the PAHs. The only compounds that would have survived such a treatment would be the very persistent polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs).

Less AhR-ligands would mostly, but not always, lead to lower BEQ levels. If Roos and co-authors were to perform bioassays with the extract before being treated with acid, they probably would have found much higher BEQ levels. Therefore, one would expect a higher BEQ in the Roos *et al.* (2011) samples if the PAHs were included, seeing that their PAHs levels they found are very similar to the concentrations of this project (Table 2.9).

In another South African study, Edwards *et al.* (2016) reported on potential impacts of organic pollutants in the lower Phongolo River and floodplain in north-eastern KwaZulu-Natal. The level of the AhR ligands in the sediment were also investigated using the H4IIE-*luc* reporter gene bioassay. Two types of extracts were prepared from the sediment of the Phongolo River study: one form of the extract contained all of the AhR ligands ("all AhR-ligands"), and the other the persistent AhR-ligands such as PCDD/Fs only. Although these extracts did not target the PAH containing fraction specifically their BEQ results were useful to compare general AhR ligand levels between the two areas: a highly urban and industrialised Soweto and Lenasia vs a remote area of the country known for its sugar cane crops and malaria vector control, both factors that could contribute to AhR-ligands. The sugar cane biomass is burnt annually from May to December and dichlorodiphenyltrichloroethane (DDT) is used in indoor residual spraying for malaria vector control. The mean of BEQs measured by Edwards *et al.* (2016) in the sediments collected during the 2012 high flow season for all AhR-ligands was 11.1 ± 3.8 pgBEQ/g and 21.01 ± 11.3 pgBEQ/g for the persistent AhR-ligand extract. In their 2013 sediments all AhR-ligand only had one sample with assay quantifiable levels of 8.98 pgBEQ/g and a mean of 13.6 ± 4.3 pgBEQ/g for the persistent compounds (Edwards *et al.*, 2016). These levels were considerably lower than those in the present study, and comparable to Dobsonville 2014 and Lenasia 2013 (Table 3.1), the sites with the lowest BEQs in the current study. This comparison shows that the urban area of Soweto and Lenasia was more polluted than the rural Phongolo area, despite of its share of AhR-ligand sources.

In a study by Keiter *et al.* (2008) following the distinct decline in fish populations in the Danube River, Germany, the AhR-agonists of sediment were tested. The lack in knowledge of the levels of organic pollutants led to the biological investigation using the H4IIE cell line's commercial version, the DR-CALUX (Dioxin Response Chemically Activated Luciferase Expression) assay. Keiter *et al.* (2008) dosed a raw extract onto the cells (which contained all AhR-ligands) as well as a second extract containing only persistent AhR-ligands. Chemical analysis of their crude extract showed a mean ΣPAH_{16} of 4 898 ng/g and a mean ΣCPAH of 2 423 ng/ (Keiter *et al.*, 2008). The BEQs calculated from the raw extracts were all above 1 000 pgBEQ/g and the mean BEQs of the extract with persistent ligands was 395 pgBEQ/g (Keiter *et al.*, 2008). Even with the high levels of halogenated aromatic hydrocarbons (HAHs) measured in the Danube River, it seems that the very high PAHs, compared to the sites in the present study, contributed greatly to the BEQs measured in this polluted river (Keiter *et al.*, 2008). Sediments from the Soweto and Lenasia sites have considerably lower levels of both ΣPAHs and BEQs when compared to this European river.

Vogt (2013) studied PCBs and PAHs in sediments and soils in the Durban Bay area, on the eastern coast of South Africa. The extracts for both the present study and that of Vogt (2013) were prepared similarly—collecting a PAH containing fraction (see section 2.2.2). The Vogt (2013) study included both marine and estuarine sites, but for comparison to the present study, only sites farthest inland, i.e. the sites least influence by the marine water, were chosen. These inland sites had BEQ values lower than our study. The Umhlatuzana River had a BEQ of 4.26 pgTCDD-eq/g (Σ PAHs: 58.7 μ g/kg) and the Umbilo River had BEQ of 7.7 pgTCDD-eq/g (Σ PAHs: 186.6 μ g/kg). Closer to the Durban harbour the BEQs were much higher (Vogt, 2013).

Louiz *et al.* (2008) studied the dioxin-like activity, along with endocrine activity, in the sediments of the Bizerta lagoon, one of Tunisia's most polluted lagoons. For comparison, the most inland sites were chosen. These sites were mainly exposed to anthropogenic stressors such as metallurgy industry (Louiz *et al.*, 2008). The authors also determined the concentrations of PAHs in the sediments with instrumental analysis. These authors determined the cytochrome P4501A1 activity by quantifying EROD after exposing PLHC-1 cells. This cell line was derived from hepatoma cells of the desert topminnow (*Poeciliopsis lucida*). The cytochrome gene activation is mediated by the AhR, the same as the H4IIE cells (Villeneuve *et al.*, 2001). Louiz *et al.* (2008) calculated BEQs (in terms of 2,3,7,8-TCDD) that was compared to our sites. The BEQs quantified by Louiz *et al.* (2008) were an order of magnitude higher than ours, ranging from 0.8–12.8 ngBEQ/g. Although these authors measured PAH concentrations, they dosed the cells with the raw sediment extracts, that was not subjected to clean-up or fractioning steps. Thus, the very high BEQs reported by Louiz *et al.* (2008), cannot wholly be attributed to the PAHs they quantified.

Yoo *et al.* (2006) published a study where the activities of AhR active compounds were characterised in sediments from Korea. These authors used the H4IIE-*luc* reporter gene bioassay. One of their sampling areas was the inland creeks and streams that flow into Lake Shihwa—known to have moderate to high halogenated aromatic hydrocarbons (HAHs), such as dioxins. Their samples were acid-washed to remove all non-persistent compounds, like PAHs. Yoo *et al.* (2006) reported BEQ values of REP50, therefore the extrapolated REP50 values (for those who do not have empirical values) of Soweto and Lenasia were used for comparison (Table 3.1). The REP50 values of 2013 from the sites in the present study ranged from 25.3 pgBEQ/g to 642.7 pgBEQ/g, and for 2014 from 1.2 pgBEQ/g to 972.1 pgBEQ/g (Table 3.1). For both years Moroka had the greatest REP50. All the REP50s of 2013 were empirical values, only Dobsonville's was extrapolated. All the REP50s of 2014 were extrapolated. Yoo *et al.* (2006) reported REP50s that ranged between 14–868 pgBEQ/g. Although the BEQs by Yoo *et al.* (2006) were all comparable to our results, these responses were elicited by persistent pollutants. In terms of the types of contaminants, Lake Shihwa is not similar to Soweto/Lenasia, but in terms of AhR mediated toxicity the results were very similar.

In another study, Hong *et al.* (2012) compared the AhR-mediated potencies determined by the H4IIE-*luc* cell line of sediments sampled from the Yellow Sea. The sediments were sampled from Chinese (Liáoning province) and South Korean (Western coast) estuaries (Hong *et al.*, 2012). The sediment

extracts were not fractioned for PAHs and the raw extract was dosed to the cells (Hong *et al.*, 2012). The mean BEQs from the Korean sediments were 4.6 pgBEQ/g (ranging <3.4–11 pgBEQ/g) and the Chinese sediments had a mean of 4.9 pgBEQ/g (<3.4–28 pgBEQ/g). Although the BEQs for Soweto and Lenasia were predominantly higher than those reported by Hong *et al.* (2012)'s BEQs, Lenasia 2013, Nancefield 2014, and Dobsonville (2013 and 2014) had very similar BEQ results (Table 3.1). Hong *et al.*, (2012) also reported instrumental analysis for potential AhR-ligands including PAHs. The mean Σ CPAHs were 86 ng/g (Korea) and 260 ng/g (China). The CPAHs present in these samples were calculated to contribute to 40% of the TEQs determined for the Chinese sediments and 12% for the Korean sediments. The CPAHs that were most prevalent—thus having the most potential AhR binding—was dibenz(ah)anthracene and benzo(k)fluoranthene (Hong *et al.*, 2012). Soweto and Lenasia had more of the 'smaller' CPAHs present in the sediment, namely benz(a)anthracene and chrysene (Table 2.8).

Hilscherova *et al.* (2001) studied dioxin activity in the Morava- and Drevnice Rivers, in the Czech Republic (Table 2.9). They isolated different pollutant groups by fractioning the sediment extract with a Florisil column. One of these fractions contained the PAHs (similar to this study). The BEQs obtained with the H4IIE-*luc* cells, were three orders of magnitude higher than the Soweto and Lenasia BEQs (mean of 9 ngTCDD-eq/g). This is to be expected as the PAHs reported by Hilscherova *et al.* (2001) were at high concentrations in the sediments (Table 2.9).

In Asia, Xia *et al.* (2014) followed the same fractioning technique as Hilscherova *et al.* (2001) to separate the different compounds in a crude extract. Xia *et al.* (2014) used the H4IIE-*luc* bioassay to quantify dioxin-like activity in the sediments of Lake Tai in China. The instrumental quantification of the CPAHs was also done and they found that the mean Σ CPAHs between the sites was 96.52 ng/g—two times lower than measured at Soweto and Lenasia (Table 2.8). The BEQs of this study was also lower than Orlando East of 2013, Protea Glen of 2014, Moroka and Eldorado Park of both years (Xia *et al.*, 2014) (Table 3.2).

Song *et al.* (2006) reported on the AhR-active compounds in the Haihe and Dagou Rivers in China's Tianjin area after also fractioning a crude extract to contain mainly PAHs—like Hilscherova *et al.* (2000). The Haihe and Dagou Rivers flow through the Tianjin City and had been exposed to industrial and domestic waste historically (Song *et al.*, 2006). From their eleven sites the four most inland sites were compared to the Soweto and Lenasia sites. The BEQs bioassay ranged between 693.6 pgBEQ/g and 6 834 pgBEQ/g (Song *et al.*, 2006), and the mean of the four chosen sites was 2 313.85 pgBEQ/g. The authors attributed these high BEQs to PAHs, literature reports PAH concentrations in the area between 800 and 1 200 ng/g in soil (Wang *et al.*, 2003). The measured BEQs in the fractioned extracts were considerably higher than the 2013 BEQs of Soweto and Lenasia (Table 3.1). The BEQs of 2014's Moroka and Eldorado Park were comparable (Table 3.1) to three of the four selected upstream sites (693.6–926.6 pgBEQ/g) (Song *et al.*, 2006).

3.4 Conclusion

The H4IIE-*luc* bioassay results confirmed that there are AhR agonists present in aquatic environment of Soweto and Lenasia. The sites that had the greatest bioassay equivalents were Moroka and Eldorado Park for both sampling years, ranging between 137.2 and 816.8 pgBEQ/g. The cytotoxicity seen during the bioassay could have been caused by the LPAHs: Nap, Acea, Acey and Phe. On comparison the 2014 BEQs were considerably greater than that of the previous year. The luciferase activity and the results of the bioassay can be attributed to the CPAHs in the extract, as there was a strong correlation ($r = 0.78$, $p = 0.0002$) between the BEQs obtained and the CPAHs quantified. For those sites that did not follow this trend, having lower BEQs than the correlated CPAHs, inhibition of the Ah-receptor was possible. For the opposite, where the BEQs were greater than its correlated CPAH concentration, activation of the AhR by other agonists is probable, resulting in higher BEQs. All the BEQs for both sampling years exceeded the sediment quality guidelines. Dobsonville was the only site to exceed the lower ISQG level, where the remaining site surpassed the upper guideline (PEL). The exceedance of these guidelines at the Moroka and Eldorado Park was extremely high—6- to 960 fold for the ISQG and 10 to 38 times the PEL.

Compared to local and international studies, we can deduce that the present study area has moderate to high pollution in terms of AhR ligands. The Soweto and Lenasia BEQs were lower than the European studies that are all subjected to heavy industrial inputs, but mostly higher and for some similar to, the Asian studies. For many of the comparative studies, the results reported were elicited from a mixture of PAHs and other AhR-ligands and in some cases not from PAHs at all. In South Africa, the BEQs reported by Roos *et al.* (2011), Edwards *et al.* (2016), and Vogt (2013), were all lower than or comparable to the lower BEQs of this study. Thus from the results presented here, it can be conclude that in terms of the biological responses to PAHs in Soweto and Lenasia, the potential risk of AhR mediated toxicity is great for the aquatic organisms in the system and potentially people exposed to the sediments.

3.5 References

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Chapter 4: Biomarker responses and fish health assessment of *Clarias gariepinus* from impoundments of Soweto and Lenasia, South Africa

4.1 Introduction

4.1.1 Health assessment of aquatic environments

The assessment and evaluation of stressor effects on ecosystem health is constantly faced with challenges such as the validation of laboratory experiments (Adams, 2001) that are done as environmental analogues. Standard laboratory toxicology tests are used to investigate single or mixed contaminants for simple but critical endpoints, such as survival, growth and reproduction potential (Adams, 2001). Test organisms usually do not function normally by the time these endpoints are reached (Larsson *et al.*, 1985), and the test conditions seldom reflect the complex conditions of a natural environment (Lagadic *et al.*, 1994). The use of laboratory studies (using environmental samples) are thus ideal for single endpoint studies such as targeted enzymatic (Aas *et al.*, 2000) or physiological changes (Villalobos *et al.*, 2000) in response to contaminants. There are shortcomings associated with laboratory studies in terms of making an interpretation or conclusion regarding environmental studies. Therefore, using organisms from the environment are ideal indicators of ecosystem health. Indicators are parameters or parameter derived values that describe or provide information on the state of the environment (UNstats, 1997). In 1954 Feibleman published a paper on the role of integrated levels in organisations of different disciplines in science: physics, chemistry, biology, psychology and anthropology and he explained that disturbances introduced at any level of organisation create reactions in all the other levels it is associated with (Feibleman, 1954). Thus the assumption can be made that indicators can be used to report on the disturbance in the organisation of the environment where organisation of the environment means: molecular, sub-cellular, cellular, tissue, organ, systems, whole organism, population, communities, and ecosystem. The health of organisms often reflects the ecosystem health and biotic integrity it occupies (Adams *et al.*, 1993). In essence this is why organisms are used in environmental studies as indicators. Biota represent different levels of organisation from molecular- up to community level and even ecosystem level if different organisms are studied together i.e. fauna and flora. Numerous studies have used aquatic biota as indicators, such as molluscs (Wootton *et al.*, 2003; Sureda *et al.*, 2013), amphibians (Bryer *et al.*, 2006; Leney *et al.*, 2006), birds (Triosi *et al.*, 2006; Herbert *et al.*, 2011), and fish (Kayal & Connell, 1995; Vives & Grimalt, 2002).

4.1.2 Using fish for health assessments

The investigation of organisms at higher trophic levels often gives a cumulative indication of the effects that act on the system as it is mediated through the food web (Newman, 2010). Fish have variable diets, changing as they grow older—they continue to grow throughout their lives—and as their dietary needs change they occupy different levels of the food web (Larkin, 1978). Fish are therefore useful as long term indicators of aquatic system health. Fish cope daily with natural

physiological stressors such as changes in water temperature and velocity, and dissolved oxygen concentrations, as well as sediment loading and food availability (Adams *et al.*, 1993). The cumulative effect of these natural factors along with introduced anthropogenic factors may lead to impairment of fish health. In order to deal with these stressors energy is required, often allocating useful energy away from critical functions of growth and reproduction (Adams *et al.*, 1993). The severity of stress can lead to the reduction of growth, limiting physiological systems and impairing reproduction, resulting in an inability to tolerate additional stress (Adams, 1990).

There are various approaches to assess the effects of stressors on fish health and the ones used in this study are discussed below. These are aimed at specific biological endpoints used to describe effects resulting from exposure to a chemical (or any other form of stressor).

4.1.3 Biomarkers and bio-indicators

There are various definitions for the term “biomarker”. Strimbu and Tavel (2010) define it as any characteristic—any biological marker—that can be measured or evaluated as an indicator of normal biological or pathogenic processes, or pharmacological responses to therapeutic intervention. Similarly, the World Health Organisation (WHO) defines it as a measurement of the interaction between biological systems and environmental hazards (WHO, 1993a). The WHO’s International Programme on Chemical Safety further defined biomarkers by adding there is a measurement involved that reflects this interaction between a biological system and a potential hazard whether it is chemical, physical, or biological in nature. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction (WHO, 1993b). Furthermore, Adams (2001) characterised biomarkers as the functional measures of exposures to stressors that are expressed at the suborganismal level of biological organisation. Adams (2001) go further and also define bio-indicators as the structural and functional entities that represent biological effects and endpoints at higher levels of organisation. In this study, the term “biomarkers” will be used to specifically refer to the lower level of organisation responses (up to subcellular), and “bio-indicators” to any responses at higher levels of organisation i.e. cellular responses and higher.

Since biomarkers and -indicators highlight discrepancies on various organisational levels of the individual organism they are often applied as an early warning or proactive tool. They are used to measure the effect of toxicants before serious permanent damage is done in an entire ecosystem because changes in the organism is generally detectable before adverse effects are seen in higher levels in the biological organisation (Newman, 2010) (Figure 4.1).

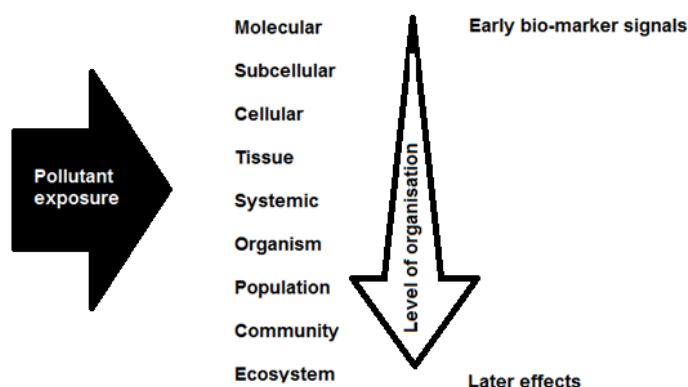


Figure 4.1: Levels of biological organisation and the order of response to pollutant stress [modified from Van der Oost *et al.* (2003)]

The technique in which biomarkers or bio-indicator endpoints are measured are very diverse and depend on the level of organisation investigated (Figure 4.1). One such a technique is using bioassays. As previously mentioned (see section 3.1.1): bioassays are investigating or monitoring tools to estimate the biological effects (Hilscherova *et al.*, 2000; Behnisch *et al.*, 2002; Koh *et al.*, 2005) in organisms. They are often designed as rapid and relatively inexpensive methods to quantifiably analyse the reaction to stressors in terms of biological responses (Behnisch *et al.*, 2002). In the previous chapter a very specific bioassay was described the reporter gene bioassay. Bioassays in this chapter also include, biochemical response assays (molecular level, Figure 4.1) and various biometric indices (tissue to community level, Figure 4.1) each investigating a different biomarker or bio-indicator endpoint.

4.1.4 Use of biomarkers and bio-indicators to assess fish health

Biomarker responses

Biochemical response bioassays have been developed to measure various biomarkers of interest. From here on, biomarker responses will refer to the biochemical response bioassays completed in the laboratory to measure each respective biomarker endpoint.

Van der Oost *et al.* (2003) categorised biomarker responses into three: (1) exposure, (2) effect, and (3) susceptibility. In this project: biomarkers of (1) exposure and (2) effect were the focus. Biomarkers of exposure measure the product of the interaction of exogenous substances or their metabolites, and xenobiotics with target molecules or cells within the body (Van der Oost *et al.*, 2003; USEPA, 2014). The biomarkers of exposure used in this project were acetylcholinesterase activity (AChE), and cytochrome P450 activity (CYP450). Some authors however consider AChE as a biomarker of effect (Rickwood & Galloway, 2004), but in this thesis the convention suggested by Van der Oost *et al.*

(2003) and others is followed. According to Van der Oost *et al.* (2003), biomarkers of effect are the measurable biochemical, physiological and other alterations within tissues and body fluids of an organism that are recognisable due to possible compromised health or disease. In this study cellular energy allocation (CEA) as well as biomarkers indicating oxidative stress and oxidative stress damage were used. Biomarkers that show oxidative stress responses are superoxide dismutase (SOD) and catalase activity (CAT), and oxidative stress damage biomarkers are protein carbonyl formation (PC), and lipid peroxidation indicated by malondialdehyde content (MDA).

Acetylcholinesterase (AChE) is a specialized carboxylic ester hydrolase that is responsible for breaking the synaptic connections of nerve firing. The enzyme hydrolyses acetylcholine into acetate and choline (Lionetto *et al.*, 2013). By deactivating acetylcholine AChE prevents constant nerve firing (Solé *et al.*, 2006, 2010). These enzymes play a crucial role in the signal transmission in animals, controlling functions such as movement, respiration, hormonal function and reproduction (Solé *et al.*, 2010). AChE is found in the brains of fish, but is also present in large quantities in the liver (Van der Oost *et al.*, 2003). AChE can be inhibited when exposed to organophosphate pesticides (Van der Oost *et al.*, 2003) and PAHs (Kang & Fang, 1997; Lau *et al.*, 2004). Payne *et al.* (1996) suggested that complex mixtures (other than pesticides) can inhibit the AChE system, such as extracts from used engine oils and wood leachate. Petroleum oils and wood preservatives are known sources of PAHs (Aprill *et al.*, 1990).

The cytochrome P450s (CYP450) are a superfamily of haeme containing enzymes that are widely diverse with regards to substrate specificity and catalytic activity (Guengerich, 2008). The P450 enzymes are generally regarded as the enzymes which are the first defence against exogenous compounds (Liska, 1998). When an organism is exposed to a toxicant, the CYP450 enzymes are expressed (Ellero *et al.*, 2010). Various subfamilies are expressed depending on the type of toxicant present.

Polycyclic aromatic hydrocarbon contamination specifically leads to the expression of the CYP1A1 gene (see section 3.1.2). This expression is the endpoint of the aryl-hydrocarbon (AhR) mediated response (Hilscherova *et al.*, 2000; Denison *et al.*, 2004). The Ah-receptors are located inside the cytoplasm of the cells. When AhR activating agents, such as PAHs, enter the cells, they bind onto the AhR complex (Figure 3.1). Upon binding, the AhR is transported into the nucleus, where it attaches onto a specific DNA sequence (called the dioxin response element, DRE), which consequently results in the transcription of the genes, such as the CYP450s (Aarts *et al.*, 1995; Denison *et al.*, 2004; Whyte *et al.*, 2004). The inhibition and activation of the P450s can be used as a biomarker of exposure as it reacts to the presence of toxicants.

Superoxide dismutase (SOD) and catalase serve as the first enzymatic defence against reactive oxygen species. SODs form the first tier of this cellular antioxidant system (Bartosz, 2005). These metalloenzymes catalyse the dismutation of reactive superoxides (O_2^-) into oxygen and hydrogen peroxide. Thus, they are an important antioxidant defence in nearly all cells exposed to oxygen and

reactive oxygen species (ROS) (Pandey *et al.*, 2003). Reactive oxygen species and superoxides are formed through natural biochemical processes, but have been found to increase during pollution exposure (Van der Oost *et al.*, 2003). An increase in SOD levels indicates high levels of ROS that need to be broken down. PAHs have been found to induce SOD levels in common carp (*Cyprinus carpio*) (Van der Oost *et al.*, 1998) and dab (*Limanda limanda*) (Livingstone *et al.*, 1993). PAHs that are phototoxic, like fluoranthene, often generate free radicals when photosensitized or photomodified (Delistraty, 1997).

Catalase (CAT) is the second enzyme of the enzymatic defence against ROS (Bartosz, 2005). These enzymes are mainly responsible to counteract the toxicity of peroxide (Lionetto *et al.*, 2003). CAT is produced in response to the increase of ROS, reducing the hydrogen peroxide formed by SOD (Pandey *et al.*, 2003). Hydrogen peroxide is produced through the reaction of superoxides and water, and is important on a cellular level to fight infections. Even though it is an important compound, high levels can cause oxidative damage to cells (Pandey *et al.*, 2003). Catalase activity is indicative of the levels of an oxidative stress compound within the organism and an induction of catalase activity by PAHs has been found in the livers of dab (*Limanda limanda*) (Livingstone *et al.*, 1993) and in shorthorn sculpin (*Myoxocephalus scorpius*) (Stephensen *et al.*, 2000).

Malondialdehyde content (MDA) and protein carbonyls (PCs), discussed below, are by-products of oxidative damage and are used to measure the extent of the oxidative stress (Parvez & Raisuddin, 2005; Üner *et al.*, 2006). Quantifying MDA and PCs are meaningful indicators of pollution in freshwater and marine ecosystems (Van der Oost *et al.*, 1994).

Lipid peroxidation prediction is important in toxicology research pertaining oxidative stress and is quantified by measuring MDA content (Üner *et al.*, 2006). MDA is formed when lipid membranes degrade due to oxidation (Solé *et al.*, 2006, 2010). Lipid peroxidation is a major contributor to cellular damage, as it can affect the cellular antioxidant system (Ferreira *et al.*, 2007). Malondialdehyde is one of the final products of the peroxidation of polysaturated fatty acids (Gawel *et al.*, 2004). MDA content is therefore used to indicate if lipid damage occurred in an organism due to oxidative stress. It is an important biomarker as the levels reflect the severity of lipid peroxidation in an organism.

The direct damage or oxidation of amino acid in proteins results in the formation of protein carbonyls. If the PCs increase it can cause damage to cellular systems and tissue and once PCs are formed they cannot be reversed (Parvez & Raisuddin, 2005). Protein carbonyls decrease enzymatic functions and can cause delayed protein regeneration (Ferreira *et al.*, 2007). It is the most commonly used biomarker of protein oxidation (Dalle-Donne *et al.*, 2003).

The energy availability and food intake of fish vary due to seasonality, food availability, flow rate, trophic level and life stages and their energy budgets are often compromised by the increase of stress and pollutant exposure (Gourley & Kennedy, 2009). Assessing the energy budget of organisms at a cellular level is a quick method of determining the effects of stress on that organism. The method

entails determining the reserve energy as well as the energy consumption of the organism because the exposures of organisms to contaminants result in a net decrease of energy budget (De Coen & Janssen, 1997).

Bio-indicators

Biological indices can be used as bio-indicators as they explain the condition or state of a biological variable/system (often at levels of organisation higher than cellular level). An index is a formula derived number that is used to describe a condition or status. It represents a single value that is made from a variety of mathematical ratios of different variables, which says something about the sample or site, in terms of the variables, to allow for comparison over time.

The fish health assessment index (FHAi) is a multi-aspect index, which incorporates many variables into one final value, to describe the overall health of individual fish as well as the population sampled. Other useful indices are the organo-somatic indices which include Fulton's condition factor (CF), the gonado-somatic index (GSI), the hepato-somatic index (HSI), and the spleen somatic index (SSI). The latter three indices incorporate the value of each index's organ mass such as mass of gonad, liver or spleen, into a percentage in terms of the body mass. The calculated value is used to describe the state of physiological systems in numerical values. These indices are used in temporal and spatial comparisons. In addition to these applications, CF is also gauged against a specific scale, expressing different conditions of fish.

Adams and co-authors (1993) described the FHAi as a rapid and inexpensive quantitative index. It was developed as a field necropsy method, where the results provide a health profile of the fish, based on ratio of abnormalities observed in the tissue and organs of individuals sampled from a population (Goede & Barton, 1990; Adams *et al.*, 1993). The variables assessed are divided into three categories: haematological assessment, external-, and internal evaluation. The haematological assessment consists of the measuring blood protein levels, haematocrit, and leukocrit. The external evaluation is the degree at which abnormalities are present in the eyes, opercula, fins, and skin. The internal evaluation consists of gauging between normal and abnormal internal organs—and if abnormal, what abnormalities or alterations are present in the gills, liver, spleen, kidney, and hindgut. In order to do quantitative statistical analysis, variables are assigned numerical ranking scores based on the degree of severity or alteration. A FHAi variable score of 30 indicates a severe abnormality or alteration for that variable, where decreasing severity is scored 20, 10 and 0. A score of zero indicates normal state or absence of abnormalities (Adams *et al.*, 1993). The FHAi score for individual fish is calculated by adding all the variable scores whereas the site FHAi is the mean of the FHAi of all the fish sampled at that site. The standard deviation of the mean FHAi score represents the variability of the health of the fish caught at the site and the coefficient of the variance indicates the level or degree of the health of the fish population. The FHAi allows for statistical comparisons of fish health between data sets (Adams *et al.*, 1993).

The general well-being of fish is described in terms of their condition (poor, good or excellent). Fulton's condition factor (CF) shows the volumetric relationship between the body mass and the total length of the fish and in the formula is represented by the letter *K*. It expresses the condition (well-being, relative robustness or fatness) in numerical terms (Mortuza & Rohman, 2006), where the general condition of the fish is proportionate to the factor value (*K*). The factor calculated is compared to a range of values that describes the condition of the fish. The condition factor was designed for fusiform fish like salmonids (Barnham & Baxter, 1998) and poses a problem when investigating dorsal ventrally flattened fish such as *Clarias gariepinus* (see section 1.4.3). Lückhoff (2005) investigated the condition factor for *C. gariepinus*. His findings showed that catfish in good condition had condition factor values above 0.85 and that the upper limit for *C. gariepinus*' condition factor range is 1.04 (excellent condition) and the lower limit, 0.6 (poor condition). Studies have shown that PAHs reduce growth and condition in fish (Dutta *et al.*, 2005; Meador *et al.*, 2008).

