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DISTRIBUTION OF PERSISTENT AND PSEUDO-PERSISTENT ORGANIC POLLUTANTS IN RIVERS DRAINING URBANISED CATCHMENTS IN SOUTH AFRICA





Chengetayi Cornelius Rimayi

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg and Faculty of Science, Vrije Universiteit, Amsterdam in fulfilment of the requirements for the joint degree of Doctor of Philosophy

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DECLARATION

I declare that this thesis is my own work, unaided work. It is being submitted for the Joint Degree of Doctor of Philosophy to the University of the Witwatersrand, Johannesburg, South Africa and Vrije Universiteit, Amsterdam, The Netherlands. It has not been submitted before for any degree or examination to any other university.



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Chengetayi Cornelius Rimayi

28 May 2018

Abstract

This research aimed to characterise the occurrence and distribution of persistent and pseudo-persistent organic pollutants in water and sediment samples from rivers in urbanised catchments in South Africa, particularly the Witwatersrand catchment area in the Gauteng Province, the Hartbeespoort Dam catchment in Gauteng and North West Provinces as well as the uMngeni River in KwaZulu-Natal Province. The objective was to highlight suitable analytical techniques for analysis of persistent and pseudo-persistent organic pollutants in the South African context where the cost of analysis is high and the procurement of high end analytical instrumentation is beyond the reach of the majority of analytical laboratories. Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) are some of the most complex, toxic and ubiquitous organic pollutants in industrialised regions and have been classified by the Stockholm Convention as persistent organic pollutants that need to be monitored in the environment in order to reduce or eliminate their release.

A Dioxin-Responsive Luciferin (DR-Luc) reporter gene assay was utilised to screen for dioxin-like toxicity in sediments from the Witwatersrand catchment, with all sediment samples from the Jukskei, Klip and Vaal River testing positive for dioxin-like activity. Total DR-Luc dioxin-like activity ranged from 16 to 37 pg World Health Organisation Toxic Equivalents per gram (WHO-TEQ g^{-1}) dry weight (dw) for the Jukskei River catchment and 1.5 to 22 pg WHO-TEQ g^{-1} dw for the Klip and Vaal Rivers. A comprehensive two-dimensional gas chromatography- micro-electron capture detection (GCxGC-μECD) method was developed to analyse seven PCDDs and ten PCDFs with dioxin-like toxicity in sediment samples. The highest concentration of 2,3,7,8-TCDD in the Jukskei River was found at the Midrand site (2.5 pg g^{-1} dw) and highest concentration of 2,3,7,8-TCDD in the Klip and Vaal Rivers (5.7 pg g^{-1} dw) was recorded at the Alberton site in Klip River. The results indicate that South Africa is at the moment at a general low risk of dioxin-like compound pollution.

Thirty one selected indicator PCB congeners, 19 alkyl polyaromatic hydrocarbons (PAHs) and 18 parent PAHs (including 16 priority PAHs) were analysed in sediment samples from the Jukskei, Klip and Vaal Rivers using gas chromatography- mass spectrometry (GC-MS) to determine their distribution and potential sources. Aroclor 1254 and 1260 were identified as possible sources of the PCBs detected as PCB signature patterns in order of abundance were 138> 180> 206> 153> 187> 149 and 138> 153> 180> 149> 187> 110> 170 for the Jukskei and Klip River sediments, respectively. A combination of pyrogenic and petrogenic PAH sources was detected in the Witwatersrand

catchment with fluoranthene, pyrene and phenanthrene being the most abundant PAH's in both the Jukskei and Klip River sediments.

Seasonal variation of triazine herbicides (as well as atrazine and terbuthylazine metabolites and degradation products), some of which are present at pseudo persistent concentrations in the Hartbeespoort Dam catchment surface water and groundwater was investigated using GC-MS and liquid chromatography-tandem mass spectrometry (LC-MS/MS). In the Hartbeespoort Dam, ∑triazine herbicide concentrations were in the order atrazine> simazine> terbuthylazine> propazine> ametryne> prometryn. Atrazine could be detected in groundwater at concentrations >130 ng L^{-1} in all seasons except spring. Triazine compound bioaccumulation in catfish (*Clarias gariepinus)* and carp (*Cyprinus carpio*) muscle analysed was negligible whilst Hartbeespoort Dam groundwater ∑triazine herbicide concentrations ranged between 527 and 367 ng L^{-1} and ∑triazine herbicide concentrations in the Hartbeespoort Dam surface water samples were >2000 ng L^{-1} in all seasons except spring. In light findings of endocrine and immune disrupting atrazine effects, the atrazine concentrations detected in the Hartbeespoort Dam groundwater and surfacewater may be a cause of concern.