Fish livers are regarded as the main site of storage, bio-transformation and excretion of pollutants (Hinton & Laurén, 1990; Velmurugan *et al.*, 2007) as well as storing energy reserves in the form of glycogen (Miranda *et al.*, 2008). The hepato-somatic index (HSI) is the relationship between the liver mass and body mass, and indicates the energy reserves of the fish or the effects of xenobiotics on the liver. The HSI may increase following exposure to pollutants due to an increase in hepatocyte size and numbers (Marchand *et al.*, 2009) in order to increase the liver's detoxification potential (Goede & Barton, 1990). In contrast pollution can also reduce the HSI—with a decrease in cell size and number or even atrophy of hepatocytes (Sanchez *et al.*, 2008; Marchand *et al.*, 2009). However, it is important to keep in mind that the size of the liver is also affected by various other variables such as energy stores and food availability, parasites and seasonal changes (Goede & Barton, 1990; Sanchez *et al.*, 2008). To interpret the HSI results, the previously mentioned factors must be considered. PAHs have previously been demonstrated to have effects on the livers of fish. The livers of Japanese medaka (*Oryzias latipes*) and guppies (*Poecilia reticulata*) exposed to waterborne benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene (DMBA) had developed hepatocellular neoplasms (Hawkins *et al.*, 1990). Liver neoplasm in the American catfish, the brown bullhead (*Ameiurus nebulosus*), was associated with high PAHs recorded in the sediments (Baumann *et al.*, 1991; Smith *et al.*, 1994). Karami and co-authors (2016) found that acute phenanthrene toxicity to *Clarias gariepinus* juveniles included many histopathological alterations to their livers. These alterations included vacuolisation, bleeding and a decrease in liver cells, together with the cellular degradation and necrosis (Karami *et al.*, 2016).

The spleen is a lymphatic organ of which the main function is to produce and store blood (Fänge & Nilsson, 1985). It also plays a role in antigen and erythrocyte degradation, and antibody production (Goede & Barton, 1990; Rohlenová *et al.*, 2011). The spleeno-somatic index (SSI) is the relationship between the spleen mass and body mass, and is used as an indication of immuno-responses (Rohlenová *et al.*, 2011). Swelling or enlargement of the spleen can be indicative of disease in the fish or related to immunological problems (Adams *et al.*, 1992). Fish immune systems are very sensitive to PAHs, which can affect either specific or non-specific immunity (Reynaud & Deschaux, 2006). Non-specific immunity effects include the decrease of lysosomes and phagocytes (Reynaud & Deschaux,

2006). Examples of specific immunity effects are the reduction of total white blood cells in Nile tilapia (*Oreochromis niloticus*) (Hart *et al.*, 1998), as well as the depression of lymphocytes in medaka (*Oryzias latipes*) (Carlson *et al.*, 2002; 2004), spot (*Leiostomus xanthurus*) (Faisal & Huggett, 1993), and common carp (*Cyprinus carpio*) (Reynaud *et al.*, 2003) that had been exposed to various PAHs.

The GSI expresses the gonad size relative to the body size to describe sexual maturity or growth. It is used as a popular, simple and instantaneous measure of reproductive effort of a fish (Fouche *et al.*, 2010). It may also indicate irregularities, such as enlargements or tumours caused by contaminants (Stentiford *et al.*, 2003) when individuals are compared with each other within a sample. This index is used to report on reproductive potential between sites and can also possibly indicate endocrine disruption within a site between individuals, based on gonad size (atrophy or hypertrophy). The potential impact that PAHs have on gonadal growth is easily measured in the field using the GSI. However, more sensitive and specialised methods exist to evaluate these effects such as gamete quality (Casillas *et al.*, 1991; Nagler & Cyr, 1997).

Applying the methods described previously in an integrated assessment, where various endpoints are investigated, would give a better representation of the overall health state of these animals, and so indirectly the ecosystem.

It is important to state that although this study is to assess PAHs in the aquatic environs of Soweto and Lenasia, the health effects seen in the fish sampled cannot be wholly attributed to PAHs only. The fish sampled for this study had been exposed to many different stressors. However, the preceding literature review contains evidence of PAH specific influences on the respective biomarker- and bio-indicator responses.

4.2 Material and methods

4.2.1 Sample collection

Fish samples were collected from the four fish sampling sites proposed for this project (Figure 1.2). These sites are Lenasia (Le), Fleurhof (Fl), Orlando East (OE), and Nancefield (Nc) [Bushkoppies WWTP's final pond]. The fish (n = 20) used as the control had been caught from the Vaal River basin and were depurated at the Water Research Group aquarium at the NWU Potchefstroom Campus for 6 months. They were kept in aquarium standard water that was replaced every fortnight. They were processed in the same manner as the fish sampled from the study sites.

4.2.2 Fish sampling and field necropsy

Clarias gariepinus was sampled during the high flow season (October) of 2013 and 2014, as described in Chapter 1 (Sampling of fish). Fish were kept in an aerated container until field analysis was performed.

The necropsy protocol followed was a modified version as described by Heath *et al.* (2004) (adaption of Goede & Barton, 1990) and the scoring system of Adams *et al.* (1993). Although collecting blood for the haematological assessment was completed, it was not included in our final FHA score, due to unforeseen circumstances which lead to the loss of sample. The euthanized fish—severed spinal cord—were measured (total length, TL) and weighed, and the values recorded. The external characteristics, which are the eyes, skin, fins, gills, opercula and number of external parasites, were macroscopically evaluated for abnormalities or injuries, and scored accordingly (Adams *et al.*, 1993). Following dissection the internal evaluation commenced: the internal characteristics which are the liver, spleen, bile (collected for chemical analysis, section 2.2.2) and internal parasites were evaluated. The gonads, liver and spleen were dissected out and weighed to calculate their respective indices—the gonado-somatic index (GSI), hepato-somatic index (HSI), and the spleeno somatic index (SSI). Subsamples of the liver and muscle were collected to determine the biomarker responses. These samples were submerged in Hendrikson's buffer (40 mM tris-HCl, 10 mM b-mercapto-ethanol, 1 mM 0.04% bovine serum albumin [BSA], 1 mM EDTA), transported in liquid nitrogen, and stored at -80 °C. It was only after the necropsy was completed that muscle samples were collected for chemical analysis (see section 2.2.2).

4.2.3 Biomarker responses

Sample preparations

Three stock batches were prepared from the samples collected. The mass of the tissue samples were noted and the appropriate buffer added. The first batch (labelled A) was for CAT, SOD, PC and CYP450 activity: a mass of 0.1 g liver tissue was added to 1 mL general homogenising buffer (GHB) [0.1 M potassium phosphate buffer (PPB), 1.15% KCl, 1 mM EDTA, 0.1 mM phenyl methane sulphonyl fluoride (PMSF), 20% glycerol]. For specific detail on the PPB see section *Acetylcholinesterase activity assay*. Batch B was for AChE and MDA, which was 0.05 g liver in 250 µL tris-sucrose buffer [25 mM tris-HCl, 250 mM sucrose]. The final batch (C) was 0.2 g muscle tissue in 400 µL electron transport system (ETS) buffer [0.1 M tris-HCl, 0.2% Triton X-100, 15% polyvinylpyrrolidone, 153 µM MgSO₄]. From these batches aliquots were taken for each respective biomarker response assays.

Protein content of each sample of each batch was determined using the Bradford (1976) method. This method is based on the binding of Coomassie brilliant blue (active ingredient in Bradford's reagent) dye to proteins and then measuring its absorbance (optical density was read at 590 nm). The sample's absorbance was compared to a protein standard curve of bovine serum albumin (BSA) at a concentration series varying between 0 and 2 500 µg/mL. Protein content is determined because the biomarkers are expressed as activity per milligram protein.

All assays (except for CYP450) were done in clear 96 well microtitre plates. The CYP 450 assay was completed using the black half area 96 well plate included in the kit. Optical density was read on a

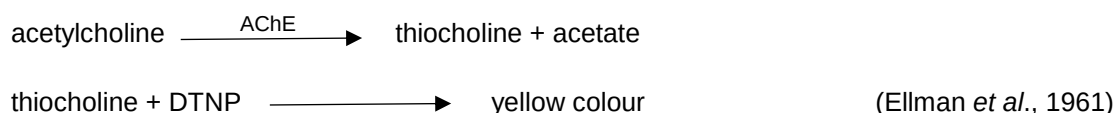
Berthold multi-mode microplate reader (LB941) and fluorescence on a BioTek multi-detection microplate reader (FLx 800).

All reagents used in the biomarker analysis were acquired from Sigma-Aldrich, unless otherwise stated.

Biomarkers of exposure

Acetylcholinesterase activity assay

The samples used in this assay was prepared according to batch B (see *Sample preparations*), which was centrifuged at 9 500 g for 10 minutes at 4°C. The procedure of determining AChE activity was adapted from Ellman *et al.* (1961). A potassium phosphate buffer (PPB) was prepared in deionised water [0.09 M K₂HPO₄] and the pH adjusted to 7.4 with 0.09 M potassium dihydrogen phosphate [KH₂PO₄]. The procedure was completed on ice. The following three reagents were added to the wells of the 96 microtitre plate: 210 µL PPB, 10 µL s-acetylthiocholine iodide (30 mM) and 10 µL Ellman's reagent (10 mM 2,2'-dinitro-5,5'-dithio-dibenzoic acid [DTNP] in methanol) and mixed by gentle tapping the plate. To ensure accurate readings only seven samples (in triplicate) were analysed at a time. The plates were incubated at 37°C for 5 minutes. After incubation 5 µL sample and blank (GHB) was added to the wells and mixed by tapping. The kinetic reaction was recorded immediately by measuring OD at 450 nm every minute for 6 minutes starting at time = 0 minutes (7 intervals). This assay is based on measuring the enzyme activity as a yellow colour is produced when thiocholine reacts with the Ellman's reagent (Ellman *et al.*, 1961).



AChE activity was calculated by determining the mean absorbance (Abs) of the readings at each time interval. The gradient for each sample was calculated using the absorbance (y-values) versus time (x-values). The tempo (absorbance per minute) was calculated by dividing the gradient by the assay time (6 minutes) and then by the sample's protein content (see *Sample preparations*). The AChE activity is expressed as Abs/min/mg protein (Ellman *et al.*, 1961).

Cytochrome P450

The cytochrome P450 activity was determined using the DetectX® P450 demethylating fluorescent activity enzyme linked immuno-sorbent assay (ELISA) kit from Arbor Assays (K011-F1) according to the package insert. The samples, which had originally been stored in the batch A buffer on the day of sampling (see *Sample preparations*) were mixed by vortex. A formaldehyde standard series [12.5–400 µM] was prepared according to the package insert. The samples, standards and blank (0.1 M PPB supplied with kit) were added (95 µL) to the half area 96 well plate in duplicate wells. After

sealing the plate and incubation the plate at 37°C for 15 minutes the reaction was started with 5 µL nicotinamide adenine dinucleotide phosphate (NADPH) solution (reconstituted according to kit instructions) and incubated (37°C for 30 minutes). The reaction was stopped with the 5 µL glacial acetic acid that was included in the kit. The DetectX® formaldehyde detection reagent was added to each well using repeat pipette (25 µL), gently tapped to ensure mixing, and incubated for 30 minutes at 37°C. The fluorescence of the samples was read at 510 nm after an initial excitation at 450 nm. Sample concentrations were determined from the standard curve and reported as nM CYP450/mg protein (protein content determination see *Sample preparations*).

Biomarkers of effect

Oxidative stress biomarkers

Superoxide dismutase activity

The SOD method was adapted from Del Maestro & McDonald (1989). Samples used for the SOD assay was from batch A. A 50 mM Tris buffer containing 1 mM diethylene triamine penta-acetic acid (DTPA) (49:1 v/v) was prepared and aerated vigorously for 20 minutes at room temperature. The pH was adjusted to 8.2 by adding HCl and aerated again for another 10 minutes. The assay was performed on ice until quantification. The samples and a Tris buffer blank (4 µL) together with 245 µL DTPA/Tris buffer was added to the wells of the 96 well microtitre plate in triplicate. The reaction was started by adding 4 µL pyrogallol (24 nM in 10 mM HCl) and the kinetic reaction recorded by measuring the OD at 560 nm every 30 seconds for 5 minutes starting at time = 0 minutes (11 intervals). This assay was performed in the dark as pyrogallol is light sensitive. This assay is based on the ability of SOD to scavenge superoxide anions (O_2^-). The removal of superoxides during the assay reduces the overall autoxidation rate of pyrogallol. One unit of SOD activity is defined as the amount of enzymes that inhibit the rate of pyrogallol's autoxidation by 50% (Del Maestro & McDonald, 1989). The absorbance gradient and tempo was determined. The sample tempos and the blank were compared to calculate the inhibition magnitude (by normalising the tempo into a percentage) and then expressed in terms of 50% inhibition (dividing by 50). This value represents the SOD activity in units. To convert SOD into a concentration value the unit SOD value is multiplied by 125 ngSOD per unit (125 ng/mL represents one SOD unit). The fraction responsible for enzymatic activity was determined multiplying the dilution factor with the buffer volume. Finally, the SOD was expressed in terms of is protein content (see *Sample preparations*) as ngSOD/mg protein (Del Maestro & McDonald, 1989)

Catalase activity

Catalase activity was measured using the method of Cohen *et al.* (1970). The CAT assay is light sensitive and was performed in the dark, on ice. The sample supernatant (batch A; centrifuged at 10 000 g for 10 minutes at 4°C) and a blank (PPB) added (10 µL) to the 96 well plate in triplicate. Similarly 102 µL PPB was added into three wells as a standard. Only 10 samples were analysed at a

time. The reaction was started by adding hydrogen peroxide [6 mM] (93 μ L) to each well (samples and blank only) and mixed gently by tapping. The plates were incubated at room temperature for 3 minute. The reaction was stopped by the addition of 19 μ L sulphuric acid (6 N). Using a repeat pipette KMnO_4 (130 μ L) was added and immediately OD was read at 490 nm. The CAT activity assay is based on the first order kinetics and linear dose response relationships of catalase and its substrate hydrogen peroxide. The colour change that is quantified by OD is from the reaction of KMnO_4 and the remaining H_2O_2 (lowering the OD). The first order kinetics of the decomposition of H_2O_2 by catalase is as follows:

$$k = \log \left(\frac{S_0}{S_3} \right) \times \frac{2.3}{t}$$

where: k is the first order reaction rate constant; S_0 is the substrate concentration at time zero (mean of standard absorbance); S_3 is the substrate concentration at 3 minutes (standard absorbance subtracted by the mean sample absorbance); 2.3 a first order kinetic conversion factor; and t is the time interval (3 minutes). After the calculation of k, the value is normalised in terms of the protein content (divided by protein content, see *Sample preparations*) and adjusted to a μmol unit (multiplied by 1000). The CAT activity is reported as $\mu\text{mol H}_2\text{O}_2/\text{min}/\text{mg}$ protein.

Biomarkers of oxidative stress damage

Malondialdehyde content

The methodology from Ohkawa *et al.* (1979) as modified by Üner *et al.* (2006) to determine malondialdehyde (MDA) content was followed. The homogenate of batch B was centrifuged at 10 000 g (4°C) for 10 minutes. A series dilution of 1,1,3,3-tetramethoxypropane (TMP) [0-3 nmol] was used as standards. A 25 μ L aliquot of each sample supernatant, the standards and tris-sucrose buffer [25 mM tris-HCl, 250 mM sucrose] as the blank was added into separate 5 mL centrifuge tubes. The following reagents were added to each of the tubes: 50 μ L sodium dodecyl sulphate (SDS) [8.1% in deionised water], 375 μ L acetic acid [20%], 375 μ L thiobarbituric acid [0.8%] (TBA), and 175 μ L deionised water. The tubes were incubated for 30 minutes at 95°C in a water bath. After incubation, the samples were allowed to cool down to room temperature. A further 250 μ L deionised water and 1 250 μ L n-butanol:pyridine solution (15:1, v/v) was added, mixed by vortexed and centrifuged at 2 700 g for 10 minutes at room temperature. The organic phase (coloured pink) was added (245 μ L) to the wells of a microtitre plate in triplicate. The absorbance was read at 540 nm and the MDA content calculated using the TMP standard curve. The MDA content was expressed in terms of its protein content (see *Sample preparations*) as nmol MDA/mg protein. This is a colorimetric assay that measures the reaction products of TBA and a degradation product of lipid peroxidation, MDA. This method has to follow very specific conditions, specifically high temperature and acidity, to generate the pink coloured adduct that is quantified.

Protein carbonyl induction

PC content was assayed as originally described by Levine *et al.* (1990) and modified by Floor & Wetzel (1998) (Parvez & Raisuddin, 2005). Samples from batch A were centrifuged at 10 500 g for 30 minutes at 4°C to prepare a supernatant containing the soluble proteins in each sample. The supernatant of the homogenates, a hydrochloric acid [2 M] blank, and a bovine serum albumin (BSA) standards [0–40 nmol] was added (500 µL) to 2 mL centrifuge tubes. An equal amount (500 µL) of 2,4-dinitrophenylhydrazine (DNPH) [10 mM DNPH in 2 M HCl] was added to the samples and standards (not blank) and allowed to react for an hour. After the reaction time proteins were precipitated using 500 µL trichloro-acetic acid (TCA) [6%] into all the tubes. From here samples, standards and the blank were processed in the same manner. The solution was centrifuged at 10 000 g for 3 minute to form a pellet of precipitated proteins.

The TCA (supernatant) was discarded carefully. The protein pellets were resuspended in 1 mL absolute ethanol/ethyl ether [1:1 v/v] and mixed by vortex. After 10 minutes the samples were centrifuged at 10 000 g for 3 minute. This washing step was repeated three times. At the end of the final wash step the samples were centrifuged for ten minutes (10 000 g) and the supernatant discarded, care taken not to lose the protein pellet. Guanidine hydrochloride [6 M in 50% formic acid] was added (400 µL) to dissolve proteins. The sample incubated for 15 minutes (37°C) and centrifuged again for 5 minutes (16 000 g) to remove any remaining insoluble materials. The supernatant was added (100 µL) to the microtitre plate in triplicate. Absorbance was read at 390 nm and carbonyl content quantified from the BSA standard curve. Protein carbonyls were quantified as nmol carbonyls/mg protein (see *Sample preparations*). This assay is dependent on the ability of DNPH to react with carbonyl groups in proteins. The 2,4-dinitrophenylhydrazine binds to carbon-oxygen double bond of the carbonyl to form an intermediate compound that then loses a water molecule and ends as 2,4-dinitrophenylhydrozone, which is the product quantified in this assay.

Energy allocation biomarkers

Cellular energy allocation

CEA analysis was adapted from De Coen & Janssen (1997, 2003) for determination of total available energy (total protein, -carbohydrates, and -lipids) and energy consumption via the electron transport system (ETS) activity.

The homogenised muscle tissue (batch C) was divided into two sets. The first was used for determination of the available energy (Ea), where 100 µL of the homogenate was further diluted with 400 µL deionised water. The samples used for energy consumption (Ec) consisted of 100 µL of the homogenate mixed with 400 µL ETS buffer.

Available energy (Ea)

The available energy is calculated by determining the total protein contents, carbohydrate contents as well as the lipid contents of each sample (batch C). Samples were mixed by vortex before the total proteins were determined following the method of Bradford (1976) (see *Sample preparations*). The total carbohydrates were quantified by using a glucose content kit (Roche, CFAS 759 350) with a glucose standard (Roche, GlucA 657 527) [1.93 g/L]. Each sample, a blank (deionised water), and the glucose standard was added to the microtitre plate in triplicate at a volume of 2.5 μ L. The glucose kit reagent was added (245 μ L) to each well and left to incubate at room temperature for 30 minutes. The ODs were recorded at 560 nm. The carbohydrate content was calculated by multiplying the standard concentration (1.93 g/L according to kit) by the result of division between the average absorbance of the samples and the standard. The final value was reported as g/L. The lipids were determined using the method adapted from Bligh & Dyer (1959). A tripalmitin series dilution served as a standard [0–6 000 μ L]. The lipids in the homogenate were extracted by adding 500 μ L chloroform to 250 μ L homogenate and mixed by vortex. To this 500 μ L methanol and 250 μ L deionised water was added and thoroughly mixed by vortex. The organic phase was separated from the watery, polar phase by centrifugation at 3 000 g for 5 minutes at 4°C. Only 100 μ L of the organic phase was transferred into glass test tubes. A chloroform blank and the tripalmitin series dilutions were prepared in the same manner (100 μ L). Sulphuric acid (500 μ L) was added to the blank, standards and samples and each test tube was covered with aluminium foil. The samples were charred at 200°C for 15 minutes. After the samples were allowed to cool down 1 000 μ L deionised water was used to reconstitute the samples and 245 μ L (representing 0.0006 g muscle tissue) was read at 405 nm to determine the optical density. The lipid content for each sample was calculated using the tripalmitin standard curve and reported as g/L.

Each available energy fraction (proteins, carbohydrates, and lipids) was transformed into their respective enthalpy of combustion values by multiplying the content values (g/L) by the enthalpy constants (17 500 mJ/mg glucose; 24 000 mJ/mg protein; 39 500 mJ/mg lipid) (De Coen & Janssen, 2003) reported as mJ. The final available energy value was calculated by multiplying the mass of the sample (0.2 g) (see *sample preparations*) and the calculated energy values (mJ) to get a mJ/g value, which is adjusted to J/g. The sum of these values (of carbohydrates, protein, and lipids) represent the available energy (Ea) for each individual.

Energy consumption (Ec)

The energy consumption was quantified using an electron transport system (ETS) activity assay. The Ec samples (see *Cellular energy allocation* for preparation) were centrifuged for 10 minutes (3 000 g, 4°C). The supernatant (25 μ L) was added to the wells of the microtitre plate in triplicate. Buffered substrate solution (BSS) [0.3% v/v Triton-X, 0.13 M Tris-HCl] (75 μ L) was also added to the wells along with 25 μ L NAD(P)H solution [1.7 μ M NADH, 250 μ M NADPH]. The reaction was then begun by adding 50 μ L 8 mM *p*-iodonitro tetrazolium chloride (INT) solution [8 nM in 1 methanol: 1 deionised

water v/v]. The absorbance was read kinetically at 490 nm for 5 minutes (11 intervals). Energy consumption is given as J/g. The kinetic reaction was recorded by measuring OD at 490 nm every 30 seconds for 5 minutes starting at time = 0 minutes (11 intervals). This assay is based on the theoretical stoichiometrical relationship that each 2 µmol formazan crystals formed by the reduction of INT, 1 µmol oxygen is consumed. The amount of oxygen consumed was transformed into energetic equivalents by an oxyenthalpic equivalent (average for a protein, carbohydrate and lipid mixture) (De Coen & Janssen, 2003). The calculation of energy consumption was as follows: the gradient of the samples was calculated using the absorbance (y-values) versus time (x-values) followed by calculating the tempo (absorbance per minute) by dividing the gradient by the assay time (5 minutes). The tempo was then divided by the amount of formazan formed. This is determined by multiplying the formazan extinction coefficient ($\epsilon = 15\,900/\text{M cm}$) with the depth of the light path length of the total volume of the well (0.7 cm) and the volume used in the well (0.075 mL) (De Coen & Janssen, 2003). As stated above for every 2 µmol formazan formed one µmol oxygen is consumed (halving the formazan content). The energy used to reduce oxygen (energy consumed, mJ) is the product of the amount of oxygen (µmol) and the oxyenthalpic equivalent (484 kJ/mol O₂) (De Coen & Janssen, 2003). Finally, the energy consumption in the organism is the product of the energy required to reduce oxygen and the inverse of the sample mass (1/0.2 g), mJ/g adjusted to J/g.

4.2.4 Bio-indicator assessments

Health assessment indices calculations

The individual fish FHA scores are calculated by adding all the variable scores. The population or site FHA score is calculated by determining the mean of the individual FHA scores (Adams *et al.*, 1993).

$$\text{FHA} = \frac{\sum_n \text{Individual FHA score}}{n}$$

A standard deviation and coefficient of variance for each site was also calculated Adams *et al.* (1993).

$$\text{SD} = \frac{\sum_{i=1}^n (V_i - X)^2}{n-1} \quad \text{CV} = 100 \times \frac{\text{SD}}{X}$$

Where n is the number of fish sampled at that site, X is the mean FHA value at that site and V_i is the FHA value for fish i.

The mass and length of each fish recorded during the necropsy was used to calculate Fulton's condition factor, by using the formula described by Bolger & Connolly (1989):

$$K = 100 \times \frac{W}{L^3}$$

Where K is Fulton's CF, W is mass in gram (g), and L is total length in centimetres (cm). The GSI, HSI and SSI were calculated by expressing the index respective organ mass as a percentage of the gutted body mass (Rohlenová *et al.*, 2011).

$$OSI = \frac{\text{Organ mass (g)}}{\text{Gutted body mass (g)}} \times 100$$

The evaluation of fish condition often requires the measurement of the parameters of the same fish over time to reveal a more accurate condition over time—as factors such as climate, food availability, and gonad maturation affect the results (Bolger & Connolly, 1989). For this project however, only single CFs were recorded to indicate the condition of the fish at the time it was sampled. These results were used in conjunction with the remaining indices to report on the health of the fish.

4.2.5 Statistical analysis

The results obtained with the biomarker- and bio-indicator assessments were tested for normality using the D'Agostino & Pearson omnibus normality test. One-way analysis of variance (ANOVA) was performed if the data were distributed normally and Tukey's multiple comparison test as a post-test. The Kruskal-Wallis test was used together with Dunn's multiple comparison test as post-test for non-parametric data sets. A $p < 0.05$ was considered significant. Significance was indicated by common conscripts on graphs for seasonal differences per site and spatial differences within a survey. Statistical analysis was done using Graphpad Prism version 5.

4.3 Results and discussion

The following results are for the biomarker and bio-indicator assessments done on the fish sampled from Soweto and Lenasia. Biometric information of *Clarias gariepinus* sampled from the study area is given in Table 4.1 and the biomarker responses summarised in (Figure 4.2–4.5) The bio-indicators such as the fish health assessment index (FHAi) is presented in Figure 4.6–4.7 and the other body indices in Figures 4.8–4.11.

4.3.1 Biometric information of *Clarias gariepinus* sampled

There were more females caught than males for most of the sampling events in both years except for Fleurhof and Nancefield 2013 (Table 4.1).

There were no significant differences between the sites for the mass of the fish sampled or their total lengths, indicating fish were all similar in size and are comparable when interpreting results. The sex of the fish also had no significant effect on the mass and length of the fish.

Table 4.1: Sex ratio, mean mass, mass range, mean total length, and total length range of *Clarias gariepinus* sampled in Soweto and Lenasia (2013 and 2014). Standard deviation in parenthesis where means are reported

	Sex ratio (F:M)	Mean mass (g)	Mass range (g)	Mean total length (mm)	Total length range (mm)
Lenasia 2013	11:9	2 363 (1 469)	740–4 800	631 (143)	420–830
Fleurhof 2013	5:5	3 024 (961)	1 720–4 840	704 (97)	570–850
Nancefield 2013	9:11	2 030 (998)	940–4 900	628 (91)	510–930
Orlando 2013	17:3	2 289 (121)	1 000–4 720	630 (121)	410–830
Lenasia 2014	7:4	2 232 (974)	780–3 460	641 (111)	495–780
Fleurhof 2014	7:3	3 120 (1 745)	1 080–6 640	704 (153)	500–980
Nancefield 2014	6:2	3 570 (1 431)	1 020–5 260	781 (128)	540–930
Control	10:10	3 337 (1 373)	1 100–6 040	736 (108)	540–910

4.3.2 Biomarker responses

The stress induced by xenobiotic stressors leads to biochemical irregularities, which results in the uptake, biotransformation and elimination of that compound (Newman, 2010). Although only PAHs have been quantified in the study area, and their metabolites detected in the fish, other contaminants might have influenced the biomarker responses. Table 4.2 summarises already known increases/decreases of the various biomarker responses as a reaction to specific exposures and effect, aiding the interpretation of the results discussed below.

Table 4.2: Summary of the interpretations of biomarker responses (Adapted from Van der Oost *et al* 2003 and Wepener *et al.*, 2011)

Biomarker	Response (increase/decrease)	Effect interpretation or exposure
Acetylcholinesterase (AChE)	↓	Due to presence of enzyme inhibitors such as pesticide exposure
Cytochrome P450 activity (CYP450)	↑	Up-regulation of <i>P450</i> usually due to presence of organic pollutants
Superoxide dismutase content (SOD)	↑	Stimulation in the presence of reactive oxygen species (ROS) and oxidative agents
Catalase activity (CAT)	↑	Produced in response to ROS formation
Protein carbonyl content (PC)	↑	Indicates damage to proteins due to ROS
Malondialdehyde content (MDA)	↑	Indicates lipid peroxidation due to ROS
Cellular energy allocation (CEA)	↑ ↓	Decrease due to stress compensation (higher energy demand). Increase due to increase in energy sources

Biomarkers of exposure

There was an inhibition of AChE in the fish at all the sampling sites during both years compared with the AChE levels in the control fish, but the 2014 AChE results were lower than those of 2013. Four of the sets of fish had AChE activity significantly lower than the control fish indicating inhibition of AChE activity (Figure 4.2A). The greatest inhibition for the 2013 fish were at Fleurhof ($5 \times 10^{-4} \pm 2 \times 10^{-4}$ Abs/min/mg protein) and Nancefield ($6 \times 10^{-4} \pm 2 \times 10^{-4}$ Abs/min/mg protein) which had 56% and 43% inhibition of AChE compared to the control. The highest AChE value was at Lenasia ($1 \times 10^{-3} \pm 4 \times 10^{-4}$ Abs/min/mg protein), and only had 14% inhibition compared to the control. All the 2014 AChE results showed that the enzyme had been inhibited more than 50%, ranging between 71 and 80% ($2-3 \times 10^{-4}$ Abs/min/mg protein) (Figure 4.2A). The inhibition of AChE activity higher than 50% may lead to obvious toxic effects, and an inhibition higher than 80% may lead to the death of those individuals (Connell *et al.*, 1999). AChE activity declined significantly ($p < 0.05$) between the consecutive years for Lenasia and Nancefield only.

PAHs have been found to inhibit acetylcholinesterase (Kang & Fang, 1997; Lau *et al.*, 2004). Kang & Fang (1997) showed that several PAHs had dose dependant inhibitory effects on the AChE activity, *in*

vitro, of the electric eel, *Electrophorus electricus*. These authors found that anthracene, benzo(a)pyrene and chrysene had the highest inhibitory effect (IC_{50}), in that order. These PAHs congeners were also found to have high competitiveness to inhibit AChE, i.e. high K_i values (Kang & Fang, 1997). The AChE activity in the livers of *Oreochromis mossambicus* exposed to sewage water was lower than their control (27% inhibition) (Al-Ghais, 2013), and although the author did not quantify any contaminants in the sewage PAHs have been found in sewage in other studies (Wild & Jones, 1989; Mo et al., 2001). The Nancefield site was the last pond of a waste water treatment plant (See section 1.3.2) and showed a similar decrease in AChE activity compared to the control fish (43% and 80% (Figure 4.2A) than the Al-Ghais (2013) study. In Tanzania, Mdegela et al. (2010) also investigated sewage exposed fish. These authors assessed various biomarkers in wild *C. gariepinus* and quantified PAH metabolites in the fish as well. The AChE activity in the brain tissue of the Tanzanian *C. gariepinus* was 33% inhibited which was higher than the AChE inhibition in the Orlando and Lenasia catfish of 2013 (24% and 13% respectively), but lower than the inhibition of the other Soweto and Lenasia sites.

Clarias gariepinus was sampled from the Phongolo River system to investigate organochlorine pesticides (OCPs) in this malaria vector control area (Edwards et al., 2016). These fish were expected to have been exposed to malaria vector control (MVC) pesticides, which include OCPs (specifically dichlorodiphenyltrichloroethane, DDT), pyrethroids, carbamates and organophosphates (Brooke et al., 2013). Of the preceding list the organophosphates, pyrethroids, and carbamates inhibit AChE activity (Lionetto et al., 2013). The AChE results of the Phongolo *C. gariepinus* showed no significant changes between the two sampling years (2012 and 2013) even though there was a significant increase in Σ OCPs (Edwards et al., 2016).

Clarias gariepinus was sampled downstream from Soweto and Lenasia, from the Lower Klip River (Region 3 & 4; Figure 1.1), by Wepener et al. (2015) in 2014. The fish sampled from these sites had metals and OCPs present in their muscle tissue (Wepener et al., 2015), contaminants known to inhibit AChE activity (Lionetto et al., 2013). The AChE activity of 0.0016 Abs/min/mg protein measured in the Lower Klip River fish, was significantly higher ($p < 0.05$) than the 2014 *C. gariepinus* results of 0.0002–0.0003 Abs/min/mg protein, of the current study. The greater AChE activity downstream supports the evidence of more and/or greater AChE xenobiotics upstream in 2014.

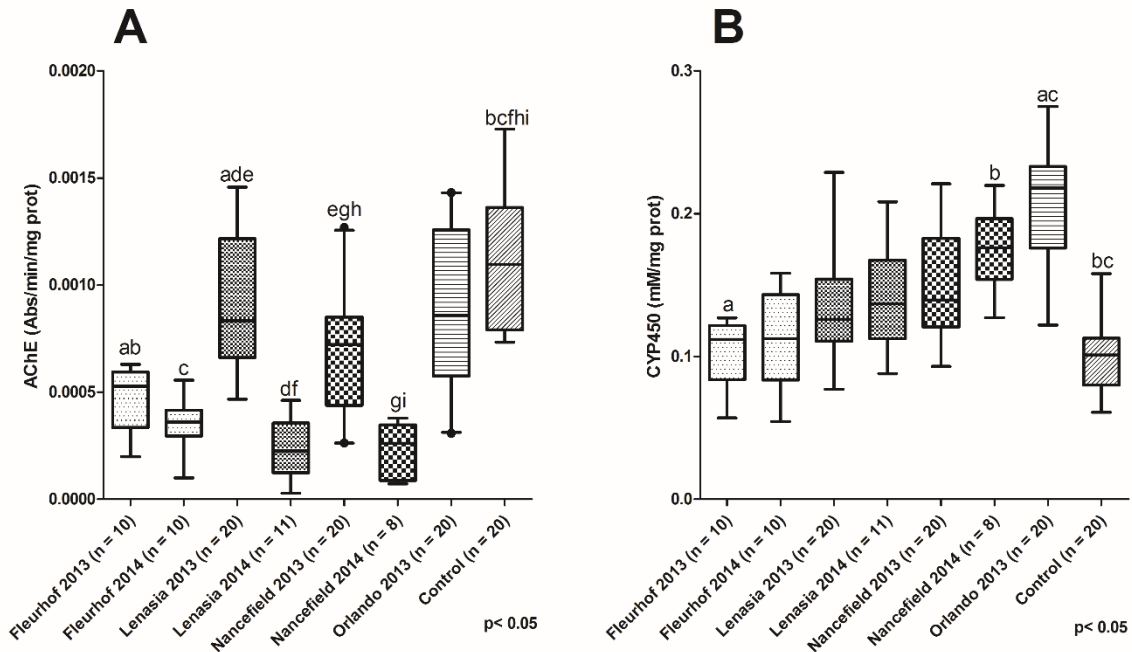


Figure 4.2: Biomarkers of exposure A) Acetylcholinesterase activity; B) and cytochrome P450 measured in *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014), and control fish. Median values indicated and whiskers set at 5th and 95th percentiles. Bars with common conscripts indicate significant differences (A Tukey's multiple comparison test; B Dunn's multiple comparison test, $p < 0.05$)

The cytochrome enzymes have detoxification responsibilities (Van der Oost *et al.*, 2003). Inhibition or activation of the cytochrome enzyme activity (CYP450) compared to a control indicates the effect of the xenobiotics present on this enzyme system. The CYP450s for the Fleurhof fish from both survey years were the least active of all the study sites and were comparable to the control with only a 4% up-regulation (Figure 4.2B). The highest CYP450 activity was measured for the Orlando fish (88% increase compared to the control), followed by Nancefield 2014 with a 63% increase. Orlando was significantly higher than Fleurhof 2013 and the control was significantly lower than both Orlando and Nancefield 2014 ($p < 0.05$). The activation of the CYP450s indicates the presence of xenobiotics at the sites.

There were visual temporal differences between fish from the different sites: CYP induction increased for Nancefield from 2013 to 2014 and decreased for the fish from Lenasia (Figure 4.2B), however these changes between the sampling years were not significant. PAHs are known AhR-ligands that induce the expression of the cytochrome enzymes (Villeneuve *et al.*, 2002) (see section 3.1.3).

The CYP450 activity measured in this project reflects the activity of all cytochromes in the sample and as explained in Chapter 2 and 3 there are a number of cytochrome *P450* dependent monooxygenase enzymes involved in first phase detoxification. These include the CYP1A isoenzyme, aryl hydrocarbon hydroxylase (AHH), and ethoxyresorufin-*O*-deethylase (EROD), all of which may be influenced by PAHs according to the literature. In order to compare the results of this study to what is reported in literature, papers on the effect of PAHs on afore mentioned three enzymes in fish had been chosen.

Padros *et al.* (2000) investigated the hepatic metabolising enzymes in brook trout (*Salvelinus fontinalis*) that had been exposed to benzo(a)pyrene interperitoneally. The activity of EROD and ethoxycoumarin-O-deethylase (ECOD) were quantified. EROD is predominantly mediated by cytochrome *P4501A* and ECOD is considered a non-specific enzyme that is catalysed by several forms of cytochrome *P450* (Padros *et al.*, 2000). These authors reported significantly up-regulated levels of EROD (170%) and ECOD (187%) compared with their control. Similarly, Levine and Oris (1999) showed that waterborne BaP increased EROD in rainbow trout (*Oncorhynchus mykiss*) by 38- and 88-fold after 24 hour exposure to 0.42 and 1.13 µg/L BaP respectively.

Vigano *et al.* (1995) also investigated liver microsomal enzyme activity in *O. mykiss*, injected with sediment extracts from sites previously proven to contain PAHs and PCBs. These crude extracts with unknown pollutant concentrations were transferred into corn oil, of which the final volume added depended on the %TOC in each extract. This allowed that the sites had the same nominal concentration of organic carbon and that the exposure range could be expressed in terms of TOC. The dosages injected into the fish were based on the dry mass of the sediments extracted per kilogram body mass (BM) of the fish which corresponded to 6, 28, 138 & 690 mgTOC/kg BM. Plain corn oil was also injected as a blank. After six days of exposure EROD and AHH activities were measured. The AHH activity of the lowest TOC dose was lower than their control (12.8% inhibited). The AHH activity increased with the increase of dosage but was inhibited at highest concentration (39% lower than control) (Vigano *et al.*, 1995), suggesting that the PAH containing extracts (as reported as the bile fluorescent metabolites) inhibited this metabolising enzymes. Likewise, the EROD system also followed the same increasing trend with the final concentration (690 mgTOC/kg BM) a decrease activity was noted. The first dosage elicited a 112% increase in EROD activity followed by a 125% and 267% increase for the next two dosages (28 & 138 mgTOC/kg BM). The decrease of activity at the highest concentration is not considered as inhibition as it still has a significantly higher activity than the control (169%) (Vigano *et al.*, 1995).

A difference in organ specific EROD activity in *C. gariepinus* was noted by Mdegela *et al.* (2006). Depurated fish were exposed to waterborne BaP and after a 4 day exposure EROD activity was determined in the gills and livers of these fish. An 11 and 17 fold EROD increase in response to BaP in the gills of laboratory exposed female and male *Clarias gariepinus* had been reported, whereas the EROD activity in the liver of both sexes increased by two fold (Mdegela *et al.*, 2006). Naicker *et al.* (2007) established a primary cell line of *C. gariepinus* hepatocytes. They validated the cell line by exposing the cells to BaP and measuring the EROD response. The hepatocytes exposed to 0.01, 0.1 and 1 µM BaP showed significant EROD activity increases: 96%, 129%, and 68% respectively (Naicker *et al.*, 2007).

In environmental studies, increases of cytochrome enzymes in PAH polluted areas have been reported. Hepatic EROD activity and the concentration of bile fluorescent aromatic compounds (FACs, ngBaP-eq/mL) were measured in brown bullhead (*Ameiurus nebulosus*) sampled from the lower Great Lakes (Arcand-Hoy & Metcalfe, 1999). The authors reported a significant increase in

EROD activity (between 120 and 200%) at sites with high levels of biliary PAHs (89–4 649 ngBaP-eq/mL). These sites, Hamilton harbour, Black- and Detroit Rivers also had high levels of Σ PAHs in the sediments (Table 2.5).

Hepatic CYP450 induction was also seen in caged channel catfish (*Ictalurus punctatus*) exposed to river water contaminated with PAHs and PCBs (Haasch *et al.*, 1993). There were slight inhibition of EROD, CYP1A1 and total CYP450 activities in these fish after one day of exposure. However, the expression of hepatic cytochromes increased as the exposure period increased. After one week's exposure EROD and ECOD were 200% and 12% higher than the control. However, the total CYPs were 44% lower than the control. When the fish were exposed for 14 days, the EROD and ECOD results increased significantly to 822% and 233% induction relative to the control (Haasch *et al.*, 1993) but the total cytochrome P450s did not show any significant fluctuation, even with the high induction of EROD and ECOD. In a study on *C. gariepinus* from sewage ponds in Tanzania there was high EROD activity increases compared to fish from their control site. An increase of more than 350% was noted in these fish (Mdegela *et al.*, 2010).

High levels of PCBs, OCPs, and polybrominated diphenylethers (PBDEs), and detectable levels of metals in the muscle of *Labeo umbratus*, from the Vaal River, have been reported by Wepener *et al.*, (2011). These fish showed an increase in EROD activity, however the site that had the greatest levels of PCBs and other organic pollutants had the lowest EROD activity (Wepener *et al.*, 2011), indicating to possible inhibition of this enzyme system in this fish species.

As mentioned earlier, the fish from the Phongolo system is known to be subjected to various pesticides used for MVC. The total CYP450 activity measured in the *C. gariepinus* of this system increased 3.75 times, as the Σ OCPs quantified increased (from 4.68 ng/g lipid mass to 479 ng/g lipid mass) (Edwards *et al.*, 2016).

Finally, *C. gariepinus* the Lower Klip River had significantly lower CYP450s ($p < 0.05$) compared to the Soweto and Lenasia sites. The highest CYP450 was 0.012 ± 0.003 nM/mg protein) (Wepener *et al.*, 2015) which is in the same range as the control of this study (Figure 4.2B). As already mentioned, fish from these sites where exposed to metals and OCPs (320.57 ng/g lipid mass) in the environment (Wepener *et al.*, 2015).

When comparing the cytochrome results of the two American catfishes, *Ameiurus nebulosus* (Arcand-Hoy & Metcalfe, 1999), and *Ictalurus punctatus* (Haasch *et al.*, 1993), as well as *C. gariepinus* from the Tanzanian study by Mdegela *et al.* (2010), to this study, keep in mind that the total cytochromes did not seem to have been affected as much as the individual monooxygenase (MOs) enzymes. The total cytochromes generally are a less sensitive assay (Bucheli & Fent, 1995). The reaction to xenobiotics by single isoenzymes may differ considerably, inhibiting one form and up-regulating another, and so not affecting the total CYP450 levels (Miranda *et al.*, 1990; Van der Oost *et al.*, 2003).

The responses of total cytochromes and the specific MOs of freshwater fishes to various pollutants have been summarised by Van der Oost *et al.* (2003) and is shown in Table 4.3. From literature (Table 4.3) it is evident that the total cytochromes do not fluctuate as regularly as the specific monooxygenases, especially when the fish were exposed to PAHs. In the instances where the total cytochromes were increased, these specific MOs were strongly induced (Table 4.3). In these cases halogenated organic pollutants, such as PCBs, dioxins, and to a lesser extent PAHs, were responsible for the observed responses (Table 3.4).

Table 4.3: Summary of the relationships of total cytochromes (CYP450), cytochrome 1A isoenzyme, aryl hydrocarbon hydrolase (AHH), ethoxyresorufin-O-deethylase (EROD) and ethoxycoumarin-O-deethylase (ECOD) responses to various pollutants in freshwater fishes reported in literature, as summarised by Van der Oost *et al.*, 2003

Fish species	Pollutant	CYP450	CYP1A	AHH	EROD	ECOD	Reference
<i>Cottus gobio</i>	Tributyltin & triphenyltin	– –			– –		Fent & Bucheli, 1994
<i>Anguilla Anguilla</i>	Tributyltin & triphenyltin	– –			– –		Fent & Bucheli, 1994
<i>Rutilus rutilus</i>	PCBs, PAHs, OCPs	–	=		=		Van der Oost <i>et al.</i> , 1994
<i>Ameiurus nebulosus</i>	Organic solvents	–			=		Gallagher & Di Giulio, 1989
<i>Salvelinus alpinus</i>	BaP	=			+	+	Wolkers <i>et al.</i> , 1996
<i>Anguilla Anguilla</i>	BaP	=		+	+		Lemaire–Goy & Lemaire, 1992
<i>Ictalurus punctatus</i>	PCB (Arochlor 1254)	=		+	+		Ankley <i>et al.</i> , 1986
<i>Oreochromis niloticus</i>	Industrial waste	=		+	+		Ueng <i>et al.</i> , 1992
<i>Oncorhynchus mykiss</i>	BKME	=		+	+		Lindström–Seppä & Oikari, 1990
<i>Perca fluviatilis</i>	BKME	=		+	+		Lindström–Seppä & Oikari, 1991
<i>Barbus plebejus</i>	PCB (Arochlor 1260)	+			+	+	Hugla & Thome, 1999
<i>Cyprinus carpio</i>	PHAHs and PAHs	+	+		+		Curtis <i>et al.</i> , 1993
	PHAHs and PAHs	+			+		Van der Oost <i>et al.</i> , 1998
	2,3,7,8 TCDD	+			+		Van der Weiden <i>et al.</i> , 1994
	2,3,7,8 TCDD	+	+		+		Van der Weiden <i>et al.</i> , 1992
<i>Lepomis auritus</i>	PCBs & Hg	+		+	+	+	Jimenez <i>et al.</i> , 1990
<i>Rutilus rutilus</i>	PCBs	+		+	+	+	Monod <i>et al.</i> , 1988
<i>Oreochromis niloticus</i>	PCBs & OCPs	+	+				Bainey <i>et al.</i> , 1996
<i>Thymallus thymallus</i>	PCBs	+	+	+	+	+	Monod <i>et al.</i> , 1988
<i>Oncorhynchus mykiss</i>	2,3,7,8 TCDD	+	+	+			Van der Weiden <i>et al.</i> , 1992

– – indicated strong inhibition (<20% to control); – shows inhibition; = shows no significant responses; + induction, + + shows strong induction (>500% to control). PHAH = polyhalogenated aromatic hydrocarbons BKME = Bleached kraft paper mill effluent

Total hepatic CYP450s of *C. gariepinus* for this study showed a visible increase and in some instances, such as Nancefield 2014 and Orlando, a significant increase of activity against the control. Although AHH and EROD were not quantified for these fish, PAHs quantified in the sediment and fish bile (Table 2.7 & 2.10), and the activation of the AhR (Table 3.1) suggest that these enzyme systems must have been induced, contributing to the total CYP450 activity. However, as stated earlier the AHH and EROD activity together do not necessarily increase the total CYPs (Table 4.3) and the increased induction seen for the fish of Soweto and Lenasia can therefore not be attributed to PAHs

alone. Pollutants such as PCBs and OCPs—previously quantified in the study area (Roos *et al.*, 2011)—together with the quantified PAHs can be attributed to the increased total CYP450 activity.

Biomarkers of effect

The biomarkers of effect results are divided into three sections: 1) the oxidative stress biomarkers 2) the biomarkers of oxidative stress damage, and 3) cellular energy allocation. The oxidative stress biomarkers are superoxide dismutase (SOD) and catalase (CAT) and are reported in Figure 4.3 A & B, respectively.

Biomarkers of oxidative stress

Superoxide dismutase forms the first tier of the anti-oxidant system responsible for converting reactive oxygen species (ROS) and superoxides into oxygen and hydrogen peroxide and therefore an increased SOD level may indicate high levels of ROS.

The SOD levels were elevated in the Lenasia fish from 2013 at 0.88 ± 0.36 ngSOD/mg protein; which was a 160% increase compared with the SOD levels of the control. This was the only increase that was statistically significant ($p < 0.05$). The smallest increase of SOD was found for the Fleurhof fish of 2014 at 0.46 ± 0.23 ngSOD/mg protein which amounted to a 39% increase (non-significant) (Figure 4.3A) Where levels were available for both years, the 2013 levels were always visually greater than that of 2014 (Figure 4.3A).

The increase in the SOD levels at Lenasia (2013) suggests that the organisms experienced oxidative stress and that the enzyme system was up-regulated to decrease these oxidative compounds to prevent cellular damage (Pandey *et al.*, 2003).

Otitoloju and Olagoke (2011) reported that crude oil—a known PAH source—has an inhibitory effect on SOD in *C. gariepinus*. These authors showed a 83% inhibition, which was significantly lower relative to their control (Otitoloju & Olagoke, 2011). Almroth *et al.* (2008) reported similar results. A 21% inhibition of SOD was seen in female corkscrew wrasse (*Symphodus melops*) from a PAH polluted site relative to a reference site. In contrast to this, the male *S. melops* from the same area had an increase of 9.6% SOD (Almroth *et al.*, 2008). This gender difference was not seen for *C. gariepinus* in the present study and SOD activity was similar between sexes.

The SOD measured in the *C. gariepinus* of the Phongolo River and floodplain increased over the two sampling surveys as the OCPs increased, specifically total DDTs and total hexachlorocyclohexanes (Σ HCH) (Edwards *et al.*, 2016). The SOD in the *C. gariepinus* from the Lower Klip River reported by Wepener *et al.* (2015), were all significantly lower ($p < 0.05$) than those upstream (present study). The maximum SOD level was 247 times lower than the Lenasia 2013 fish's SOD level. From the activation of the SOD system in the Soweto and Lenasia fish (5–160%) it is evident that there were potential SOD inhibitory compounds in the Lower Klip River.

Catalase is one of the enzymes from the second tier in the anti-oxidant system, responsible for the reduction of hydrogen peroxide formed by SOD. Catalase activity is indicative of compounds causing oxidative stress in the fish. Both the 2013 and 2014 CAT activity results were higher than the control (Figure 4.3B). *Clarias gariepinus* catalase activity for 2013 was between 4.8 and 12.7 times higher than the control, and for 2014 between 15 and 22 times higher. Temporal increase in the catalase responses was seen between 2013 and 2014 at all the sites, and the increase was statistically significant ($p < 0.05$) for Fleurhof and Lenasia. The catalase activity of the Fleurhof 2014 fish was the highest where the $148.7 \pm 38 \mu\text{molH}_2\text{O}_2/\text{min}/\text{mg}$ protein, represented a 22 fold increase (significant, $p < 0.05$) compared to the control (Figure 4.3B). Orlando had the highest CAT levels for 2013, $85.7 \pm 44 \mu\text{molH}_2\text{O}_2/\text{min}/\text{mg}$ protein, a 12.7 fold increase relative to the control (Figure 4.3B), which was statistically greater than the control group ($p < 0.05$). Similarly, fish from Fleurhof 2014, Lenasia 2014 and Nancefield of both years, were significantly greater than the control (Figure 4.3B). Catalase activity increases as hydrogen peroxide increases (formed by SOD). The temporal increase of CAT is conflicting with the overall constant levels of SOD (Figure 4.3A). This suggests that the synergistic relationship between SOD and CAT was disrupted by possible inhibition of SOD and/or a strong induction of CAT.

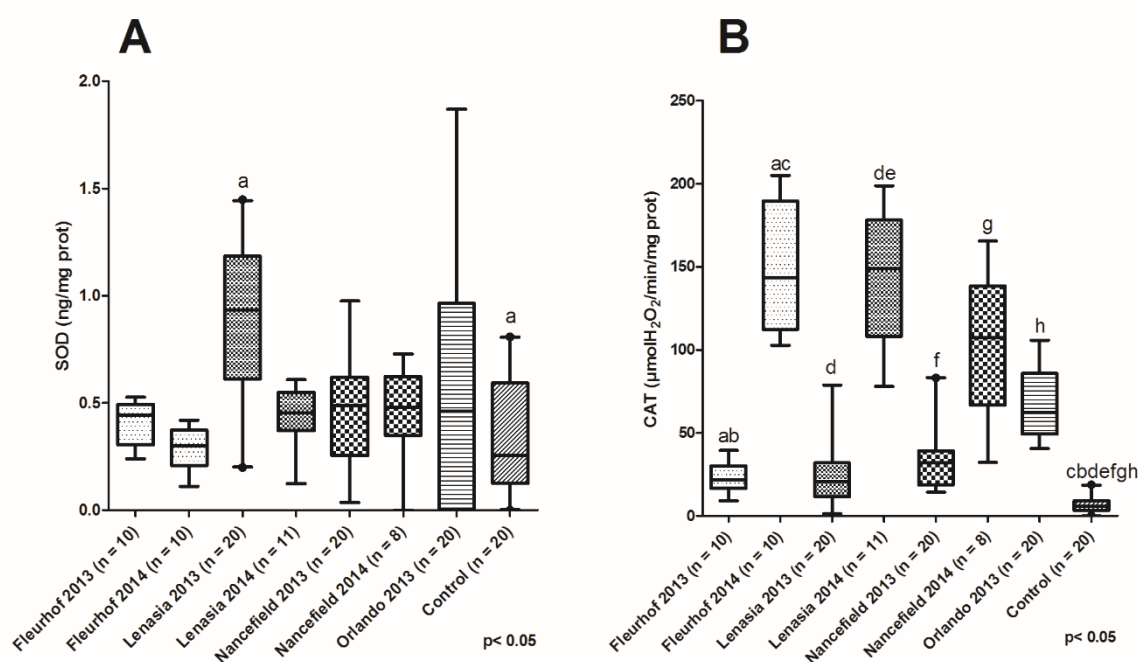


Figure 4.3: Biomarkers of oxidative stress A) superoxide dismutase; B) and catalase activity measured in *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014), and control fish. Median values indicated and whiskers set at 5th and 95th percentiles. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)

The crucian carp (*Carassius carassius*) is one of China's higher trophic level fish. Ji *et al.* (2010) transplanted laboratory-reared fish into a known PAH polluted site where PCBs and OCPs also had been detected, as part of active biomonitoring. The fish's reaction was measured using an integrated biomarker response assessment. The sites with the highest PAH concentrations also had the highest

biomarker responses. Catalase activities at the PAH polluted sites were significantly higher than their control with a 40–66% increased activity (Ji *et al.*, 2010).

Oliviera *et al.* (2008) investigated the organ specific antioxidant responses in golden grey mullet (*Liza aurata*) when exposed to phenanthrene. These fish were exposed to a concentration range of 17.8–480 ng/mL phenanthrene for 16 hours, after which oxidative stress biomarkers were analysed (Oliviera *et al.*, 2008). These authors reported significant increases of catalase activity (75%) in the gills of phenanthrene exposed fish to a control group and the CAT activity in their livers was significantly higher than in the gills. The highest hepatic CAT activity was 50% higher relative to their control (Oliviera *et al.*, 2008).

Petroleum refineries are not only sources of PAHs but also phenols, oils and metallic pollutants. Therefore, Avci *et al.* (2005) sampled wels catfish (*Silurus glanis*) up and downstream of a petroleum industry to determine the effects of the wastewater on the oxidant/antioxidant system of the fish living near the industry. They found a small increase of catalase activity in the livers of *S. glanis* between the two sites (11%) but it was not significant (Avci *et al.*, 2005). These authors also measured SOD levels and found that there was a significant increase in fish downstream. Comparing SOD and CAT responses of *S. glanis* where there was a high response of SOD and low response of CAT, which was opposite to what was seen in the *C. gariepinus* from Soweto and Lenasia (Figure 4.3 A & B).

The *C. gariepinus* of the Phongolo system's CAT levels increased similarly to the SOD levels i.e. an increase in enzymatic response as the measured OCPs increased *in vitro* (Edwards *et al.*, 2016). The catalase activity measured from the Lower Klip River *C. gariepinus* (Wepener *et al.*, 2015), was significantly lower ($p < 0.05$) than the CAT activity of the 2014 fish of Soweto and Lenasia. This shows that the fish upstream are responding to oxidative agents, such as metals and pesticides (Wepener *et al.*, 2015).

Biomarkers of oxidative stress damage

The biomarkers of oxidative stress damage measured in this study was lipid peroxidation by measuring malondialdehyde content (MDA) (Figure 4.4A) and protein damage by quantifying protein carbonyl (PC) content (Figure 4.4B).

The levels of MDA in a sample indicate the severity of lipid peroxidation due to the presence of oxidative stress compounds. None of the sites differed statistically between years or from the control (Figure 4.4A).

Lipid peroxidation in *C. gariepinus* has been reported in the Lower Klip River and the Phongolo system. The mean Lower Klip River MDA results of 1.26 and 1.82 nmol/mg protein (2013 and 2014 respectively) were significantly higher ($p < 0.05$)—200% greater—than the MDA levels of the Soweto and Lenasia fish. The levels of MDA in *C. gariepinus* from the Phongolo system, decreased temporally as the OCPs increased (Edwards *et al.*, 2016). The relative low MDA concentrations

suggest little to no lipid peroxidation in the *C. gariepinus* of the Soweto and Lenasia and that the anti-oxidant systems of these fish are functioning properly, disposing of ROS.

Similar to malondialdehyde content, the amount of protein carbonyls present indicates the severity of oxidative stress in terms of protein damage. Protein carbonyls are the products of amino acid oxidation. Orlando fish (collected in 2013) had the highest PC values (611 ± 168 nmol/mg protein) and differed significantly from all the other sites ($p < 0.05$). The other sites' levels were well below that of Orlando 2013, the lowest being for the fish caught in 2014 at Lenasia (155 ± 69 nmol/mg protein) (Figure 4.4B).

A slight temporal decrease in PC values was noted, from 2013 to 2014 (Figure 4.4B), but apart from the Orlando fish, all of the PC levels were comparable to that of the control, showing no increase in protein degradation. The 2013 fish from the Lower Klip River (Wepener *et al.*, 2015) had a similar range of PC content (277–634 nmol/mg protein) as Orlando, suggesting protein damage due to oxidative stress as seen with the other oxidative stress biomarkers (Figures 4.3 & 4.4).