The effects of a 90 day sub-chronic atrazine exposure (0.01, 200 and 500 µg L^{-1} atrazine in water) on exposed African clawed frogs (*Xenopus laevis*) was investigated to determine impact of environmentally relevant atrazine concentrations on adult male frog gonadal development as well as survival and growth of tadpoles. Tadpole mortality rates of 0, 0, 3.3 and 70% for the control, 0.01, 200 and 500 µg L^{-1}, respectively were recorded for 90-day atrazine exposure. Histochemical analysis with differential staining revealed gonadal atrophy, disruption of germ cell lines, significantly reduced seminiferous tubule diameter, seminiferous tubule structure damage and formation of extensive connective tissue around seminiferous tubules of adult frogs exposed to 200 µg L^{-1} and 500 µg L^{-1} atrazine concentrations. Ultrastructural analysis of the cellular organelles using transmission electron microscopy (TEM) revealed significant amounts of damaged mitochondria in testosterone producing Leydig cells as well as Sertoli cells of adult frogs. Morphometry showed reduced testicular volume and mass in all atrazine exposed adult frogs and an LC50 value of 343.7 µg L^{-1} was determined to cause significant mortality in tadpoles.

Contaminants of emerging concern in the Hartbeespoort Dam surface water as well as in the uMngeni River estuary surface water and sediment after an episodic hospital waste contamination event were determined using LC-MS/MS. In the Jukskei River, the main source of 11 emerging pollutants (EPs), some of which are present at pseudo persistent concentrations was identified as raw sewage overflow from communities situated adjacent to Jukskei River, with the highest ∑11 EP

concentration of 593 ng L^{-1} being recorded at the Midrand point and the lowest ∑11 EP concentration of 164 ng L^{-1} at the N14 site. Hartbeespoort Dam catfish (*Clarias gariepinus)* and carp (*Cyprinus carpio*) did not bioaccumulate EPs in muscle. Efavirenz, nevirapine, carbamazepine, methocarbamol, bromacil and venlafaxine were detected in the highest concentrations in the Hartbeespoort Dam and uMngeni River estuary and therefore require operational monitoring in urban waters.

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List of Abbreviations

1-MP	1-methylphenanthrene
2,3,7,8-TCDD	2,3,7,8-tetrachlorinated dibenzo-*p*-dioxin
2-MA	2-methylanthracene
A-2OH	Atrazine-2-hydroxy
AD-2OH	Atrazine desisopropyl-2-hydroxyl
ADD	Atrazine desethyl-desisopropyl
API	Active pharmaceutical ingredients
ASE	Accelerated Solvent Extraction
BaA	Benz(a)anthracene
BaP	Benzo(a)pyrene
BeP	Benzo(e)pyrene
DR-Luc	Dioxin-responsive luciferin reporter gene
dl-PCB	Dioxin-like polychlorinated biphenyl
dSPE	Dispersive solid phase extraction
CYP1A1	Cytochrome P450 family 1 subfamily A member 1
CYP1A2	Cytochrome P450 family 1 subfamily A member 2
CYP1B1	Cytochrome P450 family 1 subfamily B member 1
Dw	Dry weight
DEA	Deethylatrazine
DEHA	Deethylhydroxyatrazine
DIA	Desisopropylatrazine
EPs	Emerging pollutant
ESI	Electro-spray ionization
µECD	Micro-electron capture detection
Fl	Fluorene

Fw	Fresh weight
GC	Gas chromatography
GC-MS	Gas chromatography- mass spectrometry
GC-HRMS	Gas chromatography-high resolution mass spectrometry
GCxGC	Comprehensive two-dimensional gas chromatography
HA	Hydroxyatrazine
HMW	High molecular weight
K_{ow}	Octanol-water partition coefficient
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LMW	Low molecular weight
LOD	Limit of detection
LOQ	Limit of quantification
LRAT	Long range atmospheric transport
MMW	Medium molecular weight
MS	Mass spectrometry
PAH	Polycyclic aromatic hydrocarbon
PCA	Principal component analysis
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
PCDD/F	Polychlorinated dibenzo-*p*-dioxin and polychlorinated dibenzofuran
POPs	Persistent organic pollutant
Py	Pyrene
QuEChERS	Quick Easy Cheap Efficient Rugged Safe
RER	Rough endoplasmic reticulum
SPE	Solid phase extraction
TEF	Toxic equivalency factor

TEQ	Toxic equivalents
TEM	Transmission electron microscopy
TOC	Total Organic Carbon
TOF	Testicular ovarian follicule
SAWQG	South African Water Quality Guidelines
USEPA	United States Environmental Protection Agency
WWTW	Wastewater treatment works
WWTW	Wastewater treatment plant
WHO	World Health Organisation