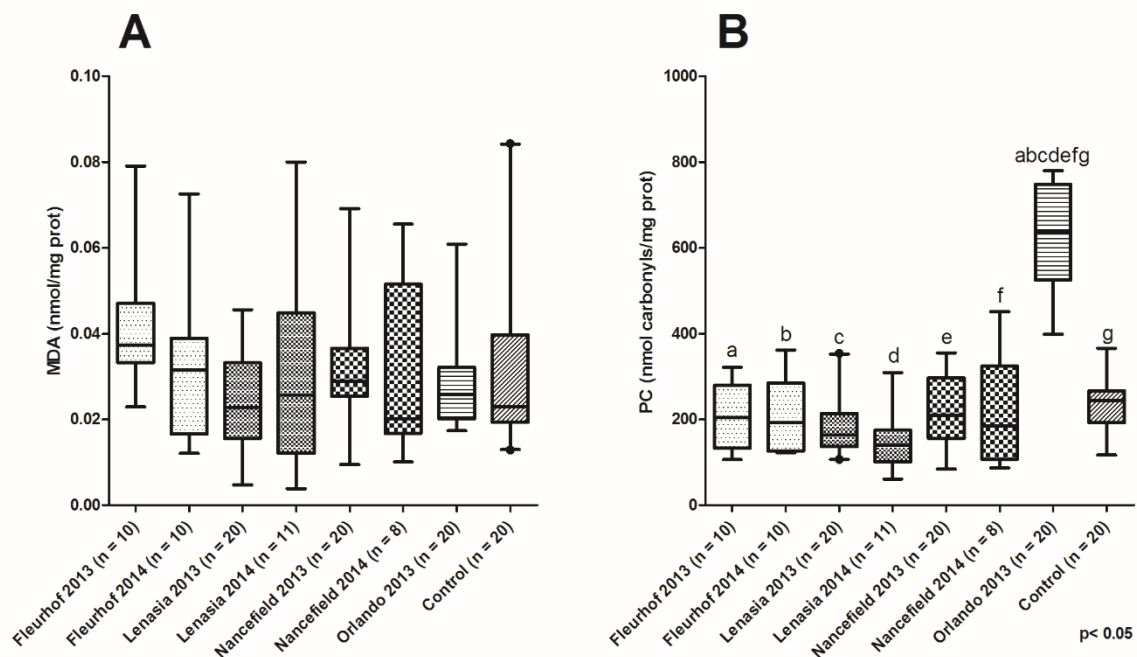


Figure 4.4: Biomarkers of oxidative stress damage A) malondialdehyde content; B) and protein carbonyl content, measured in *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014), and control fish. Median values indicated and whiskers set at 5th and 95th percentiles. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)

Cellular energy allocation

There was an overall increase in all the energy stores from 2013 to 2014 (Figure 4.5 A, B and C) and a decrease in energy consumption (Figure 4.5D). All the sites from Soweto and Lenasia had higher total carbohydrate stores than the control, ranging between 5.5% and 230%. The *C. gariepinus* sampled in 2014 from Fleurhof, Lenasia and Nancefield (both years) differed significantly from the

control ($p < 0.05$) (Figure 4.5A). A temporal increase in carbohydrates is visible between the two survey years, but only that of Nancefield showed a significant increase in carbohydrate stores from 2013 to 2014. The total carbohydrate level for the Nancefield fish from 2014 was the highest at 548 ± 23 J/g and was 230% higher than the control (Figure 4.5A). Although not the main source of energy, fish use carbohydrates to maintain growth rates, if carbohydrate reserves are depleted the main energy sources which are lipids and proteins will be catabolised for energy to provide the intermediates for the synthesis of biologically important compounds (Wilson, 1994).

Lipid storage plays an important role in fish health as it is critical for survival, fitness and reproduction (Adams, 1999). The lipid reserves of *C. gariepinus* in this study increased from 2013 to 2014 but it was only the fish from the Lenasia site for which the increase was significant (increase was significant because the levels of 2013 were low). All the 2014 fish had lipid reserves significantly higher than the control ($p < 0.05$). The Fleurhof fish from 2013 had the highest total lipid reserves of 293 ± 276 J/g, followed by Lenasia 2014 with 252 ± 265 J/g. The site with the lowest total lipid reserves was Lenasia 2013 (10.51 ± 4.4 J/g) (Figure 4.5B). The low lipid peroxidation shown by the MDA content (Figure 4.4A) coincides with the presence and high levels of total lipids specifically for 2014. Spare lipids are used as an energy source for reproductive and/or somatic tissue. Reproductive tissue uses the energy as an investment for breeding success at the time whereas, investing energy into somatic tissue ensures future reproduction at the expense of current reproduction potential (Adams, 1999).

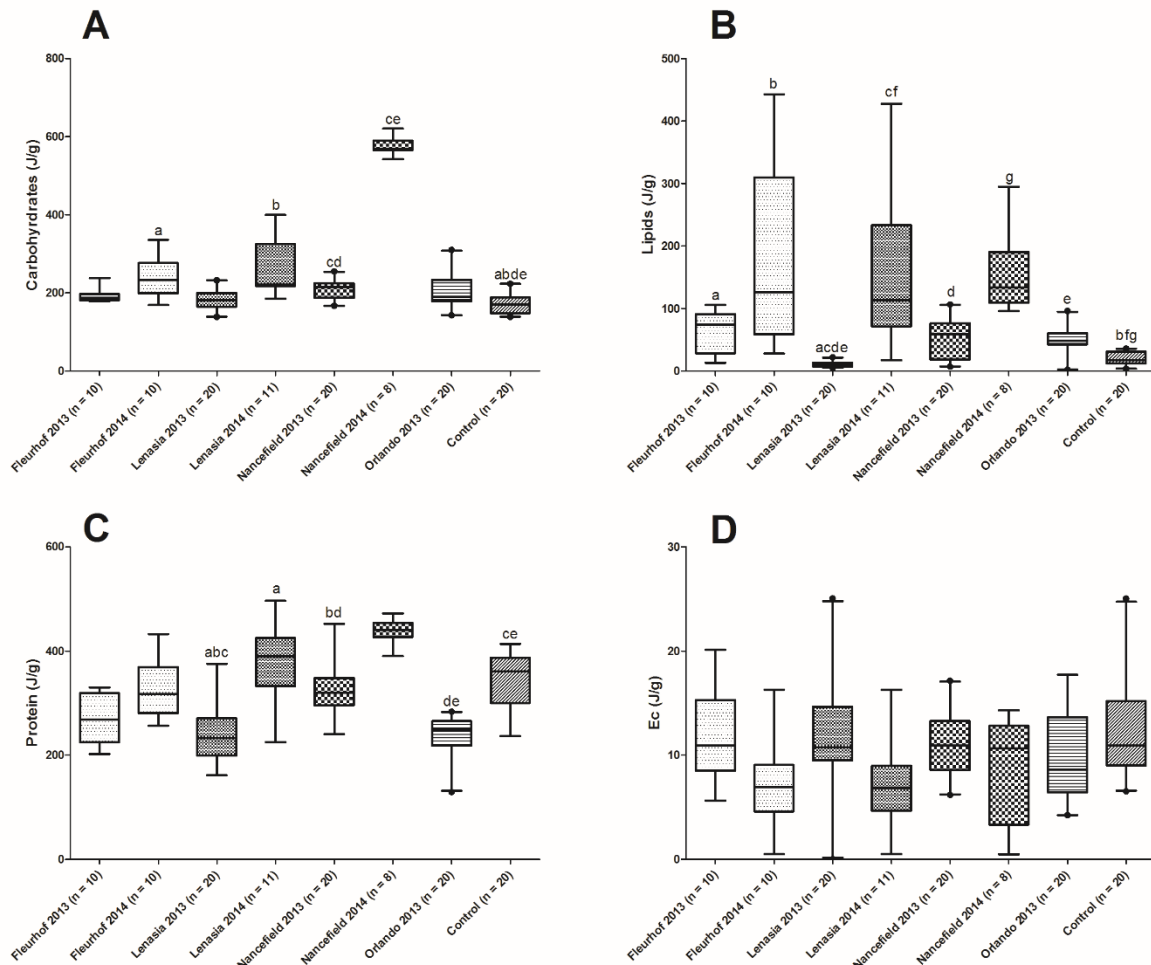


Figure 4.5: Energy reserves and consumption: A) available carbohydrates; B) total lipids; C) protein content; D) and energy consumption measured in *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014). Median values indicated and whiskers set at 5th and 95th percentiles. Bars with common conscripts indicate significant differences (Tukey's multiple comparison test, $p < 0.05$)

Protein levels were the highest of the three energy reserves in terms of energy (Figure 4.5C). As with both carbohydrates and lipids, a general increase in proteins was seen from 2013 to 2014. The highest protein levels were recorded for the fish caught at Lenasia in 2014 (377.5 ± 70.95 J/g) which was a significant increase from the previous year ($p < 0.05$) (Figure 4.5C). The control had high levels of protein reserves too, and was only significantly different from Lenasia 2013 and Orlando ($p < 0.05$) (Figure 4.5C). Orlando had the lowest protein levels at 239.7 ± 38.9 J/g. This could be due to oxidative stress damage (Figure 4.4B) as Orlando had significant levels of protein carbonyls. The overall increase in proteins could have been caused by synthesis of defence related proteins such as defensive enzymes, antigens and globulins (Smolders *et al.*, 2003) (Figures 4.2–4.3). Because these fish came from four different impoundments it is possible that differences in food availability could have contributed to the previous results. The energy consumption showed the inverse trend seen thus far—a visual temporal change in energy consumption. None of the results differed significantly. The higher consumption of energy in 2013 can be attributed to the preparation for spawning or active use of energy to mitigate environmental stressors.

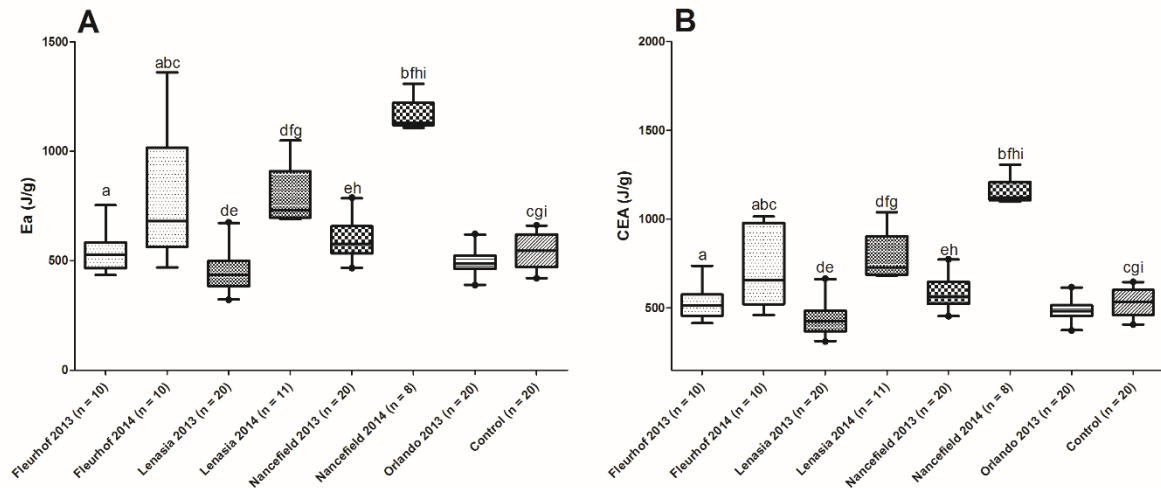


Figure 4.6: Cellular energetics: A) available energy; B) cellular energy allocation measured in *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014). Median values indicated and whiskers set at 5th and 95th percentiles. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)

Energy storages enable the fish to survive for a period of time when environmental resources become scarce (Adams, 1999). When an environment is significantly altered or disrupted, energy allocation in fish change. This disruption in natural energy allocation is to provide energy to maintain homeostasis in battling environmental stressors causing the disruption. This disruption may compromise the success of a species (Adams, 1999). The information in the available energy graph (Figure 4.6A) and cellular energy allocation graph (Figure 4.6B) corroborated each other. The fish from the 2013 survey had less energy reserves than the following year and there was significant temporal differences between all the sites ($p < 0.05$) (Figure 4.6B). As with the individual reserves, compared to the control, the fish from Nancefield of 2014 had the highest CEA of 1158 ± 73.5 J/g. The energy demand of homeostasis and fighting stressors, some of the drivers of energy consumption, was not high enough to significantly affect the cellular energy allocation in *C. gariepinus* from Soweto and Lenasia. Thus, the stressors that elicited responses in the other biomarkers were being successfully neutralised by the biochemical defence systems, protecting the energy reserves of the fish.

4.3.3 Bio-indicator assessment

The fish health assessment index (Figure 4.7 & 4.8), and organo-somatic indices (Figures 4.9–4.12) were determined during the bio-indicator assessment of *C. gariepinus*.

Fish health assessment index (FHAI)

The FHAI allows for the judgement of the general health and condition of a fish population and was developed as a comparative, but relative, biomonitoring tool to compare the same site over time and/or different sites at the same time (Heath *et al.*, 2004). The higher the FHAI score shows that the fish are in poorer health.

All the sites sampled for both events were significantly ($p < 0.05$) in poorer health compared to the control fish except the fish from Fleurhof in 2014 (Figure 4.8). The Nancefield fish of 2014 had the poorest health for both years (74 ± 46) (Figure 4.8), followed by fish from Fleurhof caught in 2013 (65 ± 30) (Figure 4.8). The variability of the health of the fish within these populations was high as shown by the coefficients of variance (CV; 62.3% and 46.6%). The fish sampled from Nancefield (both sampling events) had high FHA scores. Of the 2013 and 2014 surveys, 80 and 90% of the fish sampled had liver abnormalities such as discolouration and deformations (Figure 4.7A), and the majority of the fish had abrasions on their skin and fins. A small percentage of the 2013 fish (15%) also showed deformities within the body cavity ranging from altered gonad structures (Figure 4.7B), fusion of organs to the muscle, and an increase of dense connective tissue around organs (Figure 4.7C). All the fish sampled at Lenasia in 2013 had abnormalities including liver abnormalities such as discolouration and fatty deposits (Figure 4.7D & E). The fish had no external damage, but 80% of them had gill damage: frayed gill filaments (35%), and pale gills (45%).

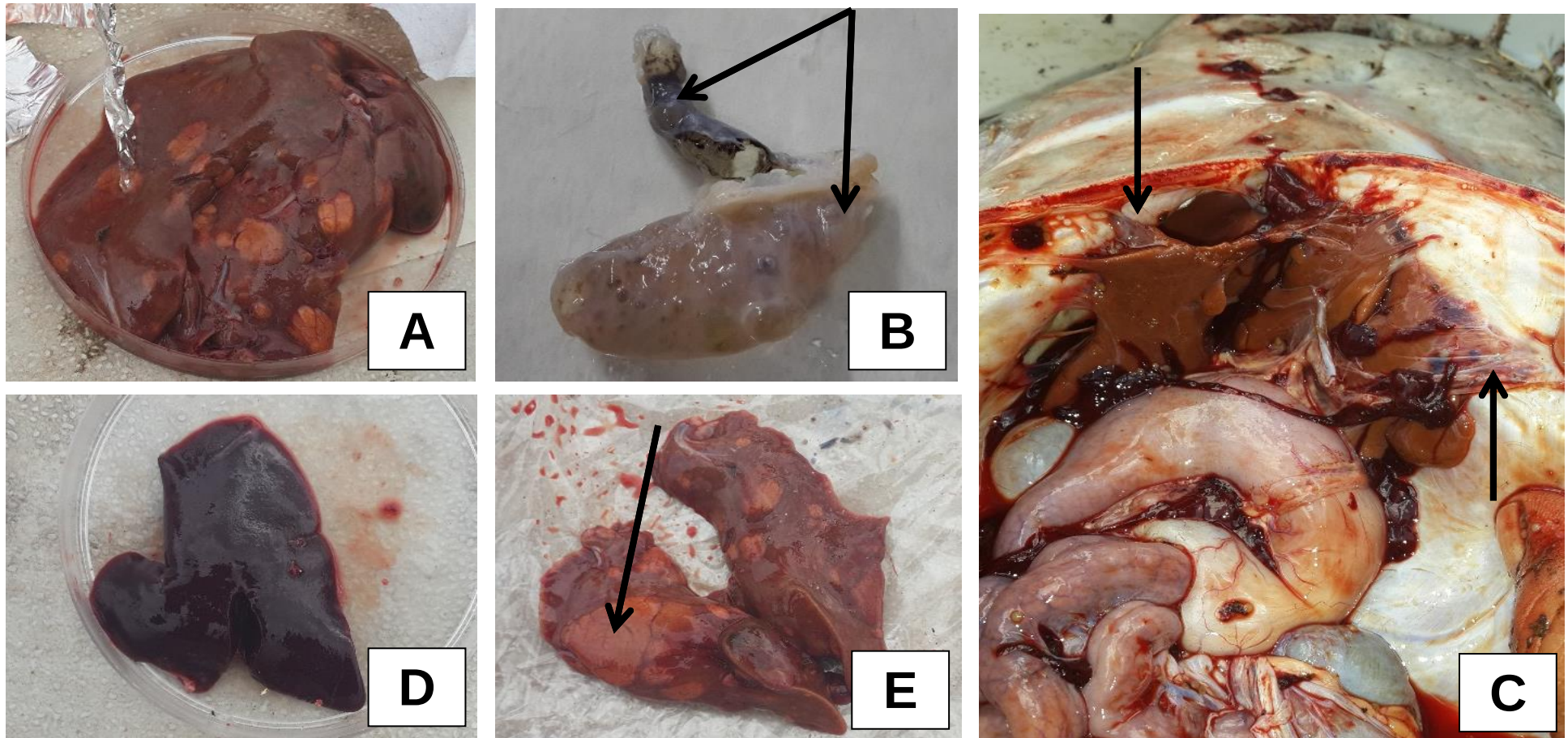


Figure 4.7: Observed abnormalities during necropsy: A) liver enlargement and darker discolouration; B) altered testes containing vesicles [arrows]; C) increase of connective tissue and fusion [arrows]; D) liver discolouration; E) increased fatty deposits in liver

The 2014 fish from Fleurhof had relatively the best health of all the environmental sites for both years as the FHAi was the lowest at 45 ± 14 (Figure 4.8) and can be considered to be in fair health. However, they were still in poorer condition than the control fish whose FHAi score was even lower at 22 ± 20 (Figure 4.8). Of the fish caught at the Lenasia site in 2014, 82% had discoloured and/or nodular livers and 36% presented enlarged spleens. Similarly, the fish from Nancefield in the same year had 75% liver abnormalities, 38% enlarged spleens, and 38% clubbed gills.

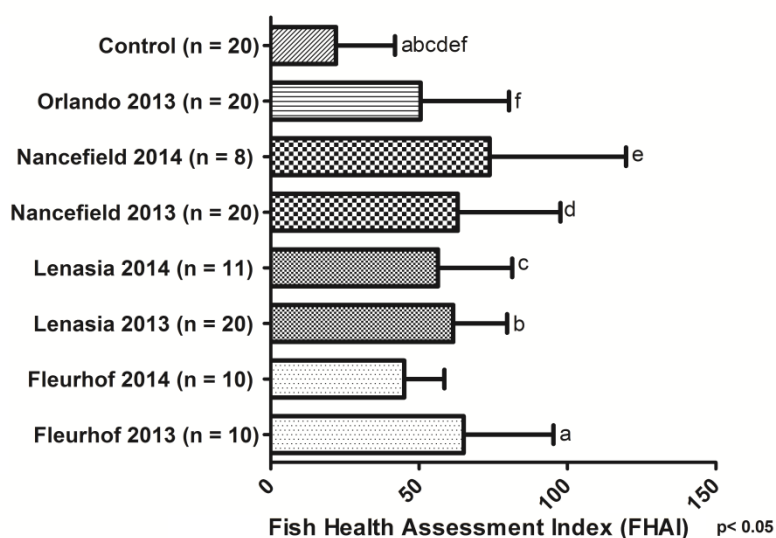


Figure 4.8: Fish health assessment index values for *Clarias gariepinus* sampled from sites from the greater Soweto and Lenasia area for 2013 and 2014, and control fish. Error bars are SD. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)

The FHAi scores reported in literature cannot be directly compared to one another if they are not from the same system or for the same fish species. Comparisons made will be relative to each study's control or reference sites and the final mean FHAi scores. In doing so, the effect of known/measured stressors and pollutants on fish can be interpreted. Watson *et al.* (2012) reported on a health assessment conducted on *C. gariepinus* from Loskop Dam and Bronkhorstspuit Dam, within the Olifants River system in the Mpumalanga Province. The latter dam was used as the control for their study and Loskop Dam used as the experimental site as it is known to be a polluted dam. *Clarias gariepinus* sampled from Loskop Dam in October 1996 yielded a FHAi score of 101.8 ± 10 (Watson *et al.*, 2012). This score is greater than for the current study (Figure 4.8). However, compared to their control (72 ± 13) Loskop dam's FHAi score was only 1.4 times greater. Nancefield (2014) and Fleurhof (2013) were 3.4 and 3 times higher than the control from this study (Figure 4.8), double the relative increase of the FHAi of Loskop Dam. In a similar study, Madanire-Moyo *et al.* (2012) also incorporated the FHAi in a study on *C. gariepinus* from three dams in the Limpopo and Olifants River systems. They investigated the relatively unpolluted Luphephe–Nwanedi Dams (reference site) in the Limpopo catchment, which receives its water from the streams of the Luphephe and Nwanedi mountains. The Flag Boshielo Dam is located in the Olifants catchment and is considered as a moderately polluted site. The Flag Boshielo Dam receives inputs from rural and urban communities, as well as from the established agricultural and mining sources in the catchment. The final and

severely polluted site was the Return Water Dam, found in the Limpopo River catchment. This dam receives its water from a platinum mining complex, which uses wastewater in its processes. Discarded mining water finds its way into the Return Water Dam (Madanire-Moyo *et al.*, 2012). The FHA scores of these sites were 42.7 ± 20.7 , 84 ± 37.2 , and 93.3 ± 27.3 respectively. These polluted sites had high FHA scores compared to their control (2 and 2.2 times higher), indicating the fish were in poor health. The most prevalent abnormality seen in Flag Boshielo- and Return Water Dams were anomalies in the gills, liver and blood parameters (Madanire-Moyo *et al.*, 2012). However, compared to their control these sites did not have as a high health difference as seen in the present study. The reference site of Madanire-Moyo *et al.* (2012), had similar FHA scores as that of the fish of the current study and was lower than the control (Figure 4.8). Thus, in terms of health relative to the control the Soweto and Lenasia fish seem to have been in poorer health, but contradictory to this the mean FHA values suggested that the fish from Soweto and Lenasia were in better health.

The Pongolapoort Dam is located in northern KwaZulu-Natal, in the MVC area. It has been reported that fish from this site are exposed to organohalogenes such as OCPs and polybrominated diphenyl ethers (PBDEs) (McHugh *et al.*, 2011; Wepener *et al.*, 2012). McHugh *et al.* (2013) assessed the health of *C. gariepinus* from Pongolapoort Dam. The fish sampled had a FHA score of 51.6 and mainly had anomalies of the liver (fatty and discolouration) as well as swollen kidneys and enlarged spleens in some of the fish (McHugh *et al.*, 2013). The scores of the Pongolapoort Dam *C. gariepinus* was similar to the FHA scores of the current study, but lower in comparison to Fleurhof 2013 and Nancefield 2013 and 2014's fish (Figure 4.8).

Clarias gariepinus along with three other ecological and economically fish species from the Okavango Delta were sampled and their health status determined by Van Dyk *et al.* (2009) to establish a baseline for future toxicological studies. *Clarias gariepinus* sampled in the Okavango Delta panhandle were expected to be in good health as this site is considered as a pristine site. The fish sampled had a mean FHA score of 10.6 (Van Dyk *et al.*, 2009). These fish had no external abnormalities. Nematodes were present in 33% of *C. gariepinus* sampled by Van Dyk *et al.* (2009). No parasites were observed in and on the fish collected from Soweto and Lenasia aquatic systems. The Okavango fish had a lower FHA score than all the sites of the current study including the control (Figure 4.8).

The fish sampled by Wepener *et al.* (2015) from the Lower Klip River had significantly lower FHA scores than Soweto and Lenasia of both surveys with 19.5 ± 22.5 for 2013 and 13.3 ± 21.1 for 2014 ($p < 0.05$). The FHA of these fish was mainly caused by pale or discoloured gills (10%) and discoloured livers (25%), and no other abnormalities were noted in these fish (Wepener *et al.*, 2015).

From literature we can deduce that the fish sampled from Soweto and Lenasia were in poor health. The wide gap between the health of the sampled fish and that of the aquarium control, and that the difference margin was greater than for studies in known polluted sites, it is clear that the stressors heavily affected the health of *Clarias gariepinus* from the study area.

Organo-somatic indices

The organo-somatic indices included Fulton's condition factor (Figure 4.9), the hepato-somatic index (Figure 4.10), spleeno-somatic index (Figure 4.11), and gonado somatic index (Figure 4.12) are reported on in the next section

Fulton's condition factor

As mentioned previously, the condition factor expresses the condition of the fish, its well-being, relative robustness or fatness at the moment that they were sampled. Lückhoff (2005) established a condition factor range specifically for *C. gariepinus*. If the condition factor for natural occurring catfish is above 0.85, the fish is in a good condition and if it is below 0.6 it is in a poor condition and between these values condition is referred to as fair. Aquaculturists strive for a condition factor of 1.04 in fed fish. The condition factor values of the fish sampled for both 2013 and 2014 seasons are reported in Figure 4.9.

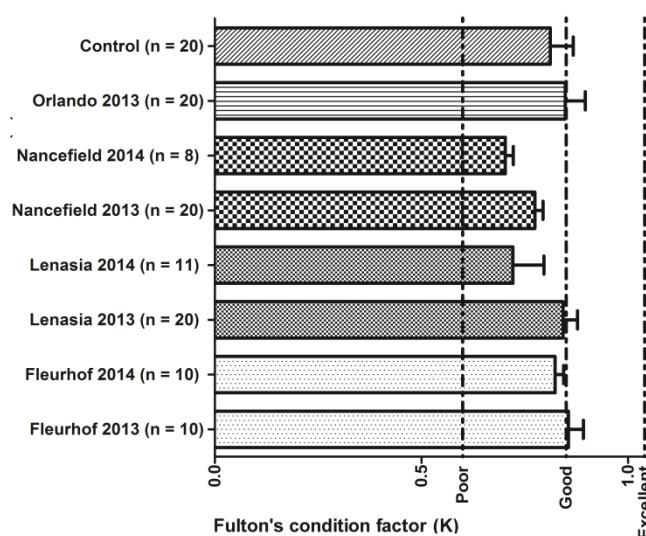


Figure 4.9: Fulton's condition factor for *Clarias gariepinus* sampled from sites from the greater Soweto and Lenasia area for 2013 and 2014, and control fish. Error bars are SEM

All the fish sampled in both seasons were in fair condition (between 0.6 and 0.84) with exception of the fish from Orlando collected in 2013 (0.85 ± 0.12) and Fleurhof also from 2013 (0.86 ± 0.22) which were in good condition (Figure 4.9). The lowest CF scores were reported for fish from Lenasia and Nancefield from 2014 (0.72 ± 0.24 and 0.7 ± 0.05 respectively; Figure 4.9). The condition factor results indicate that the fish were not short on food supply and that a decline in overall health could not be attributed to malnutrition. This is corroborated by the colour and volume of the bile sampled during the necropsy. Whenever bile was found in the gall bladder, it was light and dark straw coloured. This is an indication that the fish fed within a few days (yellow bile) or hours (empty gall bladder) of sampling (Goede & Barton, 1990). The fish sampled from the Lower Klip River during the same sampling years, had comparable (no significant differences) condition factors (Wepener *et al.*, 2015). *Clarias*

gariepinus from the Loskop Dam and Bronkhorstspuit Dam in the Olifants River system had condition factors similar to our findings with 0.84 ± 0.06 and 0.86 ± 0.1 respectively (Watson *et al.*, 2012). The fish sampled in Flag Boshielo Dam and Return Water Dam had higher condition factors, 1.2 ± 0.6 and 1.4 ± 0.8 respectively (Madanire-Moyo *et al.*, 2012). Lower condition factors were calculated for *C. gariepinus* from sewage ponds, CF ranging between 0.61 ± 0.01 and 0.69 ± 0.02 (Mdegela *et al.*, 2010). The before mentioned results of Mdegela *et al.* (2010) were lower than the CF for the fish from the sewage ponds at Nancefield (0.77 ± 0.08 in 2013 and 0.7 ± 0.05 in 2014).

Barnhoorn *et al.* (2004) investigated intersex in wild sharptooth catfish exposed to endocrine disrupting chemicals, such as *p*-nonyl phenol. There was a noted difference between the condition factors calculated for both sexes and the intersex specimens from the Marais Dam site, Gauteng South Africa. The intersex catfish had a CF of 1.05, higher than the males and female CFs of 0.9 and 0.88—this trend was however not seen at their other site, Rietvlei Dam (Barnhoorn *et al.*, 2004). The condition factors for the intersex fish were higher than all the fish from the current study (Figure 4.9). Van Dyk *et al.* (2009) reported condition factors of 0.8 ± 0.3 for *C. gariepinus* from the Okavango Delta Panhandle, and McHugh *et al.* (2013) CF of 0.77 ± 0.15 for *C. gariepinus* from Phongolopoort Dam. These results were slightly lower than the condition factors of Soweto and Lenasia, especially those determined for the fish from Fleurhof in 2013 (0.85 ± 0.11), 2014 (0.82 ± 0.06), and Lenasia from 2013 (0.84 ± 0.15) (Figure 4.9).

Hepato-somatic index (HSI)

The HSI indicates the energy reserves of the fish or the effects of xenobiotics on the liver. An increase in HSI may be seen after exposure to pollutants due to hyperplasia or hypertrophy (Marchand *et al.*, 2009) to increase the liver's potential to metabolise toxicants (Goede & Barton, 1990). Pollution may also lower the HSI due to atrophy of hepatocytes or necrosis (Marchand *et al.*, 2009; Sanchez *et al.*, 2008), but it is important to keep in mind that the size of the liver is also affected by various other variables (Goede & Barton, 1990; Sanchez *et al.*, 2008). Van Dyk *et al.* (2012) showed that the HSI values of *C. gariepinus* of Southern Africa—sampled from various aquatic systems—are close to 0.6%.

The HSI values are shown in Figure 4.10. The control fish HSI value is $0.61 \pm 0.27\%$ (Figure 4.10). Orlando had the highest HSI value for both 2013 and 2014 (Figure 4.10), and was consistent with liver anomalies seen during the health assessment. Lenasia 2013 had the lowest HSI at $1.26\% \pm 0.25$ (Figure 4.10). The HSI value of fish from Orlando was significantly higher than that for the fish from Lenasia and Nancefield in 2013 and the control ($p < 0.05$) (Figure 4.10). All the sites, except Nancefield 2014, were significantly higher ($p < 0.05$) than the control. The range of the HSI means in this study ranged between $1.16 \pm 0.48\%$ and $1.51 \pm 0.48\%$ and was higher than the average 0.6% HSI of southern African catfish in general as was determined by Van Dyk *et al.*, (2012). These results were similar to the polluted sites reported by Van Dyk *et al.* (2012), situated in northern Gauteng (Roodeplaat-, Rietvlei- and Marais Dams) and the North West Province (Hartebeespoort-, Bospoort- and Klipvoor

Dams). The dams from the North West Province and Roodeplaat Dam are hyper-trophic dams had HSI values of $1.2\% \pm 0.6$ (Van Dyk et al., 2012). These impoundments had very similar HSI values to Lenasia 2013 and 2014's results, the lowest HSI from this study (Figure 4.10). The remaining impoundments in Gauteng had HSI values of 1.4 ± 0.5 (Van Dyk et al., 2012), which was comparable to the Fleurhof site for both surveys (Figure 4.10). Mdegela et al. (2010) reported significant differences in HSI of the *C. gariepinus* from the different sewage ponds ($1.1\% \pm 0.07$ – $1.4\% \pm 0.12$) compared to their reference site ($0.5\% \pm 0.03$). Fish from Nancefield 2014 (sampled from sewage maturation ponds) did not show similar differences in HSI relative to the control, as seen with the former mentioned study (Figure 4.10). The fish from the Okavango ($0.5 \pm 0.1\%$) (Van Dyk et al. 2009) had lower HSI values than the current study, including our control—although the Okavango site is regarded as pristine, it is still a natural situation with its wide influences on fish condition, whereas the control of this study were aquarium kept fish with its stable environment and therefore a better HSI could be expected. The HSI of the fish sampled from the Pongolapoort Dam ($0.93 \pm 0.005\%$) (McHugh et al., 2013) had lower HSI values than all the environmental sites from Soweto and Lenasia, but greater than the control (Figure 4.10).

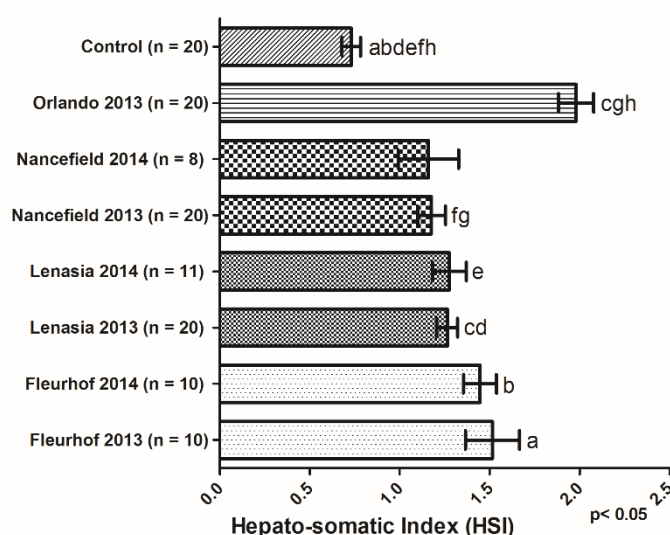


Figure 4.10: The hepato-somatic indices values for *Clarias gariepinus* sampled from sites from the greater Soweto and Lenasia area for 2013 and 2014, and control fish. Error bars are SEM. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)

Spleeno-somatic index (SSI)

The spleen is a lymphatic organ which main function is to produce and store blood (Fänge & Nilson, 1985). It also plays a role in antigen- and erythrocyte degradation and antibody production (Goede & Barton 1990; Rohlenová et al., 2011). Swelling or enlargement of the spleen can be indicative of necrosis and relate to immune problems such as infection and/or disease in the fish (Adams et al., 1992; Goede & Barton 1990).

The SSI results show the immune responses of the fish sampled in this study (Figure 4.11) and an increase in the size can be ascribed to an increase in immune responses (Rohlenová et al. 2011).

There were no significant differences between the sites and the control (Figure 4.11). There were no parasites observed in or on any of the fish sampled, at any of the sites and thus the SSI values could not have been attributed the presence of parasites (Rohlenová *et al.*, 2011) and toxicants suspected for causing the effect.

The SSI reported by Bester (2013) calculated for *C. gariepinus*, that had been sampled from Roodekopjes ($0.18\% \pm 0.11$), Vaalkop ($0.13\% \pm 0.05$) and Marico-Bosveld Dam ($0.2\% \pm 0.09$), were similar to this project. The SSI of *C. gariepinus* from the Okavango Panhandle ($0.11\% \pm 0.03$) and Pongolapoort Dam ($0.13\% \pm 0.07$) (McHugh *et al.*, 2013), were lower than all fish sampled during the current study, as well as our control (Figure 4.10). It is probable, but not statistical, that the stressors in the Soweto and Lenasia study area had an effect on the spleen, and so possibly on the immune system of the fish.

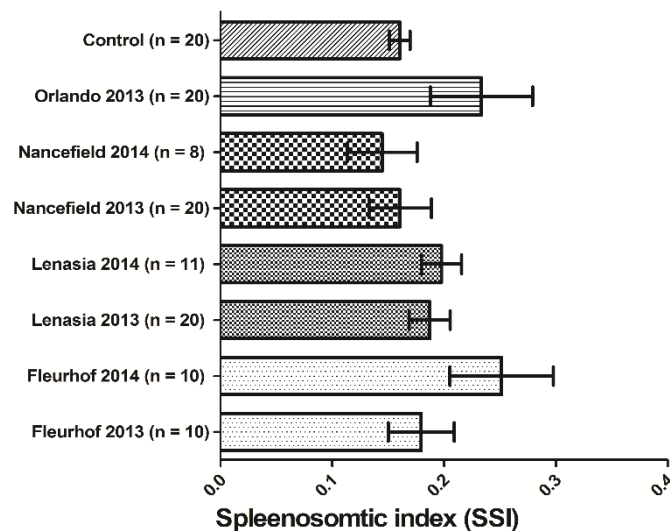


Figure 4.11: The spleeno-somatic index results for *Clarias gariepinus* sampled from sites from the greater Soweto and Lenasia area for 2013 and 2014, and control fish. Error bars are SEM

Gonado-somatic index

The gonado-somatic index provides information on gonadal health and developmental stages, in response to changes, such as environmental stressors and/or seasonal changes (McDonald *et al.*, 2000). The sampling took place during the pre-spawn season of *C. gariepinus*, and a majority of the female fish were in the maturing stage for spawn season, except for 4 fish from Orlando with underdeveloped ovaries (these fish were excluded from the GSI calculations). There is a high variation in the GSI, standard errors ranged from 0.98–8.6 (Figure 4.12A). Fish caught in 2013 at the Nancefield site had the highest GSI value followed by the 2014 values of the same site (11.19 ± 4.29 and 10.25 ± 5.44 respectively), and was the only site of which the GSI was significantly higher than that of the control ($p < 0.05$) (Figure 4.12A). There was no correlation between the total lipids available (Figure 4.5B), suggesting that the lipids were not utilised as energy for gonadal development. Fish from Nancefield (both surveys) and Orlando had the only GSI significantly higher than the *C.*

gariepinus from the Lower Klip River (Wepener *et al.*, 2015). The control fish had the lowest GSI of all the fish sampled (Figure 4.12A & B). The reason for this is that the fish were processed in April 2014, when they have not yet become gravid with eggs as they would have been during October. Bruton (1979) mentioned that the gonads of *C. gariepinus* are in a resting period during April (in southern Africa,). Maturation of gonads begins in August and by October (spring and early summer in the southern hemisphere) the bulk of the population are sexually mature (Bruton, 1979). The female fish from all the sites (both surveys) were in the developing and maturing stages (IV and V stages) (Bruton, 1979). The lowest GSI from the sampling area was Fleurhof 2013 (5.78 ± 4.81 , Figure 4.12A) and this site had a high temporal increase to 2014's GSI value of 9.29 ± 2.93 (Figure 4.12A).

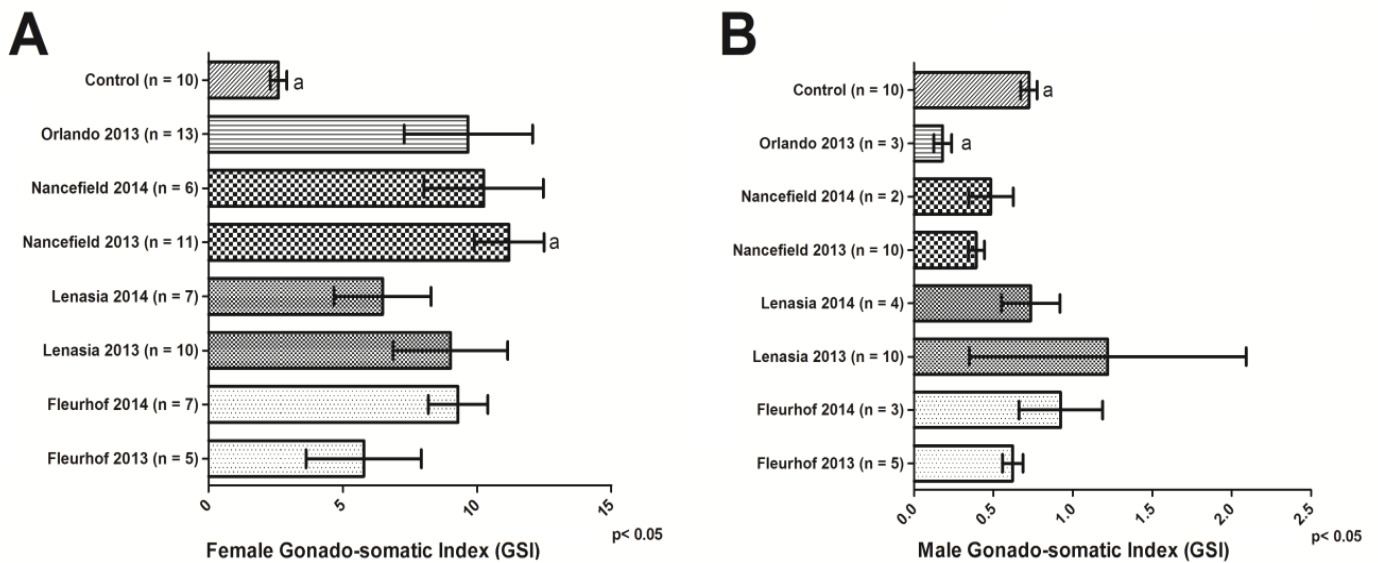


Figure 4.12: The gonado-somatic index for A) female and B) male *Clarias gariepinus* of Soweto and Lenasia (2013 and 2014), and control fish. Error bars are SEM. Bars with common conscripts indicate significant differences (Dunn's multiple comparison test, $p < 0.05$)

The female fish from Orlando that had been excluded from the calculations due to underdeveloped ovaries that were not necessarily juveniles. *Clarias gariepinus* reach maturity at a length of 330–340 mm, or within two years (Bruton, 1979). When referring to their size (510–640 mm) and mass (1 000–2 000 g), there were fish within the population smaller than these individuals that had developed ovaries and that were gravid with eggs (assuming that all individuals in the population would be of approximately the same developmental stage). According to the description of the gonad development by Bruton (1979), these fish's gonads fall in Class I (immature virgin) and II (developing virgin) of the gonad maturity stages. This underdevelopment indicates to possible endocrine disruption in these individuals and warrants further investigation.

The male fish from Lenasia 2013 had the highest GSI (1.22 ± 2.61 , Figure 4.12B) and was the only population that showed decrease in GSI over the two years. The second highest GSI was from the 2014 Fleurhof fish, with a 0.92 ± 0.46 value (Figure 4.12B). The Orlando males had the lowest GSI (0.18 ± 0.1), which was significantly lower than that of the control (0.63 ± 0.14) (Figure 4.12B), indicating

that these male fish had smaller testes. Potential feminization was observed in one male from Nancefield, which had sac like vesicles growing within a deformed testis (Figure 4.7B)—this needs to be confirmed with histology. Feminization and decrease in testes size is indicative of hormonal imbalance possibly from exposure to endocrine disrupting chemicals (Mills & Chichester, 2005). Intersex in *C. gariepinus* was seen in fish from the Rietvlei- and Marais Dams. The intersex was linked to endocrine disruption from anthropogenic pollution such as wastewater treatment plant effluent, agriculture or industry (Barnhoorn *et al.*, 2004). The *C. gariepinus* exposed to sewage effluent had a significant difference in GSI between ponds and reference site. This may be from exposure to compounds in the sewage causing enlargement of the gonads (Mdegela *et al.*, 2010).

4.4 Conclusion

From the fish biomarker data it seems that there were environmental stressors that affected the health of the fish on a biochemical level. An inhibition of the acetylcholinesterase system was noted in the fish from Soweto and Lenasia. Apart from this inhibition, exposure responses also included an increase in cytochrome P450 activity. Both the activation of the detoxification system (CYP450), along with the up-regulation of the SOD-CAT defences is evidence that the fish from the study area had been defending against xenobiotic stressors. The lower SOD (relative to CAT) showed that this enzyme system definitely was working against compounds; however inhibition or the sheer overwhelming of SOD could be seen in the high activation of the CAT defences. The low concentrations of malondialdehyde showed that the antioxidant defences had been successful in stopping lipid peroxidation, which were also corroborated by the higher lipid stores. Conversely, the lower protein stores and the higher protein carbonyls showed that there had been damage to proteins in the fish and the increase of enzymatic defences. The energy budget of the fish showed that there had been an increase in available energy over the two surveys, indicating that although there were stressors activating the fish's defences that it had not either yet affected the energy budget of the individuals, or that the defences were successfully in mitigating the threats these stressors pose.

The overall health, on a systematic level, of the Soweto and Lenasia fish was visibly affected by environmental stressors. The Soweto and Lenasia fish were in poorer health than fish sampled in the Lower Klip River. The overall health of the fish from Soweto and Lenasia was also lower than fish from other heavily polluted or affected aquatic systems, such as the Olifants River system or Rietvlei Dam. The condition factor indicated that the fish were in a fair condition, thus indicating that the health effects observed were not due to malnutrition. The significantly higher HSI at Orlando showed that fish from this site have definitely been exposure to xenobiotics. The livers in the fish from this study were larger than other studies *C. gariepinus*. The SSI did not vary significantly between sites, but the slight enlargement of the spleen was not attributed to parasites, as none were found during the necropsy. The GSI showed that many of the fish were preparing for spawning; however there were individuals at Orlando that were classified as first time spawners, suggesting that these adult fish had endocrine stressors acting on them.

Although it is difficult to pinpoint the specific causative agents of the stressors it is clear from the results presented in this chapter that the fish in the Soweto and Lenasia at the sites where we sampled were under stress and in poorer health when compared to the control fish. Of all these sites *C. gariepinus* from the Orlando site proved to be the worst affected, followed by those from Nancefield and Fleurhof.

4.5 References

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Chapter 5: Human health risk assessment for matrices of the Klip River of Soweto and Lenasia

5.1 Introduction

It has been confirmed by numerous studies that polycyclic aromatic hydrocarbons (PAHs) have adverse effects on both wildlife (Hawkins *et al.*, 1990; Black & Baumann, 1991; Karami *et al.*, 2016) and humans (Obana *et al.*, 1981; Farmer *et al.*, 2003; Binková & Šrám, 2004; Kim *et al.*, 2013). For this reason the USEPA singled out 16 PAHs (USEPA, 2008a) to be monitored in the environment (Srogi, 2007; Pies *et al.*, 2007; Pieters *et al.*, 2015; Sun *et al.*, 2015), foodstuffs (Falcó *et al.*, 2003; Perelló *et al.*, 2009; Rose *et al.*, 2009), and in the human workplace (Zhao *et al.*, 1990; Petry *et al.*, 1996; Väänänen *et al.*, 2005; McClean *et al.*, 2007). When these compounds are present in the environment they pose a threat to human health. In order to determine if the PAHs in a given environment may pose a threat, a human health risk assessment is completed. The goal of a human health risk assessment is to estimate the likelihood of adverse effects in a population at defined exposures to (a) target compound/s.

Calculating the threat based on the concentrations in sediments, water and fish tissue, can provide the ability to estimate the probability of health effects in a specific area. The risk from the exposure to PAHs is predicted by evaluating the toxicity of these chemical contaminants over different exposure times, at different concentrations and exposure routes—which are measured and identified at each site. The World Health Organisation (WHO) suggests four steps for the risk assessment process: 1) hazard identification, 2) hazard characterisation, 3) exposure assessment, and 4) risk characterisation (WHO, 2010).

Hazard identification is the first step in a health risk assessment. It involves the identification of the underlying toxicological properties of chemical, i.e. its ability to cause various health effects to humans (enHealth, 2002; Heath *et al.*, 2004; EA, 2009). Chemical characteristics like physico-chemical properties of the chemical, metabolic properties, routes of exposure, toxicological effects, chronic- and acute animal exposure studies are used in these identifications (USEPA, 2000).

Hazard characterisation mainly consists of the description of inherent properties of the compounds and its potential to have adverse health effects (WHO, 2010). This involves the understanding of how the mode of action or the mechanism of toxicity of the compound works. Hazard characterisation often employs dose-responses in the assessment to investigate toxic effects (EA, 2009). These responses evaluate qualitative and quantitative toxicity information to estimate frequencies at which negative effects may occur in humans, at different exposure concentrations (enHealth, 2002). The exposure dose, the likelihood, as well as the magnitude of health effects are characterised. The dose-response dynamic is therefore the functional relationship between the exposure and the observed health effects. Hazardous chemicals can be grouped into groups with non-threshold effects (cancer risk) and threshold effects (non-cancer risk). The assessment of the toxicity of hazardous chemicals is

usually done with animal toxicity data, as data for human exposure to most of the contaminants is unavailable (USEPA, 2000; Newman, 2010). International databases and organisations, such as the WHO, Food and Agriculture Organization of the United Nations (FAO) and the Integrated Risk Information System (IRIS) exist to supplying information on relative risk values and health effect endpoints, to help characterise the hazards of the compound of interest (OEHHA, 2001; WHO, 2010; IRIS, 2016).

Exposure assessment is evaluating and quantifying the exposure of the humans to the target compounds (EA, 2009). This involves the determination of the intensity, magnitude, frequency, and duration of exposures (USEPA, 2000; enHealth, 2002; Newman, 2010). In such assessments the exposures are determined for different sub-populations (e.g. children, adult, and elderly) by specific exposure pathways (e.g. oral ingestion, dermal absorption or inhalation) (USEPA, 2000; Newman, 2010; WHO, 2010).

The final part of the assessment is risk characterisation, which is a quantitative statement derived from comparing estimated exposures to guidelines or calculating the life-time risk estimated exposure values for a specific compound (WHO, 2010). The risk statement created describes the overall risk for individual and population health risks (Heath *et al.*, 2004; Newman, 2010). The non-carcinogenic or hazardous risks that the compounds pose are defined by calculating the hazard quotient (HQ). However, the HQ is not useful alone-standing, if there are multiple chemicals of concern. Therefore, the hazard index (HI) is calculated which takes into account the different compounds' hazard quotients (Newman, 2010). Carcinogenic risk (CR) is calculated assuming a multistage model of carcinogenicity. If the risk is greater than 1 in 100, the calculations should be replaced with a one-hit risk model for that compound. When calculating a total cancer risk the individual CRs are added together, assuming independence of effects (Newman, 2010).

Human health risk assessments are often used to bolster the ecotoxicological results generated in a study. These theoretical scenarios are used to model the risks that potentially pose negative effects to a population, risks that are calculated using empirical- or extrapolated data from the environment. Risk assessment is useful as technical support for decision making and management, knowing that the future is unpredictable (Newman, 2010). Thus, the implementation of a risk assessment is crucial in a study, to estimate possible effects a contaminant, quantified in the area of concern, may have on a human population (Newman, 2010).

In South Africa, the lifetime risk of developing a cancer—in all forms—is one in eight for men, and one in nine for women as determined by National Cancer Registry of 2010 (CANSAs, 2015). According to Stefan *et al.* (2015) the incidence of South African children manifesting cancers from 1987–2007 was 50 and 40 per million, for boys and girls under the age of 14 years. The majority of cancers in South-Africans are attributed to environmental and lifestyle factors (CANSAs, 2016). The hazardous and cancerous risks were calculated for both adults and children of Soweto and Lenasia. These risks were determined for physical contact with contaminated water and -sediments while bathing, collecting

water, fishing, or swimming. PAH concentrations in the water were extrapolated from the quantified sediment concentrations using a cross media transfer formula (Galassi & Migliavacca, 1986; Provini *et al.*, 1989). Risk from oral ingestion was also calculated for consumption of water and fish, and sediments from the area. Geophagia is a form of pica—specifically the intentional consumption of earth, soil, mud or clay (Woywodt & Kiss, 2002). In South Africa geophagia has been noted in many parts of the country such as the Free-State (Ekosse *et al.*, 2010; Mogongoa *et al.*, 2011), Limpopo Province (Ekosse *et al.*, 2010), KwaZulu Natal (Saathoff *et al.*, 2002), rural Eastern Cape (George & Ndip, 2011), and urban Gauteng (Mathee *et al.*, 2014). In a study conducted by Mathee *et al.* (2014) at the Rahima Moosa Mother and Child Hospital—a hospital serving an area that includes our study area—20% (n=60) of the women in that study were geophagic (Mathee *et al.*, 2014) and had significantly higher blood lead levels. These pregnant women were not only exposing themselves to toxicants by consuming muds and clays, but also their unborn child. Thus, the risks linked to geophagia were also calculated for pregnant women.

5.2 Materials and Methods

5.2.1 Site selection

Human health risk was calculated for the sampling sites proposed for this project (Figure 1.1). These sites are Protea Glen, Lenasia, Fleurhof, Moroka, Eldorado Park, Orlando West, Orlando East, Nancefield and Dobsonville. The water and sediment risks were calculated for all the sites and for both survey years (2013 & 2014). The risks from the consumption of fish were calculated for those fish from the fishing sites (Lenasia, Fleurhof, Orlando East, and Nancefield (Nc) [Bushkoppies WWTP's final pond]).

5.2.2 Hazard identification

The hazard identification of PAHs in the study area has been identified and discussed in the previous chapters (Chapters 2–4), as well as in these chapters' literature reviews other studies' findings are also discussed. The physico-chemical properties and carcinogenicity of the PAHs were summarised in Table 2.1.

5.2.3 Hazard characterisation

The 16 priority PAHs are divided into two groups for the hazard characterisation: the carcinogenic PAHs (CPAHs: BaA, Chr, BbF, BkF, BaP, InP & DBA) and the non-carcinogenic PAHs (nCPAHs: Nap, Acea, Acea, Flu, Phe, Ant Fla, Pyr & BgP)—the nCPAHs are the remaining PAHs that are not classified as CPAHs. Cancer slope factors (CSF) and reference doses (RfD) are not available for all the PAHs (calculations were done accordingly). Since no reference dose values are available for sediment, the available soil values were used. The appropriate reference doses and slope factors of the PAHs are summarised in Table 5.1. No CSFs or RfDs are available for the metabolites, thus their hazard characterisation was omitted.

Table 5.1: Available cancer slope factors and reference doses for polycyclic aromatic hydrocarbons (USEPA, 1999; CIDA, 2015)

PAH	Cancer slope factors (mg/kg/d)		Reference Dose for non-carcinogenic effects (mg/kg/d)		
	Oral	Dermal	Soil	Water	Fish
Nap			0.02	0.02	0.02
Acea			0.06	0.06	0.06
Flu			0.04	0.04	0.04
Ant	0.23		0.30	0.30	0.30
Fla	0.0023		0.04	0.04	0.04
Pyr			0.03	0.03	0.03
BaA	0.73	2.4			
Chr	0.023	0.024			
BbF	0.73	2.4			
BkF	0.23	0.24			
BaP	7.3	24			
InP	0.73	2.4			
DBA	7.3	24			
BgP	0.023				

5.2.4 Exposure assessment

The exposure assessment parameters for Soweto and Lenasia are shown in Table 5.2. The application of the human health risk in this study is used from an ecotoxicological perspective, thus the exposure parameters was selected from literature—chosen to represent the population as best possible.

Table 5.2: Exposure parameters for human health risk assessment for adults and children (USEPA, 2004; Health Canada, 2004) adapted to represent the population in the study area

Formula Abbreviation		Population condition	Population variables Adults Children ^a	
BM	Body mass (kg)	Normal ^b	66.5	31.8
		Pregnant ^c	80	
LE	Life expectancy (years) ^d		62.5	62.5
D1	Days per week		7	7
D2	Weeks per year	Normal	52	52
		Pregnant	27 ^e	
D3	Years exposed	Normal	30	6
		Pregnant ^f	1.75	
SE	Skin exposed (cm ³)	Sediment	6610	4 050
		Water	18 000	4 350
AT	Average timing (LE x 365 days)		22 812.5	22 812.5
T_{event}	Event duration (hours/event)		0.58	0.58

a = USEPA, 2008b

b = Pouane *et al.*, 2002c = Rasmussen *et al.*, 2009

d = StatsSA, 2015

e = Two trimesters of pregnancy

f = Total years pregnant: Average 2.33 children per South African woman (CIA, 2016)
[2.33 x 9 months/12 months]

5.2.5 Risk characterisation

During this step of the assessment the risk is calculated for different matrices through different exposure pathways. Both carcinogenic risk (CR) (for the CPAHs) and hazardous risk (for the nCPAHs) were calculated. The risk of PAHs in Soweto and Lenasia was characterised for sediments, water and fish. The risk characterisation variables used in the various calculations are summarised in Table 5.3.

Table 5.3 Risk characterisation variables for dermal- and oral exposures to soils, water and fish (USEPA, 2004; Health Canada, 2004)

Formula abbreviation		Population variables		
		Pregnant women	Adults	Children ^a
SL	Soil loading factor		1×10^{-7}	
AF skin	Dermal absorption factor		0.2	
AF gut	Gastro-intestinal absorption factor		0.2	
EF	Exposure frequency (days per year)		350	
EV	Event frequency (event per day)		1	
D3	Exposure duration (years)		30	6
IR water	Ingestion rate of water (L/day)		1.41	0.48
IR sed	Ingestion rate of sediments (mg/day)	0.05 ^b		
		0.001 ^c		
IR fish	Ingestion rate of fish (kg/day)		0.054	0.003

a = USEPA, 2008b

b = Geophagia ingestion rate (worst case)

c = unintentional ingestion rate (conservative)

Calculating non-carcinogenic and carcinogenic risk

Hazardous risk was described using the hazard index.

Hazard quotient (HQ):

$$HQ = \frac{\text{Dose}}{\text{RfD}}$$

where RfD is the reference dose of non-carcinogenic effects

(Newman, 2010)

Hazard index (HI):

$$HI = \sum_{i=1}^n HQ_i$$

(Newman, 2010)

Cancer risk (CR) for each PAH was calculated by the following formula:

Multistage risk model:

$$CR = \text{Dose} \times \text{CSF}$$

where CSF is the cancer slope factor

(Newman, 2010)

In the case where the calculated risk is greater than 0.01, the one-hit risk model is applied:

$$CR = 1 - e^{-(\text{Dose} \times \text{CSF})}$$

(Newman, 2010)

Total cancer risk:

$$CR_{\text{total}} = \sum_{i=1}^n CR_i$$

A hazard index value calculated smaller than 0.1 indicates no hazard. A HI value between 0.1 and 1 shows low hazard risk, whereas if a HI is between 1.1 and 10 there is a moderate hazard risk. Finally, if the HI is greater than 10 there is a risk for high hazardous effects (Lemly, 1996).

A cancer risk calculated for ingestion, which is less than 1×10^{-4} or 1 in 10 000 is considered acceptable risk. Similarly, a risk less than 1×10^{-6} or 1 in 1 000 000 is considered negligible for dermal exposures. Once these “acceptable risks” are exceeded the population may be more prone to manifest cancers from the exposure to PAHs

The hazard index and cancer risk calculations were used to assess the chance of carcinogenic and non-carcinogenic effects to manifest due to exposure to the PAHs quantified from the specific study sites in Soweto and Lenasia. Assessment of the different matrices and exposure routes only differed in the calculation of the dosages (as daily average dose, DAD). Each dose calculation is shown below and uses variables and parameters set out in Tables 5.2 and 5.3.

Risk characterisation from exposure to PAHs in water

Potential risk from PAHs from water was characterised for dermal exposure and oral ingestion. The fact that for this project water was not analysed, a cross media transfer equation was used to extrapolate concentrations. Accepting there is an equilibrium condition between the sediments and water, the concentrations of the PAHs were calculated (Galassi & Migliavacca, 1986; Provini *et al.*, 1989):

$$C_{\text{water}} = \frac{C_{\text{sediment}}}{K_p}$$

where C_{sediment} is the concentration of PAHs in the sediment and K_p is the absorption coefficient onto soil (in terms of the organic fraction of the sediment)

$$K_p = K_{oc} \times OC$$

where K_{oc} is the organic carbon-water partition coefficient and OC the fraction of measured organic carbon per site.

The setting for dermal exposure includes activities such as swimming, playing, and bathing in the water as well as water collection. The parameters were set at one event a day—35 minutes per event—for 350 days of the year. The total skin surface area is the sum of the feet, legs, trunk, arms and hands (USEPA, 2004).

The dose (mg/kg/day) for calculating the risk from dermal exposure to PAH contaminated water was derived from:

$$\text{Dose}_{\text{dermal water}} = \frac{DA_{\text{event}} \times ED \times EF \times ED \times SA}{BM \times AT}$$

(For explanation of abbreviations refer to Tables 5.2 and 5.3)

(USEPA, 2004)

Where the DA_{event} is the absorbed dose per event (mg/cm³/event), which is calculated by:

$$DA_{\text{event}} = K_p \times C_{\text{water}} \times T_{\text{event}}$$

(For explanation of abbreviations refer to Tables 5.2 and 5.3)

(USEPA, 2004)

K_p in this case is the dermal permeability coefficient which is chemical specific, and is derived using the water-octanol partition coefficient (K_{ow}) (USEPA, 2004).

The ingestion of water follows the set parameters in Table 5.3. The daily intake of water ingested is accepted as the water that originated from the contaminated sites.

$$\text{Dose}_{\text{ingested water}} = \frac{C_{\text{water}} \times IR_{\text{water}} \times AF_{\text{gut}} \times D_1 \times D_2 \times D_3}{BM \times LE}$$

(For explanation of abbreviations refer to Tables 5.2 and 5.3)

(USEPA, 2004)

Risk characterisation from exposure to PAHs in the sediment

A population in the vicinity of rivers and dams often comes into regular contact with contaminated sediments while walking along the river bank, swimming, bathing, or water collection for domestic use. The skin surface area exposed used for the calculations was for the hands, legs and feet. The remaining parameters are set out in Table 5.3

$$\text{Dose}_{\text{dermal sediment}} = \frac{C_{\text{sediment}} \times SE \times SL \times AF_{\text{skin}} \times EV \times D_1 \times D_2 \times D_3}{BM \times LE}$$

(For explanation of abbreviations refer to Tables 5.2 and 5.3)

(USEPA, 2004)

The risk from sediment ingestion was calculated using the soil ingestion formula, the amount of sediments ingested was set at a conservative and worst case amount (Table 5.3)

$$\text{Dose}_{\text{ingested sediment}} = \frac{C_{\text{sediment}} \times IR_{\text{sed}} \times AF_{\text{gut}} \times D_1 \times D_2 \times D_3}{BM \times LE}$$

(For explanation of abbreviations refer to Tables 5.2 and 5.3)

(USEPA, 2004)

Risk characterisation from consuming PAH contaminated fish

The ingestion of fish follows the set parameters in Table 5.3. The daily intake of contaminated fish is accepted as the fish caught from the study sites.

The dosage for calculating risk from consuming fish contaminated with PAHs was derived using the formula below:

$$\text{Dose}_{\text{ingested fish}} = \frac{C_{\text{fish}} \times IR_{\text{fish}} \times AF_{\text{gut}} \times D_1 \times D_2}{BM \times 365 \times LE}$$

(For explanation of abbreviations refer to Tables 5.2 and 5.3)

(USEPA, 2004)

5.3 Results and discussion

The results of the characterisation of risk for the PAHs in Soweto and Lenasia are reported in Figures 5.1–5.6. The exposure routes used in the risk characterisation were the dermal and ingested pathways for water, sediments and fish. Both carcinogenic and non-carcinogenic risks were calculated. The extrapolated PAH concentrations in the water used in the risk calculations are reported in Table 5.4. In order to compare the results of this study to that of Roos *et al.* (2011) as mentioned previously, this study was conducted in Soweto and Lenasia, and was the precursor of the current study (see sections 1.2 & 1.3.2). The concentrations of PAHs in sediments reported by Roos *et al.* (2011) were used to calculate cancer risks following the same assessment parameters as for this study. Additionally, their sediment concentrations were also extrapolated to determine the prospective concentrations in the water for which CRs were also calculated. These values were used to compare relative risk between the studies.

Table 5.4: Extrapolated concentrations (ng/mL) of the polycyclic aromatic hydrocarbons in the water from the nine sites in the greater Soweto and Lenasia area for 2013 and 2014

2013	Nap	Acey	Acea	Flu	Phe	Ant	Fla	Pyr	BaA	Chr	BbF	BkF	BaP	InP	DBA	BgP	ΣPAH
Protea Glen	1.969	0.062	0.285	0.074	0.117	0.025	0.051	0.056	0.006	0.006	0.002	0.001	0.0004	0.0004	0.0002	0.001	2.656
Lenasia	4.560	0.138	0.674	0.195	0.129	0.045	0.020	0.032	0.002	0.002	0.001	0.001	0.001	0.0001	0.0004	0.0004	5.801
Fleurhof	8.562	0.139	0.625	0.226	0.763	0.192	0.412	0.453	0.051	0.024	0.007	0.005	0.003	0.002	0.001	0.003	11.467
Moroka	2.500	0.348	0.205	0.271	0.825	0.165	0.720	0.682	0.078	0.041	0.018	0.009	0.002	0.004	0.002	0.009	5.879
Eldorado Park	6.777	0.243	0.917	0.145	0.321	0.068	0.159	0.174	0.019	0.012	0.005	0.003	0.002	0.001	0.001	0.003	8.850
Orlando West	3.360	0.367	0.149	0.214	0.771	0.153	0.450	0.493	0.049	0.027	0.009	0.006	0.003	0.002	0.001	0.004	6.058
Orlando East	3.573	0.236	0.559	0.188	0.216	0.048	0.073	0.086	0.007	0.004	0.001	0.001	0.001	0.0004	0.0003	0.001	4.995
Nancefield	2.070	0.026	0.254	0.039	0.047	0.013	0.005	0.006	0.001	0.001	0.0001	0.0001	0.001	0.00004	0.0003	0.00002	2.462
Dobsonville	3.505	0.067	0.302	0.046	0.111	0.029	0.029	0.016	0.004	0.004	0.001	0.001	0.002	0.0003	0.001	0.001	4.117
2014	Nap	Acey	Acea	Flu	Phe	Ant	Fla	Pyr	BaA	Chr	BbF	BkF	BaP	InP	DBA	BgP	ΣPAH
Protea Glen	2.843	1.496	0.129	0.141	0.123	0.027	0.050	0.054	0.006	0.004	0.002	0.001	0.001	0.0004	0.0003	0.001	4.878
Lenasia	6.715	3.790	1.222	1.128	0.324	0.071	0.133	0.171	0.021	0.012	0.002	0.001	0.001	0.001	0.0003	0.003	13.594
Fleurhof	5.583	2.298	0.884	0.200	0.174	0.041	0.059	0.096	0.007	0.004	0.002	0.001	0.001	0.001	0.0004	0.001	9.352
Moroka	17.648	19.558	3.622	1.633	0.837	0.220	0.746	0.781	0.067	0.040	0.003	0.001	0.003	0.003	0.001	0.007	45.172
Eldorado Park	12.374	6.002	2.278	0.367	0.633	0.181	0.463	0.501	0.051	0.029	0.004	0.002	0.003	0.002	0.001	0.005	22.896
Orlando West	8.996	11.555	1.248	1.266	0.266	0.097	0.151	0.170	0.018	0.011	0.005	0.003	0.002	0.001	0.001	0.002	23.790
Orlando East	6.448	0.078	0.350	0.137	0.184	0.049	0.051	0.060	0.005	0.005	0.005	0.003	0.002	0.0003	0.001	0.001	7.377
Nancefield	3.039	0.043	0.193	0.090	0.252	0.060	0.079	0.089	0.009	0.006	0.002	0.002	0.001	0.0004	0.0004	0.001	3.867
Dobsonville	1.801	0.064	0.086	0.145	0.201	0.046	0.075	0.049	0.008	0.007	0.004	0.002	0.002	0.0005	0.001	0.001	2.491

5.3.1 Non-carcinogenic risk characterisation (Hazard Index)

Non-carcinogenic, or hazard risk was calculated for Soweto and Lenasia using the hazard index (HI). The HI was calculated for the ingestion of water (adults and children) (Figure 5.1 A–D) and the ingestion of sediments by pregnant women (geophagia and unintentional ingestion) (Figure 5.1 E–H) for both 2013 and 2014.

The hazard risks from ingesting PAH contaminated water calculated for adults from the study area of 2013, had all low hazard scores, except for Protea Glen and Nancefield. The greatest HI was at Fleurhof, followed by Eldorado Park (Figure 5.1A). The fact that Moroka did not have the greatest HI—thus far the most responsive site (see Chapters 2 & 3) may be attributed to its overall lower concentrations of nCPAHs (Table 5.4). The reference doses of acenaphthene and fluorene calculates the majority of the hazard index score—the RfD is the denominator in calculating the HQ, and when the denominator is low, the calculated quotient will be high. Thus, the low concentrations of fluorene at Moroka (0.205 ng/mL) relative to Eldorado Park's high concentration (0.905 ng/mL) (Table 5.4) decreased Moroka's hazard quotient. In contrast, the 2014 hazard index had Moroka as the site with the highest HI value, followed by Eldorado Park. Dobsonville had a temporal decrease with its HI value, being the only site with no risk for 2014 (Figure 5.1B). There was no hazardous risk to children during the 2013 year (Figure 5.1C), and only Moroka had low hazard risk for the 2014 survey (Figure 5.1D). The drivers for the HI of water ingestion were the naphthalene, acenaphthene and fluorene concentrations (Table 5.4).

The hazardous risks of sediment by unintentional ingestion (Figure 5.4 E & F) and by geophagia (Figure 5.4 G & H) show a similar temporal increase of HI, which is expected as both risk characterisations utilised the same chemical data. The HI for geophagia had no hazard risk for both survey years at all of the sites (Figure 5.4 G & H). However, from the results a temporal increase was seen at Lenasia, Moroka and Eldorado Park, indicating an influx of hazardous PAHs. The drivers that increase HI for sediment ingestion was also naphthalene, acenaphthene and fluorene. Compared to the intentional consumption of sediments (geophagia) the unintentional ingestion HIs were all very low compared to the no hazard guideline (0.1) (Figure 5.4 E & F).

The hazard risks calculated for water ingestion was dominated by the 2- and 3-ring PAHs, naphthalene, acenaphthene and fluorene. These congeners have been found to have different health effects including reproductive and developmental as well as aplastic anaemia. The Agency for Toxic Substances and Disease Registry (ATSDR) reviewed the health effects of PAHs and according to their findings naphthalene decreases metabolism in rat livers, apart from being cytotoxic. Acenaphthene increases liver cholesterol levels and fluorene decreases red blood cells and haemoglobin, and increases hemosiderin deposits in the spleen. All three these PAHs also increased the liver mass of exposed rats (Mumtez *et al.*, 1996).

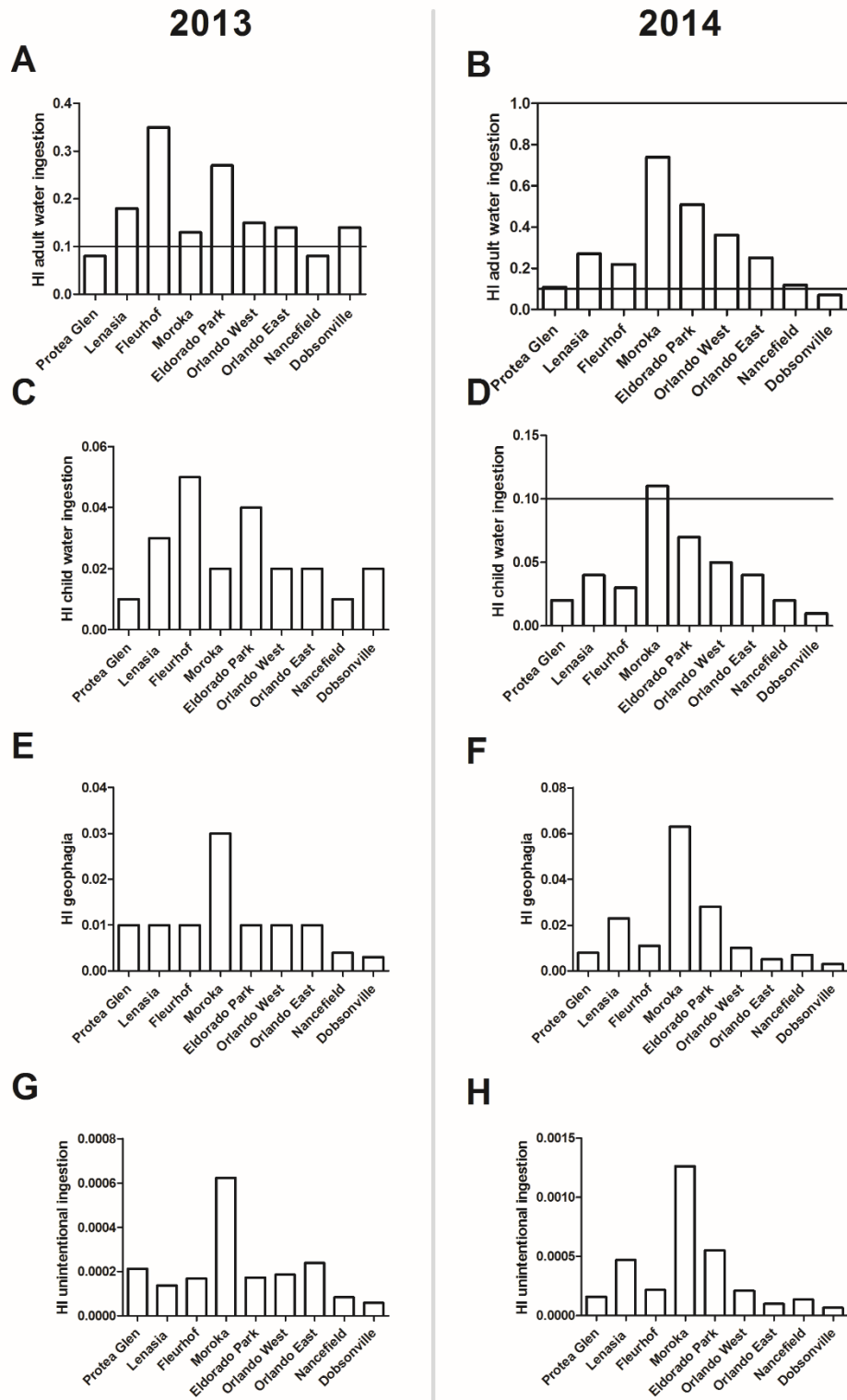


Figure 5.1: Hazard index reporting non-carcinogenic risk of polycyclic aromatic hydrocarbons from Soweto and Lenasia for: Water ingestion for adults during A) 2013 and B) 2014, as well as water ingestion hazard risk for children of C) 2013 and D) 2014. The hazard risk for pregnant women ingesting sediments by geophagia during E) 2013 and F) 2014, as well as unintentional ingestion of sediments during G) 2013 and H) 2014. No hazard: $HI < 0.1$; low hazard: $0.1 < HI < 1$; moderate hazard: $1.1 < HI < 10$; high hazard: $HI > 10$, where applicable indicated by a line

5.3.2 Carcinogenic risk characterisation

Cancer risk from water exposure

The characterisation of cancer risk was implemented for exposure through water, sediment and fish consumption. The exposure pathways selected were dermal and ingestion. Oral cancer slope factors were available for all the CPAHs as well as anthracene, fluoranthene and benzo(g,h,i)perylene, whereas only CPAHs had dermal slope factors (Table 5.1). The results for these assessments are reported in Figures 5.2–5.5. A total cancer risk (sum of all CRs calculated) was also calculated and is reported in Figure 5.6.

The PAH concentrations in the water that had been extrapolated from the sediment levels, (Table 5.4) were used for the water dermal and ingestion risk assessment. The dermal cancer risk from water exposure is reported in Figure 5.2. All the 2013 sites had dermal CR values higher than the acceptable risk level (1 in 1 000 000). The greatest risk was at Moroka (16 in 1 000 000), then Fleurhof (13 in 1 000 000) and Orlando West (11 in 1 000 000) (Figure 5.2A).

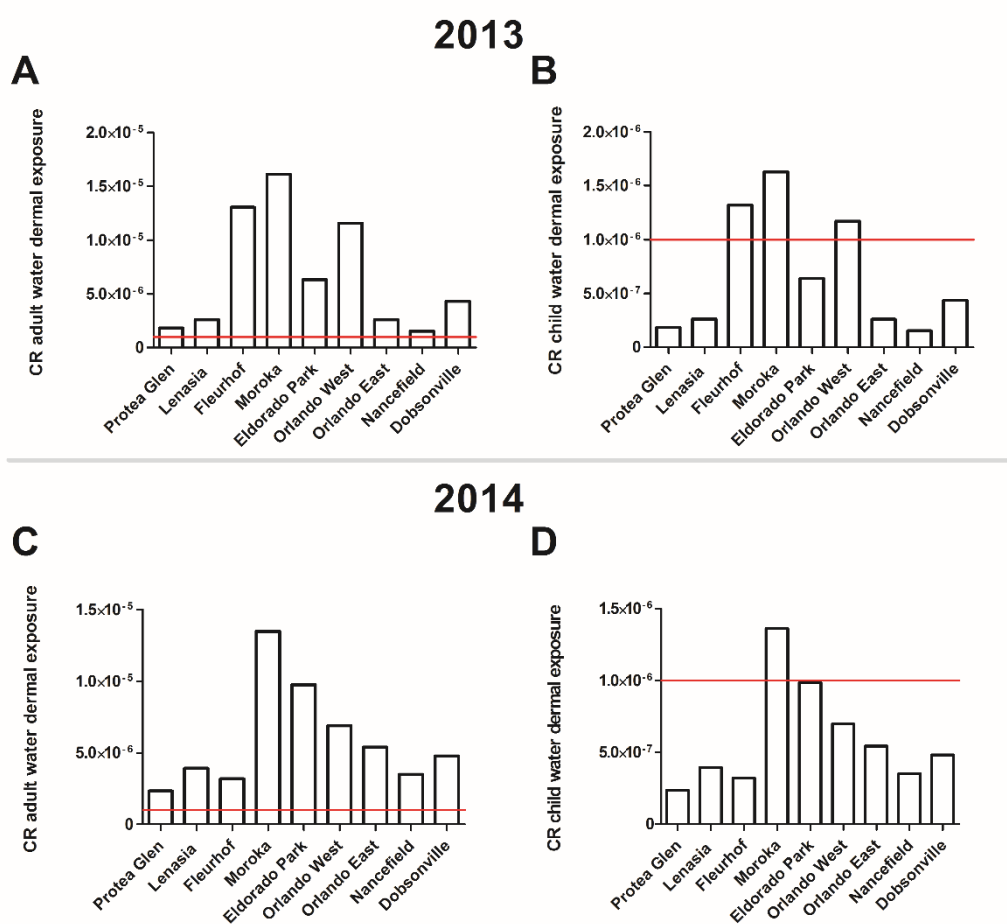


Figure 5.2: Carcinogenic risk of dermal exposure to polycyclic aromatic hydrocarbon contaminated water for A) adults and B) children during 2013, as well as C) adults and D) children during 2014. Unacceptable risk for dermal exposure is greater than 1 in 1 000 000 (1×10^{-6}) (red line)

The children CR determined from the 2013 data were similar to that of the adult assessment. These sites—Moroka, Fleurhof and Orlando West—were the only sites exceeding the acceptable risk level (1.6, 1.3, and 1.1 in a 1 000 000 CR, respectively) (Figure 5.2B). A temporal decrease in the adult CR was noted for Fleurhof, Moroka and Orlando West, but all the other sites had increases. Moroka still had the greatest risk (13 in 1 000 000), followed by Eldorado Park (9.7 in 1 000 000) (Figure 5.2C). The CR calculated for the children (2014) showed the same increases/decreases as the adult assessment (Figures 5.2C & D). Moroka was the only site to have unacceptable risk for 2014 (1.3 in 1 000 000). Eldorado Park's CR increased 1.5 times from 2013, but was still within the acceptable cancer risk range (0.98 in 1 000 000) (Figure 5.2D). Only three of the Roos *et al.* (2011) sites exceeded the unacceptable risk for dermal exposure. The CRs calculated using Roos *et al.* (2011)'s extrapolated water concentrations were lower for both adults and children compared to our study. The highest Roos *et al.* (2011) CR from dermal exposure for adults was 9 in 1 000 000.

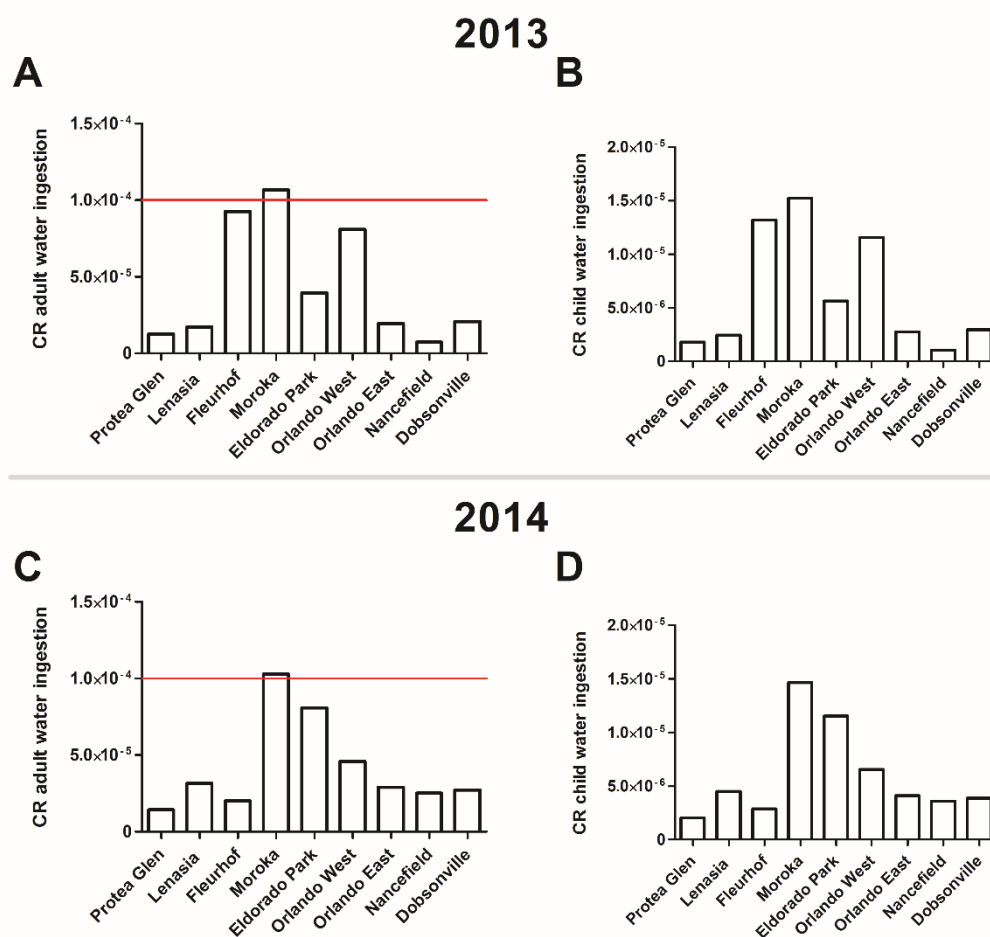


Figure 5.3: Carcinogenic risk to ingestion of polycyclic aromatic hydrocarbon contaminated water for A) adults and B) children during 2013, as well as C) adults and D) children of 2014. Unacceptable risk for ingestion is greater than 1 in 10 000 (1×10^{-4}) (red line)

The CPAHs that posed the greatest threat were benzo(a)anthracene, benzo(a)pyrene and dibenz(a,h)anthracene and subsequently influenced the CR through water exposure the most. The risks from ingesting PAH contaminated water are reported in Figure 5.3. Only Moroka exceeded the 1 in 10 000 risk level (1.07 in 10 000) in 2013, all the remaining sites had negligible risk (Figure 5.3A).

The CR for children was below the acceptable risk level for all the sites for both years (2013 & 2014) (Figure 5.3A & B). The same temporal decrease/increase trend in the adult risk assessment was noted for the water ingestion characterisation as with the dermal assessment (Figures 5.2A & C and 5.3 A & C). Again only Moroka exceeded the acceptable risk level (1.03 in 10 000) (Figure 5.3C). The cancer risks calculated from the extrapolated PAH concentrations in water for the Roos *et al.* (2011) study were lower for both adults and children compared to our study. The highest CR calculated using the Roos *et al.* (2011) data was a risk of 5 in 100 000.

The CPAHs responsible for Moroka's CR barely exceeding the acceptable risk level was fluoranthene, anthracene, benzo(a)anthracene and chrysene.

Cancer risk posed by exposures to sediments

The cancer risks associated with dermal exposure to the sediments of the study sites are reported in Figure 5.4. All the sites for both surveys exceeded the acceptable 1 in 1 000 000 risk level, for both the adult- and child assessments (Figure 5.4A–D). The greatest CR for adults in 2013 was at Moroka (1 100 in 1 000 000), followed by Orlando East and Orlando West (593 and 342 in 1 000 000, respectively) (Figure 5.4A). The same trend was seen with the child assessment, Moroka having a CR of 297 in 1 000 000 (Figure 5.4B). In the following year Moroka still had the greatest CR (1 070 in 1 000 000) for adults, only slightly lower than for 2013 (Figures 5.4C). Orlando East and Orlando West also had a temporal decrease along with Protea Glen and Fleurhof, but there was increased risk for Lenasia, Eldorado Park, Nancefield and Dobsonville. Eldorado Park and Lenasia were the sites for second and third highest CR for 2014 (508 and 387 in 1 000 000) (Figure 5.4C). The dermal cancer risk calculated for children in 2014 followed the same trend as the adults (Figure 5.4D). Moroka posed a risk of 275 in 1 000 000 (Figure 5.4D).

Cancer risks from dermal exposure to the sediment sampled by Roos *et al.* (2011) were higher at two sites, S/L 8 and 7 (3 250 and 1 290 in 1 000 000, respectively). These sites are in the same area as our Nancefield site, indicating a great decrease in cancer risk in this area between the two studies. The PAHs that drove the dermal exposure assessment was dibenz(a,h)anthracene and to a lesser extent benzo(a)pyrene.

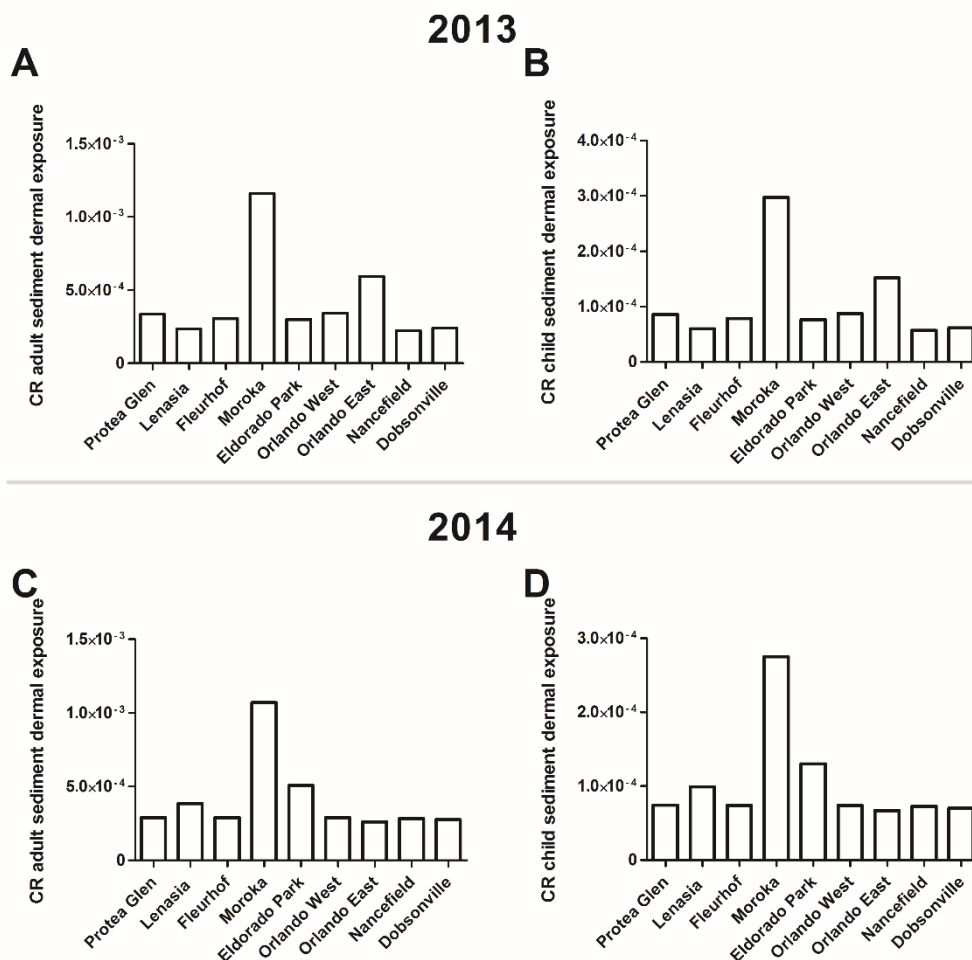


Figure 5.4: Carcinogenic risk of dermal exposure to polycyclic aromatic hydrocarbon contaminated sediments for A) adults and B) children of 2013, as well as C) adults and D) children of 2014. Unacceptable risk for dermal exposure is greater than 1 in 1 000 000 (1×10^{-6})

The carcinogenic risks associated with ingestion of contaminated sediments are reported in Figure 5.5. The intentional ingestion of soils and sediment by geophagia for both survey years had unacceptable risk for carcinogenic effects, whereas the unintentional ingestion was below the acceptable risk levels (Figure 5.5A–D). Geophagia CR for 2013 was the greatest at Moroka, followed by Orlando West (99 and 29 in 10 000, respectively) (Figure 5.5A). A temporal decrease in risk—concurrent with the chemical concentration seasonal trends—was noted for Moroka, Protea Glen, Fleurhof, Orlando West and Orlando East (Table 2.8) while an increase was noted for Lenasia, Eldorado Park, Nancefield and Dobsonville (Figure 5.5A & C). The sites with the greatest cancer risks for 2014 was at Moroka (91 in 10 000) and Eldorado Park (43 in 10 000) (Figure 5.5C). As already mentioned, the unintentional ingestion of sediments had no cancer risk (Figure 5.5B & D). This emphasises the danger of geophagia of contaminated sediments and soils, especially to pregnant woman—potentially exposing the unborn child to contaminants, in this case PAH congeners such as dibenz(a,h)anthracene, benzo(a)pyrene, benzo(k)fluoranthene and benzo(a)anthracene.

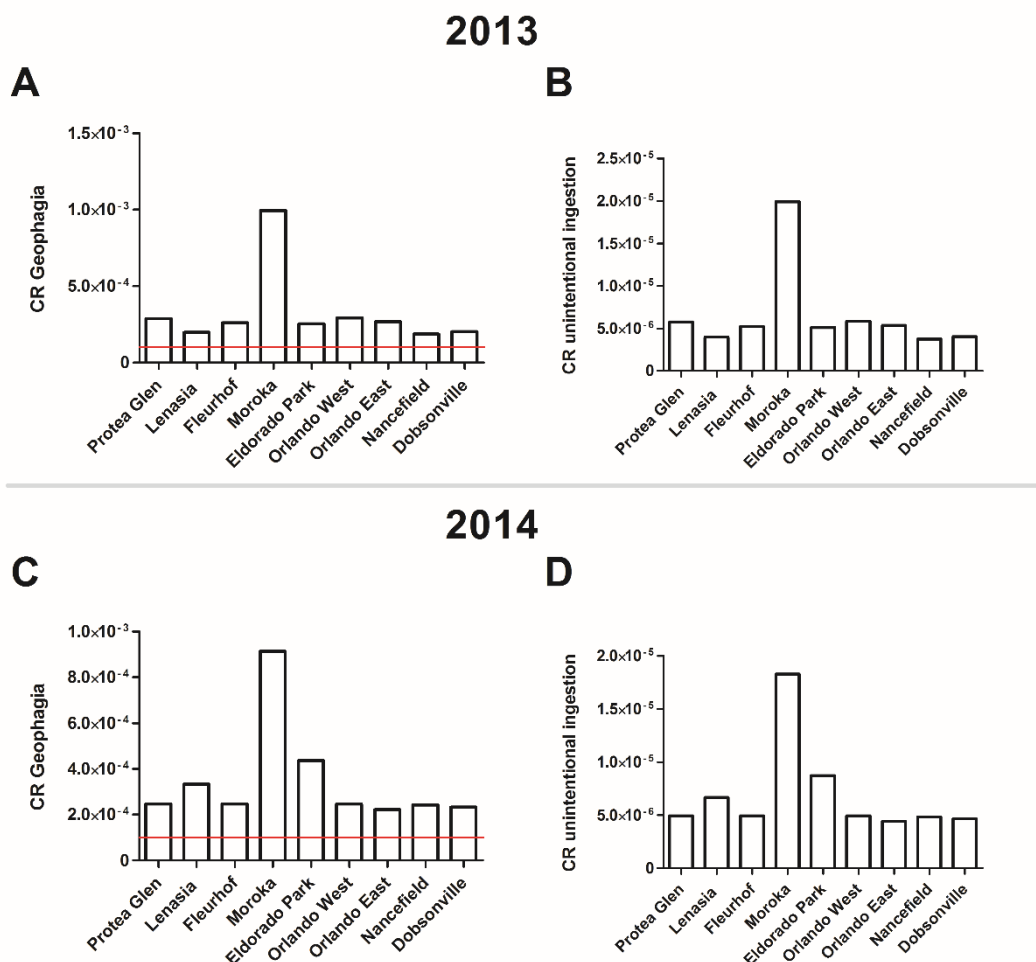


Figure 5.5: Carcinogenic risk to geophagia of polycyclic aromatic hydrocarbon contaminated sediments for pregnant women of Soweto and Lenasia: A) geophagia-, and B) unintentional ingestion during 2013, as well as C) geophagia- and D) unintentional ingestion during 2014. Unacceptable risk for ingestion is greater than 1 in 10 000 (1×10^{-4}) (red line)

Cancer risk from consuming fish from a PAH polluted system

Since fish metabolise PAHs, the level of the PAH metabolites quantified represent the fish's environmental exposure to these pollutants. The metabolite data was used as the value for the worst case scenario when consuming the fish from the area. The only biliary PAH that was quantified in this study that has a cancer slope factor is benzo(a)pyrene BaP. Using the BaP concentrations (Figure 2.2) a cancer risk for adults and children was calculated for the 2013 survey. The mean CRs for Soweto and Lenasia were well below the 1 in 10 000 risk level. The mean CR for adults was 2 ± 0.4 in 100 000 000. It is clear from the results that the fish does not contribute the most risk towards human health (because fish metabolise PAHs). PAHs, however, pose a greater threat to humans through other exposure pathways, as shown in the results earlier in this chapter.

Total cancer risk from the PAH pollution in Soweto and Lenasia

The total cancer risk posed to the population of Soweto and Lenasia is shown in Figure 5.6. This risk level was calculated as the sum of the CRs for the various pathways. Unacceptable risk was set at

the lower level—1 in 10 000—as a sensitive threshold. As seen in the previous results, temporal variations at the sites were all the same for each assessment, because they were calculated using the same chemical data set.

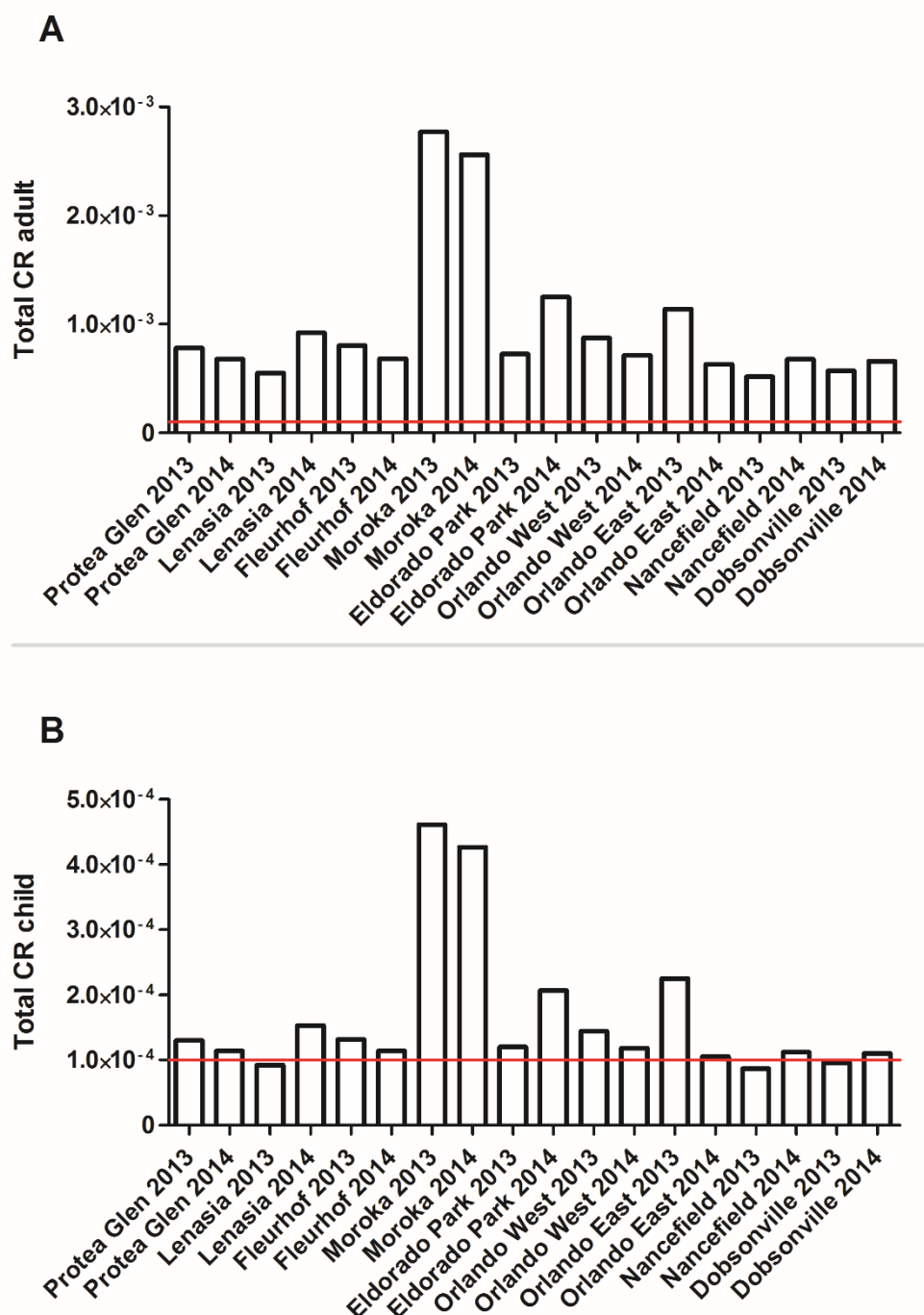


Figure 5.6: Total carcinogenic risk at Soweto and Lenasia from multiple exposures for A) adults and B) children for both survey years (2013 and 2014). Unacceptable risk is greater than 1 in 10 000 (1×10^{-4}) (red line)

In Figure 5.6 these trends are clearly visible. These seasonal changes, however do not affect the total CR for the adult population in Soweto and Lenasia, exposed to the water and sediments of our study area (Figure 5.6A). Once more, the site that posed the greatest risk was Moroka for both survey years

(277 and 256 in 10 000 risk respectively for 2013 and 2014) (Figure 5.6 A). This was followed by Eldorado Park (2014) and Orlando East (2013) (73 and 63 in 10 000) (Figure 5.6A). The cancer risk to children in the population is reported in Figure 5.6B. Similarly, the same sites that had the highest CR for the adults, were the same ones for the children as well (Figure 5.6B). Moroka posed a CR of 46 and 43 in 10 000, for the 2 consecutive survey years. There were also sites that had an overall negligible risk, below the 1 in 10 000 level. These sites were Lenasia and Nancefield, both for 2013 sampling year (Figure 5.6B).

The PAHs that were the main drivers of the carcinogenic risk associated within the Klip River system of Soweto and Lenasia were benzo(a)anthracene, benzo(a)pyrene, dibenz(a,h)anthracene, and to a lesser extent fluoranthene, anthracene and chrysene. In laboratory studies these congeners have been found to have various carcinogenic effects at different doses and exposure pathways (Mumtaz *et al.*, 1996; IRIS, 2016). These studies have allowed for the classification of the different PAHs into carcinogenic classes. The International Agency for Research on Cancer (IARC) has classified the CPAHs (Table 2.1) into carcinogenic groupings: Group 2A—which are probable human carcinogens and include benzo(a)anthracene, benzo(a)pyrene and dibenz(a,h)anthracene, while benzo(b)fluoranthene, benzo(k)fluoranthene, chrysene and indeno(1,2,3-cd)perylene are part of Group 2B—possibly carcinogenic to humans (OEHHA, 2001) (see section 2.1.5).

From all the exposure routes investigated in this assessment, dermal exposure to the sediments posed the greatest cancer risks of which the highest CR was 1 100 in 1 000 000 at Moroka (2013) (Figure 5.4A). The dermal exposure cancer risks calculated for our study were similar to those quantified by Roos *et al.* (2011), also for PAH exposure. However, the CRs of two sites from the Roos *et al.* (2011) study, which were very close to each other, were considerably greater than our study's results. Overall, the CR determined for the current study is slightly lower than the Roos *et al.* (2011) study. In contrast to this, the cancer risks due to dermal exposure to water showed an increase between the two studies, and the risk from ingestion of the PAH contaminated water (extrapolated) was the greatest for our study.

From all the different assessments Moroka has shown to be the site with the greatest risk, followed by Eldorado Park and Orlando East (Figures 5.2–5.6). These results are supported by the quantified PAH concentrations in the environment (Table 2.8 & Table 3.1).

5.4 Conclusion

The PAHs quantified in the Soweto and Lenasia study area pose unacceptable risks to the population that are exposed to the Klip River system. The human health risks were modelled using instrumentally determined levels in the sediment and extrapolated concentrations in water, along with parameters and exposure routes applicable to the area and human population. It is important to note that the human health risk modelled here is dependent on the various exposure factors, thus a site with high concentrations of certain PAHs may not necessarily have high risks. This shows the importance of completing these risk assessments, to identify risk in areas where pollutant concentrations alone would have not indicated risk. In the case of this study, the sites with the greatest concentrations had the greatest risks. The hazardous risk expressed by the hazard index of ingesting PAH-contaminated water posed the greatest non-carcinogenic risk to both the adult- and child population in the study area. Dermal exposures were the most potent pathways in the cancerous risk assessment, where the exposure to water and fish were negligible. A small decrease in risk was noted between the two surveying years of 2013 and 2014. This study also had relatively lower cancer risk compared to the risk calculated from Roos *et al.* (2011)'s PAH data—data obtained in the same study area but from an earlier period. The CR to children was lower than the adults'. The site with the greatest CR for both years was Moroka, exceeding the acceptable risk level for each of the assessments. The most notable CRs of this site were for dermal exposure to sediments (1 100 in 1 000 000) and total cancer risk (277 in 10 000). The overall risk assessment of the Soweto and Lenasia area has shown that there are both carcinogenic and non-carcinogenic risks to the human population exposed to the Klip River flowing through the area.

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Chapter 6: Statistical integration of results, the study conclusion, and recommendations

6.1 Introduction

In this chapter the findings discussed in the previous chapters will be compared to each other to provide an overview of the PAHs in the aquatic ecosystem of the Klip River system in Soweto and Lenasia. The comparative integration of results for the discussion presented here was achieved using descriptive and multivariate statistics. Following the general comparative discussion are the thesis' conclusion and final recommendations for future studies.

6.2 Data analysis

6.2.1 Correlation analysis

Normality was tested using the D'Agostino & Pearson omnibus normality test. The monotonic relationships between data sets were inspected using Spearman's correlation analysis. Spearman's correlation test was used where data sets were not distributed normally and the Pearson's correlation coefficient was used for parametric data. Correlation strength was interpreted using the following categories: $|r| = 0.9-1$ (very strong correlation), $|r| = 0.7-0.9$ (strong correlation), $|r| = 0.5-0.7$ (moderate correlation), $|r| = 0.3-0.5$ (weak correlation), and $|r| = 0.0-0.3$ (no or negligible correlation) (Mukaka, 2012). Statistical analysis was done using Graphpad Prism version 5.

6.2.2 Multivariate statistics

The multivariate statistical techniques used in this chapter were principle component analysis (PCA) and redundancy analysis (RDA). Principle component analysis is based on a linear response model that explains the variation between species data and environmental variables (Scott & Clarke, 2000; Van den Brink *et al.*, 2003). In doing so new combinations of variables that explain the greatest variation in the data set are created (principal components, which are linear combinations of the original variables) (Fowler *et al.*, 1998). The first principal component (Factor 1) explains the greatest amount of information in the data set. The following principal component (Factor 2) is as different as possible from the first and explains the second largest portion of the data (Fowler *et al.*, 1998). PCAs are used in ecotoxicology to determine differences and similarities between environmental datasets looking for underlying variables that might explain the patterns observed in the PCA (Quinn *et al.*, 2009; Malherbe *et al.*, 2015; Gerber *et al.*, 2015).

Redundancy analysis is a multivariate analysis technique that uses two sets of variables—an explanatory and dependant variable set (Israëls, 1992). Although an RDA is based on similar principles as a PCA, an RDA allows the driving or explanatory variables to be selected, which then allow focus on the part of the variance that is explained by these selected external explanatory

variables (Van den Brink *et al.*, 2003). The RDA chooses principle components (factors) for only the explanatory variables selected for maximal association with the dependant variables (Scott & Clarke, 2000). RDAs are used to determine similarities or differences between datasets based on specific variables.

The biplots (PCA) and triplots (RDA) were interpreted as described by Šmilauer & Lepš (2014). The angle between the vector arrows indicate the correlation between the individual environmental variables and/or species data arrows: an angle close to 0° indicates a positive correlation between the variables; and angle closer to 90° shows no correlation; and an angle approaching 180° indicates negative correlation between variables. The perpendicular line between a sample symbol and a particular species arrow can be used to estimate the value of that sample in terms of the variable it is perpendicular to.

6.3 Polycyclic aromatic hydrocarbons in the aquatic environment of Soweto and Lenasia

6.3.1 PAHs in the sediment

The concentrations of the PAHs in the sediments of the study area were discussed in chapter 2. The greatest ΣPAHs for both years was at Moroka, followed by Protea Glen in 2013 and Eldorado Park in 2014 (Table 2.8). These levels were comparable to levels reported in literature (Table 2.9). According to a scale set by Baumard *et al.* (1998) the degree of PAH contamination in the sediment of Soweto and Lenasia ranged between 'moderate' and 'high' with the exception of Moroka 2014 that was 'very high' (Table 6.1).

In 2013 the sites with a 'high' rating were those situated in the centre of the study area (Figure 6.1A) and a decrease in concentrations was seen downstream from these sites: Protea Glen to Lenasia; Orlando West, Orlando East, and Moroka to Eldorado Park (Figure 6.1A)

Table 6.1: Levels of total polycyclic aromatic hydrocarbons in the sediments (ng/g) of Soweto and Lenasia against the scale by Baumard *et al.* (1998)

Site	2013		2014	
Protea Glen	1 077.2	High	757.8	Moderate
Lenasia	487.0	Moderate	2 089.7	High
Fleurhof	818.1	Moderate	902.8	Moderate
Moroka	3 684.2	High	5 369.5	Very high
Eldorado Park	805.5	Moderate	2 412.9	High
Orlando West	1 025.6	High	946.7	High
Orlando East	1 017.5	High	421.3	Moderate
Nancefield	274.3	Moderate	676.3	Moderate
Dobsonville	275.7	Moderate	435.5	Moderate
Low			0-100 ng/g	
Moderate			<100-1 000 ng/g	
High			<1 000-5 000 ng/g	
Very high			>5 000 ng/g	

Baumard *et al.*, 1998

In 2014 the situation between Protea Glen and Lenasia was reversed from that in 2013 with an increase in concentration downstream from Protea Glen (Figure 6.1B). The 2014 relational distribution of PAHs between Moroka and Eldorado Park was the same as in 2013, with a downstream decrease relative to Moroka, despite these two sites' overall higher levels in 2014 than in 2013 (Figure 6.1B). The levels at Nancefield were always lower than the two upstream sites, Lenasia and Eldorado Park, for both sampling years after the confluence of the Klip River and Klip Spruit. The most probable reason for this large decrease in PAH concentrations may be due to the filtering capability of the large wetland system (Liu *et al.*, 2008; Wang *et al.*, 2012) that stretches from both Lenasia and Eldorado Park to Nancefield (Figure 1.2 & 6.1) of which the impact was greater in 2014 than in 2013 (Table 6.1).

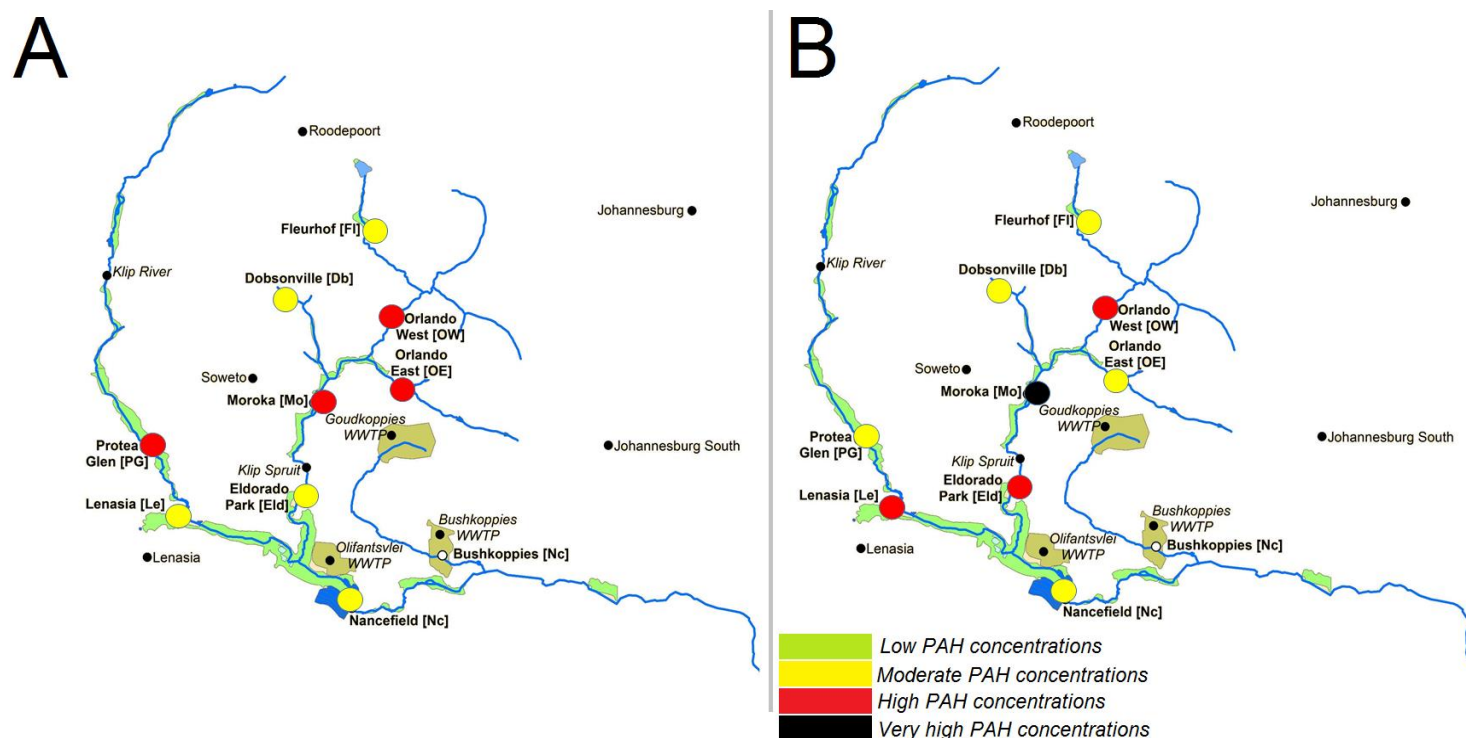


Figure 6.1: Temporal and spatial representation polycyclic aromatic hydrocarbons in the sediments of Soweto and Lenasia during A) 2013 and B) 2014. Colour scale derived from scale by Baumard *et al.* (1998)

Other spatial and temporal variations of the PAHs in the sediments of Soweto and Lenasia were investigated using a PCA. In this analysis, the concentrations of the individual congeners, LPAHs, HPAHs, CPAHs and ΣPAHs were included (from Table 2.8). Values of samples that were below the limit of detection were replaced with half LOD values to avoid using zero values. The data set was log transformed during the analysis.

Factors 1 and 2 describe 92% of the variation in the data (Figure 6.2). Factor 1 (74.46%) distinguishes between sites with 'high' and 'very high' PAH contamination (see Table 6.1) on the positive side of the factor and those with 'moderate' PAH levels on its negative side

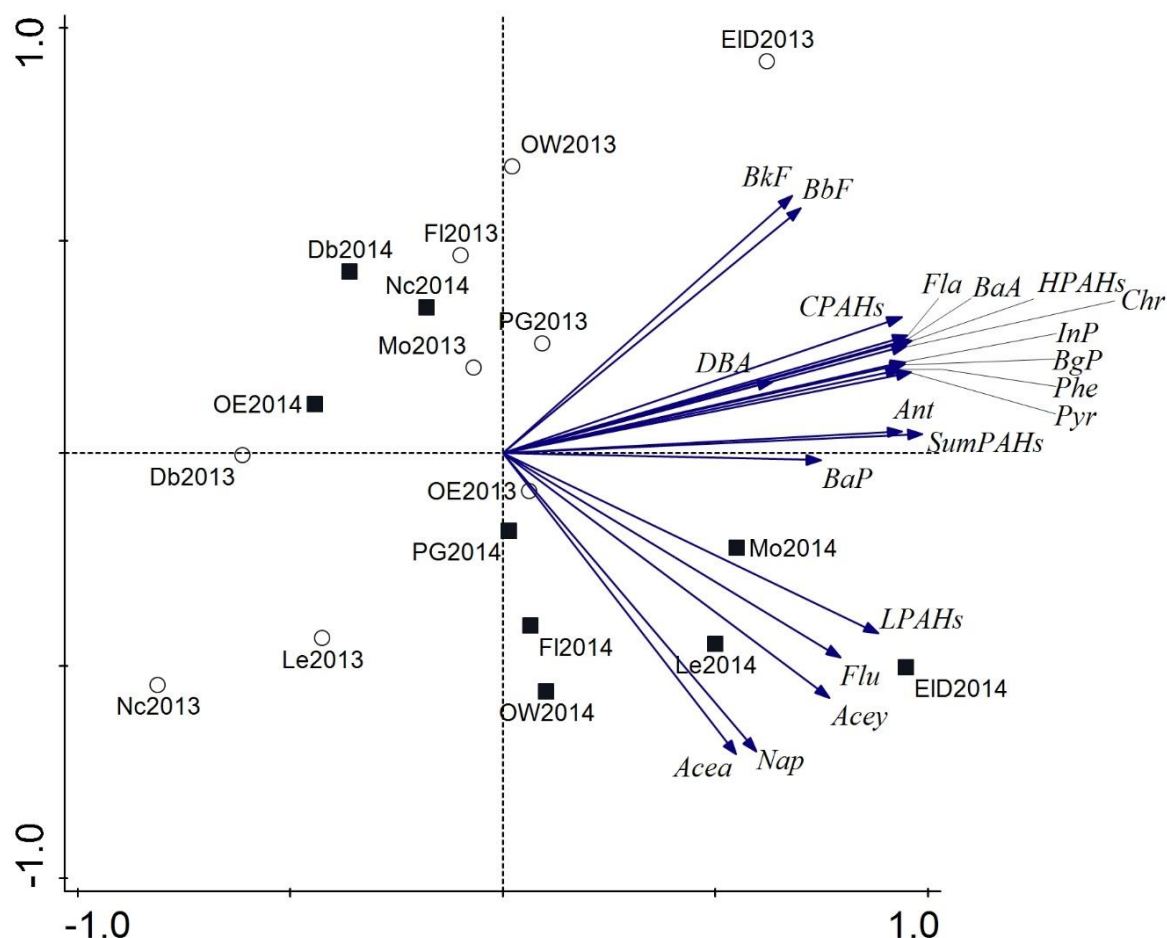


Figure 6.2: PCA biplot of polycyclic aromatic hydrocarbons in the sediments of Soweto and Lenasia during 2013 and 2014. The ordination explains 92% of the variance in the data with 74.46% by factor 1 and 17.5% by factor 2

Factor 2 (17.5%) contrasts the sites with the higher HPAHs (BkF and BbF) on its positive side to those with higher LPAHs (Acea and Nap) on its negative side (Figure 6.2). The co-linearity between CPAHs and HPAHs can be explained by the fact that the carcinogenic PAHs form part of the heavier congeners.

6.3.2 Relationship between biliary PAHs in *Clarias gariepinus* and the PAHs in the sediments

The deconjugated PAH metabolites were quantified in *Clarias gariepinus* from three sites of 2013 and the control (Table 2.10). A redundancy analysis was completed to establish whether the PAH metabolites and the native compounds found in the sediments were associated with one another (Figure 6.3). Of all of the hydroxyl PAHs that were analysed, only those that had quantifiable levels in most of the fish were included in the RDA as explanatory variables. These were: 2-OH Nap, 2,3-OH Flu, 9-OH Flu (as Σ OH Flu), 2-OH Phe, 4-OH Phe (as Σ OH Phe), 1-OH Pyr, and 9-OH BaP. Half LOQ values were assigned to those OH-PAHs below the LOQ where applicable. Those metabolites that were all below limit of quantification for all samples were not included in the analysis to prevent skewed results. The corresponding native PAHs in the sediment (Nap, Flu, Phe, Pyr and BaP) were

used as the species variables. The control fish were not included in this analysis as there were no corresponding sediment values for them.

The explanatory variables account for 57.47% of the variation in (Figure 6.3). The fish from the three sites were grouped separate from each other on the graph. This pattern shows that the OH-PAHs present in the individual fish corresponds sufficiently enough for them to ordinate according to their sites. Factor 1 (43.74%) distinguished between the fish with OH-PAHs (positive side) and those with little or no OH-PAHs (negative side).

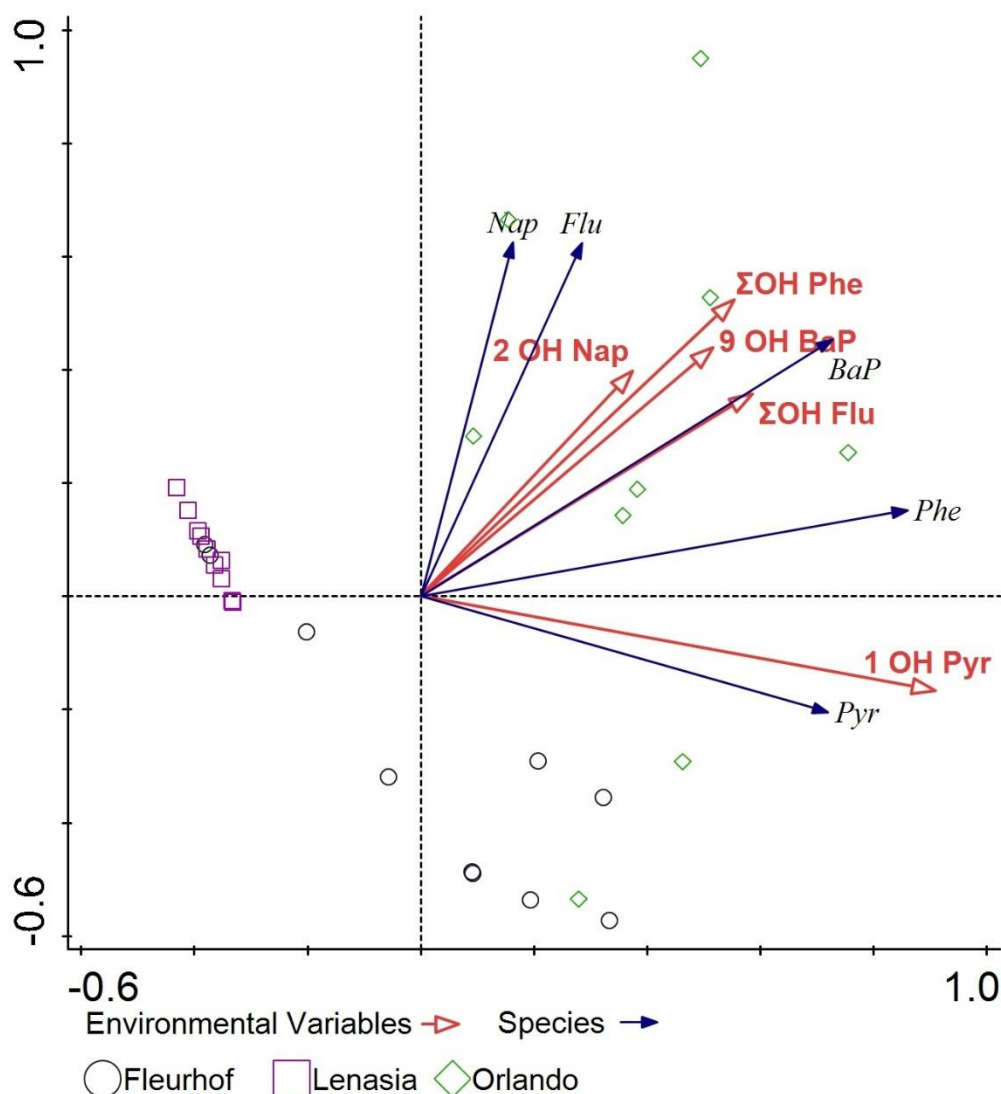


Figure 6.3: RDA triplot of the biliary polycyclic aromatic hydrocarbons in *Clarias gariepinus* and the native polycyclic aromatic hydrocarbons in the sediment from Fleurhof, Lenasia and Orlando, 2013. The ordination explains 57.47% ($p = 0.002$) of the variance, 43.74% by factor 1 and 13.73% by factor 2

Factor 2 (13.73%) separated fish with more 'lighter' OH-PAHs (positive side) from those with the 'heavier' metabolites (negative side) (Figure 6.3). The angles the vector lines of the metabolites and the native PAHs are all less than 90° (Figure 6.3). The strong correlation between the OH-PAHs and

their native forms indicates that the *C. gariepinus* must have been exposed to the native PAHs in the sediment. This can be largely attributed to the fact that *C. gariepinus* are bottom dwelling fish and are associated with sediments (Bruton, 1988). This confirms that the PAHs quantified in the sediments of Soweto and Lenasia transfer to *C. gariepinus*.

6.4 Sediment toxicity

6.4.1 Sediment toxicity in terms of instrumental data and sediment indices

The potential toxicity posed by the PAHs in the sediment was determined by using various toxicological assessment indices (see Chapter 2). These included the sediment quality guideline index (SQGI), sediment quality index (SQI) and toxic equivalent quotients (TEQs) (see section 2.2.4).

Both the SQI and SQG-I use existing guidelines to express different toxicological endpoints. The SQI indicates the quality of the sediment as calculated by magnitude of exceedance of each guideline per congener. The SQG-I calculates probable toxicity to benthic organisms based on the lower and upper levels of the guidelines. Thus, one would expect that these indices would report similar results. The Canadian SQGs were used for comparisons because they are the more protective guideline set (Table 2.15 & 2.16). The correlation between these two indices were determined and there was a very strong negative correlation ($r = -0.98$, $p < 0.0001$) for the sediments of Soweto and Lenasia during 2013 and 2014 (Figure 6.4). This negative correlation confirms what was expected: as the sediment quality decreases in terms of PAHs, the toxicity will increase.

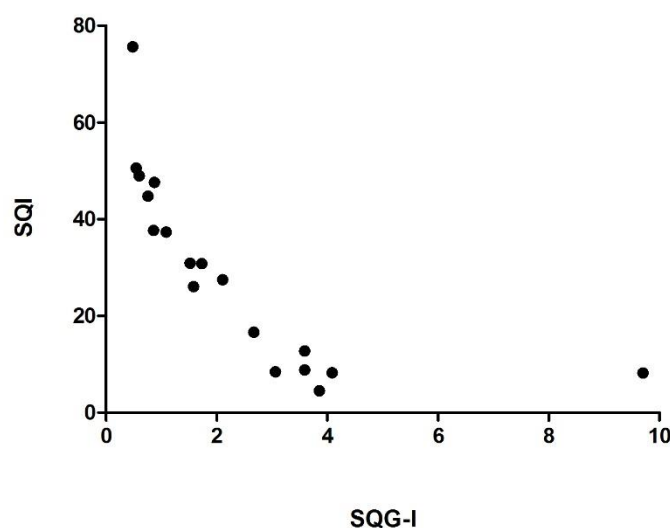


Figure 6.4: Spearman's correlation scatterplot for sediment quality index (SQI) vs sediment quality guideline index (SQG-I) for 2013 and 2014.

Another method where toxicity is predicted using instrumentally analysed concentrations is by means of toxic equivalent quotients. As described in section 2.2.4 (Toxic equivalent quotient calculation) this method incorporates a toxic equivalent factor that helps rank the toxicity of a sample in terms of 2,3,7,8-TCDD. Correlations were drawn between the SQG-I and the TEQs to compare the toxicity of

the PAHs in the sediment to different organisms, and whether these showed similar results. There was a strong correlation ($r = 0.8$, $p < 0.0001$) between the SQG-I and the TEQs based on the fish potency factors (TEQ_{FPF}) (Figure 6.5 A). The TEQs based on mammalian TEFs (TEQ_{TCDD}) correlated less than those for fish (moderate correlation, $r = 0.56$, $p = 0.0147$) (Figure 6.5 B). The TEQ_{TCDD} and the TEQ_{FPF} showed strong correlation ($r = 0.81$, $p < 0.0001$) (Figure 6.5 C).

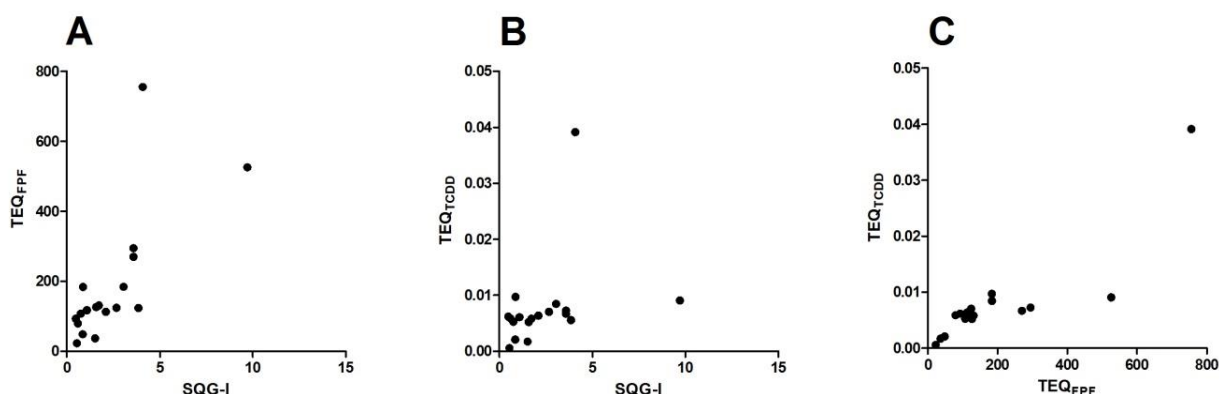


Figure 6.5: Spearman correlation scatterplot for: A) the sediment quality guideline index (SQGI) vs toxic equivalence using the fish potency factors (TEQ_{FPF}); B) the SQG-I vs toxic equivalence using toxic equivalent factors derived by Villeneuve *et al.* (2002) (TEQ_{TCDD}); C) the TEQ_{TCDD} vs TEQ_{FPF}

The correlations seen above show that the overall prediction of toxicity using different organismal endpoints (benthos, fish and mammalian) are constant. It was expected that the predictions of benthos toxicity (which is based on overall toxicity; see section 2.1.6) and the toxicity using the mammalian system (specifically AhR-mediated toxicity; see section 2.1.6) would not correlate strongly, as they are derived from separate toxicity endpoints. Analogously, both TEQ data sets were derived from the same mode of action and corresponded highly. The fish potency factors used to calculate the TEQ_{FPF} were derived by using the same mode of action—AhR mediated toxicity—but specifically for fish (see section 2.1.6, Toxic equivalent quotient calculation) and both (TEQ_{TCDD} and TEQ_{FPF}) are expressed in terms of TCDD.

6.4.2 Sediment toxicity in terms of biological responses

The toxicity of the PAHs in the sediment was also tested using the H4IIE-*luc* reporter gene bioassay (see Chapter 3). This method uses the Ah-receptor to express PAH toxicity of a sample (see section 3.1.3). The results obtained are therefore biological responses to the PAH containing fraction of the sediment extract and is expressed in terms of TCDD equivalency (see section 3.2.5). The maximum elicited responses and BEQs for both years were reported in Table 3.1. The viability assay showed that the low or below LOD responses in the H4IIE-*luc* bio-assay were due to cell death and not the absence of AhR agonists (Table 3.1). This cytotoxicity was attributed to the LPAHs in the samples—those PAHs that do not bind to the AhR—as shown by Schirmer *et al.* (1998). Only the Σ LPAHs and acenaphthylene (Acey) of 2013 correlated significantly with the MTT viability test. The Σ LPAHs showed moderate negative correlation with the cell viability ($r = -0.61$, $p = 0.0429$), and Acey showed

strong negative correlation ($r = -0.7$, $p = 0.0216$). The cytotoxicity of the LPAHs seems to be linked to lower concentrations. The correlating levels of LPAHs and Acey were lower in 2013 than 2014.

The AhR-mediated toxicity of the sediments from Soweto and Lenasia was from the CPAHs in the extracts, as shown by the strong correlation ($r = 0.77$, $p = 0.0002$) (Figure 3.5). Comparison between the BEQs and the TEQs (calculated with TEF values derived from the H4IIE-*luc* cell line (Villeneuve *et al.*, 2002)) illustrated that the AhR-mediated toxicity quantified by both the instrumental and biological analysis were similar. These results correlated strongly ($r = 0.78$, $p = 0.0001$) (Figure 6.6A). Similarly, the BEQs correlated strongly with the TEQ_{FPF} ($r = 0.77$, $p = 0.0002$) (Figure 6.6B), signify that the fish potency factors (Barron *et al.*, 2004) are equally sensitive to predict AhR mediated toxicity of PAHs using instrumental data.

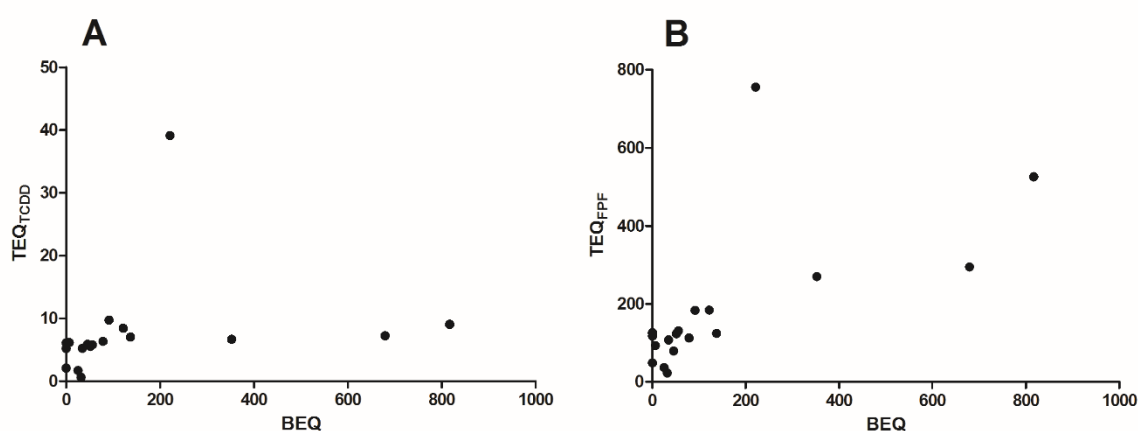


Figure 6.6: Spearman correlation scatterplot for: A) the biological equivalence (BEQ) and toxic equivalence (TEQ_{TCDD}); B) BEQ and the concentrations of the carcinogenic polycyclic aromatic hydrocarbons (CPAHs)

Finally, the prediction of sediment toxicity to benthic organism (SQG-I) was compared to the BEQs (Figure 6.7). The result was that the BEQ and the SQG-I strongly correlated ($r = 0.74$, $p = 0.0005$), proving that the toxicity predicted for benthic organisms and the toxicity quantified by the H4IIE-*luc* bio-assay posed similar threats, each in terms of their own modes of toxicity.

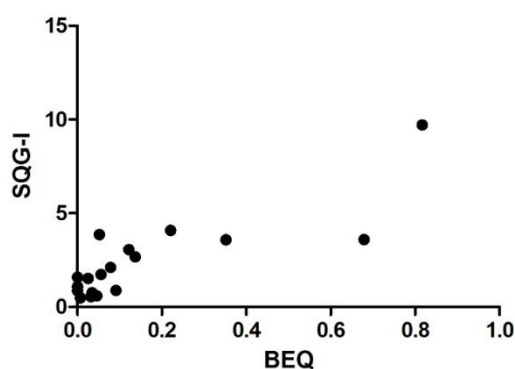


Figure 6.7: Spearman correlation scatterplot for the biological equivalence (BEQ) and the sediment quality guideline index (SQG-I)

6.5 Biomarker and bio-indicator responses to polycyclic aromatic hydrocarbons

6.5.1 Effects of PAHs on the biomarkers and fish health indices

As previously stated, the PAHs in the sediment associate with the biliary PAHs (Figure 6.3), together with the fact that bile was only sampled in 2013, the PAHs in the sediments were ordinated against the biological responses in the fish for both 2013 and 2014 (Figure 6.8). The PAHs in the sediment were used as the explanatory variables in the redundancy analysis. Half LOQ values were assigned where applicable. All the biomarker responses and selected fish health indices (CF, FHAI, HSI and SSI) were used as the species variables. In order to prevent data skewing (because Canoco treats zeros as “missing values” consequently treating the data point differently from one which was measured but resulted in no values) species values which had zeros were replaced with a value equal to a tenth of the blank (biomarkers) or a tenth of the lowest sample value (FHAI). Control fish were included in this analysis, however, because they do not have corresponding sediment values, zero values were assigned as the control fish were not exposed to sediments (thus “missing values”). The inclusion of “missing values” did not affect the ordinations of explanatory and species data compared to a similar PCA but without the congener dataset (graph not shown).

Due to strong co-linearity of the environmental data (PAHs in the sediment) the forward selection option was chosen in the RDA, to reduce overestimation of the amount of explained variance. Forward selection is a method where a parsimonious subset of explanatory variables is objectively selected. This results in a data set with fewer variables that explain the same amount of variance as the original dataset (Blanchet *et al.*, 2008).

The explanatory variables explain 44.53% of the variation in Figure 6.8. Six of the 16 PAHs were chosen during the forward selection, these were: BaP (explains 29.3% of the 44.53%, $p = 0.002$), DBA (12.8%, $p = 0.002$), Acey (7.8%, $p = 0.002$), Acea (4%, $p = 0.002$), Ant (2%, $p = 0.008$) and BaA (1.5%, 0.028).

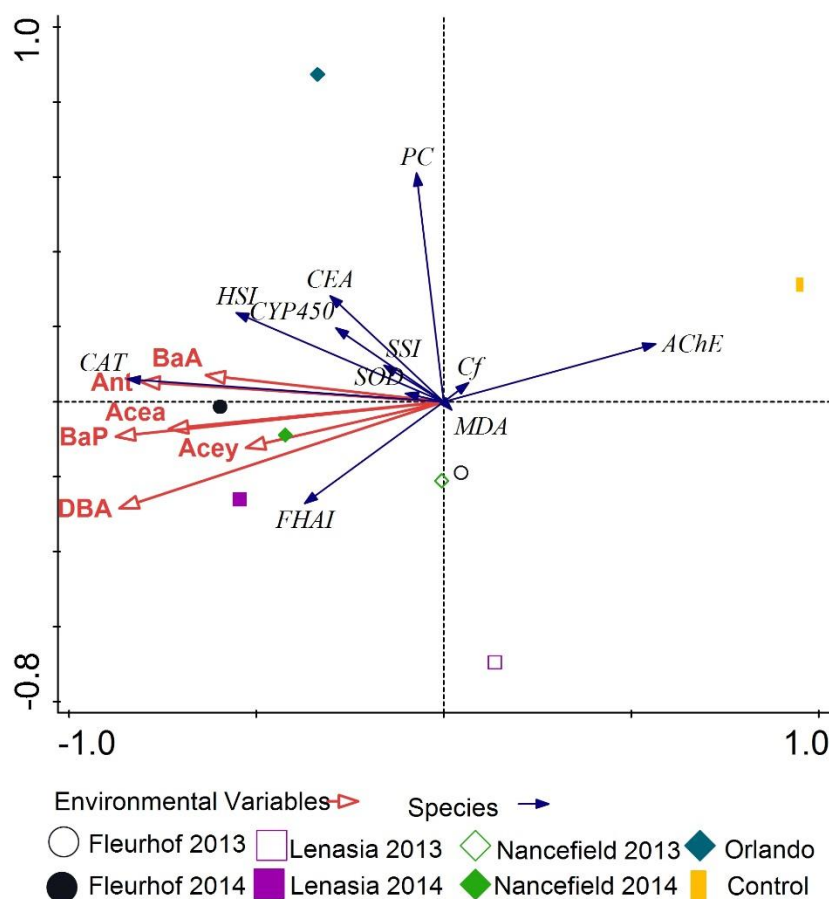


Figure 6.8: Forward selected RDA triplot of polycyclic aromatic hydrocarbons in the sediments of Soweto and Lenasia, biomarker responses and fish health indices of 2013 and 2014. The ordination explains 44.53% of the variance in the data with 36.97% by factor 1 and 7.55% by factor 2

Factor 1 (36.98%) distinguishes between the control fish on the positive side and the environmental fish on the negative side based on the ordination of the biological response in the fish: Decrease in AChE, but increase in the remainder biomarkers and health indices, arranging them on the opposite sides of Factor 1. The inhibition of AChE relative to the control showed in Figure 4.2A is supported in Figure 6.8. AChE strongly negatively correlated (180° angle) with DBA and Acey. Factor 2 explains very little variance (7.5%) and is based on the presence of protein carbonyls on the positive side and the absence thereof on the negative side. Orlando was the only site that ordinated with PCs and this is supported by the results in Figure 4.4B. The protein carbonyls did not correlate with the PAHs (90° angle), suggesting that other xenobiotics may be responsible for the PC formation in the study area. This suggests that Orlando's biomarkers and health indices did not associate strongly with the PAHs. The sites that were best explained by environmental and species data were those from 2014 (Figure 6.8). The SSI, SOD, MDA and the condition factor did not contribute to the explained variation in the data set, as shown by the short vector lines. CAT strongly correlated with anthracene and to a lesser extent benz(a)anthracene ($<0^\circ$). The strong association of the CAT (long vector) to the sites compared to low association of the SOD (short vector), showed that the SOD-CAT system is out of balance: the SOD is suppressed rather than inhibited, and CAT is over expressed to compensate for

the decreased SOD and to regain homeostasis. The HSI correlate mostly with BaA and Ant, while FHAi mostly with DBA. Effects on the lower levels of organisation (such as the molecular level) suggest brief contaminant influences. Higher in the levels of organisation the effects seen are caused by multiple stressors over a longer time period (Munkittrick & McCarty, 1995). Although the organ/systematic level (represented by the health indices) are also on a lower level of the biological organisation, it seems that the PAHs in the study area do not have such a strong effect on the overall fish health (cellular, organs and individuals) as they have on the molecular level (Figure 6.8).

6.6 Conclusion

In a previous study (Roos *et al.*, 2011) PAHs were identified as one of the most widely spread and abundant pollutant class in South Africa. One region that specifically had a high PAH burden was Soweto and Lenasia. The findings of Roos *et al.* (2011) were the main motivation for the current project: a further, in-depth investigation of the PAHs and their potential effects in the area.

The previous chapters of this thesis aimed to determine the levels of the 16 priority PAHs in the sediment and biota from the study area; in addition to the measured PAH levels, the pollutant profile was determined using composition percentages and source appointment ratios; and finally, the toxicity posed to fish and human health by the PAHs in the study area were assessed (see section 1.2.2).

It is evident that PAHs are ubiquitous in the study area. Levels present in the sediments, of which the dominant 3- and 4-ring congeners were mainly from biomass combustion, were comparable to international studies. Evidence of PAH presence in the biota was seen: low levels of Nap, Acea and Phe were found in the wetland bird eggs; and PAHs metabolites in fish bile.

The central region of the study area had the most PAHs (not exclusively): Moroka had the greatest Σ PAH levels; similarly, Eldorado Park and Orlando East had high levels of PAHs in the sediments; and the fish with the greatest Σ OH-PAHs were at Orlando (Orlando East).

The toxicity predicted for benthic organisms, fish and mammalian systems, based on the instrumentally derived PAH sediment concentrations (International SQGs, sediment indices, TEQs) were accurate compared to the biological responses generated by the H4IIE-*luc* reporter gene bio-assay (BEQs).

The inhibition of AChE together with the activation of the detoxification system (CYP450), and up-regulation of the SOD-CAT defences, is evidence that the fish from the study area had been resisting against xenobiotic stressors. Although the fish might have been exposed to a much broader variety of xenobiotics there was strong indication that the biomarker responses were due to PAHs: like acenaphthylene, acenaphthene, anthracene, benz(a)anthracene, benzo(a)pyrene and dibenz(a,h)anthracene (Figure 6.8). Literature supports that biomarkers respond to PAH exposures.

Even though the biomarker response were up-regulated, the fish could cope with the stress as was evident from the low levels of MDA and PC and the fact that the energy budget was not depleted.

The overall health, on a systematic level, of the Soweto and Lenasia fish was visibly affected by environmental stressors. The fish from Soweto and Lenasia proved to be in poorer health (relative to control) than other South African studies on *C. gariepinus*. The fish were in a fair condition, showing no malnutrition. The HSI of the fish from this study were greater than other studies investigating *Clarias gariepinus*. The SSI and GSI showed no significant difference from the control. The underdeveloped ovaries of three adult fish from Orlando suggested endocrine stressors. Regardless of what was causing the stress in the fish, the fish from Soweto and Lenasia were clearly under stress compared to the control fish. Of all these sites Orlando proved to be the worst affected, followed by Nancefield and Fleurhof.

The potential of PAHs to harm human health was shown with a human health risk assessment applied to different matrices and exposure pathways. Of these exposure pathways the intentional ingestion (geophagia) and dermal exposure of sediments posed the highest cancer risk in the study area. Moroka, Eldorado Park and Orlando East proved to be the sites with the greatest cancer risk. A total cancer risk of 277 in 10 000 was calculated for Moroka, which was the highest for the whole project.

From all the results obtained it can be concluded that the areas of most concern are Moroka and Orlando East, based on the fact that Moroka had the greatest Σ PAH and highest human health risk; and Orlando East because of the biomarker and fish health results that were the highest for 2013, and that here were no fish available to sample in 2014, most likely due to high pollution because of power station collapse. By completing the objectives, and meeting the aims of this project, the hypothesis that the humans and wildlife of Soweto and Lenasia, dependant on the Klip River, are exposed to the 16 priority PAHs is therefore accepted.

6.7 Recommendations

- The target compounds of this study were the 16 priority PAHs. The effective metabolism of these compounds by biota resulted in very low levels of native PAHs in the biota. The quantification of PAH metabolites has shown very effective in establishing the exposure of these chemicals in biota. Therefore, an in-depth chemical analysis of PAH-metabolites is suggested for future studies regarding biotic matrices for example mussel tissue, bird eggs and fish bile.
- The presence of other pollutants is seen in the results of this study and that of the precursor study by Roos *et al.* (2011). Thus for future studies a broad spectrum screening for a much larger variety of organic and inorganic chemical contaminants/pollutants is encouraged (if finances permit). Organic compounds that can be considered include: polychlorinated biphenyls, brominated flame retardants, organochlorine and -phosphate pesticides,

plasticisers, bisphenol-A/B, pharmaceuticals and personal care products and perfluorinated compounds. Inorganic compounds that may be involved are metals such as mercury and chromium.

- The biochemical responses were sufficient to show that the fish in the study area was under stress. It is recommended that a broader array of biomarkers be used in the future to further investigate the effects of the environmental stressors on the target biota. These may include: more specific cytochrome activity biomarker assays such as ethoxyresorufin-O-deethylase (EROD) and aryl-hydrocarbon hydrolase (AHH); phase II biotransformation biomarkers such as glutathione S-transferase (GST); additional oxidative stress biomarkers like reduced glutathione (GSH) and glutathione peroxidase (GPOX); metallothioneins for metal exposure; vitellogenin content assays for endocrine disruption in male fish; DNA adducts and the comet assay for DNA damage.
- On a systematic and organism level, the FHA and OSIs indicated deviation in fish health in the study area. A histopathological assessment is recommended to link the molecular level changes with the higher level changes determined by the health indices, and determine changes on a cellular level.
- It is also suggested that macro-invertebrates, such as molluscs and aquatic insects be included in future studies. The inclusion of invertebrates would fill the gap of biotic sampling where fish cannot be sampled. The evaluation of invertebrate and fish species composition and numbers can further describe pollution effects in the system and would further the ecological assessment of the area.
- A wide variety of bio-assays that are able to detect a variety of mechanisms of actions through which biota can be harmed can be employed in future research. The effectiveness of these bio-assays has been proven in this study. The array of bio-assays can be broadened to include assays capable of detecting various endocrine disruptive effects, as well as genotoxicity.
- A more frequent sampling regime for abiotic matrices (include water sampling to sediments) is proposed. This allows for a higher frequency of testing for bio-assay responses and possibly screening for contaminants.
- The last aim of this study, the theoretical human health risk assessment indicated to real carcinogenic risks. Due to this result, it would be advisable to investigate exactly how much fish from the aquatic system is consumed by the local inhabitants, as well as the rate of water and sediment exposure. This can be addressed by implementing questionnaires and interviewing the local people, as well as sampling hair and blood for chemical analysis.

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