Chapter 1

Introduction and objectives

Organic pollutants in the South African environment

South Africa's environmental pollution is related to industrialisation, urbanisation and agriculture (Adler et al., 2007; du Preez et al., 2005; Roux et al., 1999; Menyah & Woude-Rufael, 2010). The release of industrial, domestic and agricultural pollutants into water, air and land, particularly in populated urban areas has a profound effect on water quality due to precipitation, run-off, seepage and leakage from sewer systems (Fisher et al., 2016; Hebig et al., 2017; James et al., 2016; Polidoro et al., 2017). Air and water have been identified as the most significant migration vectors for toxic organic pollutants in different parts of the world (Hebig et al., 2017; Malina & Mazlova, 2017; Mekonen et al., 2016; Schriever et al., 2007; Schulz, 2001; Zhang et al., 2008). Urban areas are threatening human and environmental health and need to be monitored for both legacy and emerging contaminants. With more than 50% of the South African population living in urban areas, socio-economic factors strongly influence pollution of urban waters. The South African society has been described as consisting of two parallel societies, i.e. one society living in conditions comparable with other African developing nations and a second affluent society enjoying economic and social amenities on par with the developed world (Anderson et al., 2007). Numerous informal settlements in the former society have been described as one of the most significant contributors of pollution due to poor water, sanitation and power infrastructure. An example is the Jukskei River flowing through the Alexandra Township in Johannesburg, which has often been described as similar to raw sewage in the dry season (Nastar & Ramasar, 2012; Wiberly & Coleman, 1993). Within communities having limited or no access to sanitation, raw sewage and excreta often contaminate urban rivers such as the Jukskei River (Madikizela et al., 2017).

Poor households which rely primarily on the use of coal for cooking and heating have been singled out as one of the major sources of air pollution due to low thermal efficiencies of the stoves and burners used (Balmer, 2007). They release hazardous polycyclic aromatic hydrocarbons (PAHs) through combustion. Polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/Fs), often called "dioxins" are some of the most ubiquitous toxic compounds and are mostly detected in urban areas (Liu et al., 2013; Pemberthy et al., 2016). They are primarily emitted from combustion processes that include incineration of municipal solid waste containing chlorine as well as plastics containing polyvinyl chloride (PVC), metal smelting, chlorine bleach pulping of wood and operations of coal fired power stations and cement kilns (Braune & Mallory, 2017; Deziel et al., 2017; Pemberthy et al., 2016; Squadrone et al., 2015). The World Health Organisation (WHO)

declared 2,3,7,8-tetrachlorinated dibenzo-*p*-dioxin (2,3,7,8-TCDD) the most potent PCDD compound, with six other 2,3,7,8-substituted PCDDs and ten PCDFs being considered to have similar, but lower toxicity compared to 2,3,7,8-TCDD (Braune & Mallory, 2017; Rocha et al., 2016). A concept of toxic equivalency factors (TEFs) was developed by the WHO for dioxin-like (dl)-compounds with 2,3,7,8-TCDD having the highest TEF of 1 and the sum total of TEFs of dl-compounds present in a sample being called toxic equivalency (TEQ) (Braune & Mallory, 2017; Rocha et al., 2016).

Polychlorinated biphenyls (PCBs) are contaminants of major concern due to (i) their widespread distribution (ii) toxicity of 12 planar congeners which are structurally similar to 2,3,7,8-TCDD, called dioxin-like PCBs (dl-PCBs) and exhibit dioxin-like toxicity (Braune & Mallory, 2017; Malina & Mazlova, 2017; Martinez-Guijarro et al., 2017). The Stockholm Convention on persistent organic pollutants (POPs) was established in 2001 by the United Nations (UN) with the aim of eliminating POPs such as PCBs and PCDD/Fs entirely. However, current forecasts tell that many countries will not meet the 2025 and 2028 Stockholm Convention targets, respectively to eliminate PCB containing equipment and implement environmentally sound PCB waste management practices (Stuart-Smith & Jepson, 2017). For example, and contrary to the statutes of the Basel and Bamako Conventions on the control of transboundary movements of hazardous wastes and their disposal, developing coastal countries such as Nigeria are often used as dumping grounds for redundant ships and electronic waste, which are a source of PCBs (Terada, 2012; van Erp & Huisman, 2010). This is counter-productive for the aims of the Stockholm Convention.

Chloro-s-triazines and in particular atrazine are the most ubiquitous herbicides in the South African environment. Triazine herbicides are applied to control broad-leaf weeds, particularly in corn (du Preez et al., 2005). Atrazine is also the only organic pollutant for which the South African Water Quality Guidelines (SAWQG) for domestic use has specified a target water quality range of 0-2 µg L^{-1}. Even when applied at very low levels, triazines and their metabolites end up in our natural waters and the environment due to the nature of their persistence (Zhou et al., 2006). Atrazine has been successfully banned in some European countries with no decline in corn yields, proving that it use is not indispensable (Ackerman, 2007). However, it is still extensively used in other parts of the world.

Emerging pollutants are unregulated compounds that are not regularly monitored in the environment and for which there is limited knowledge about their toxicity and behaviour in the environment (López-Doval et al., 2017). They encompass a wide range of anthropogenically made chemicals, including pharmaceuticals, pesticides, personal and household care products which have now become indispensible for human health and comfort (Gavrilescu et al., 2015; Klatte et al., 2017).

The extent of distribution of emerging pollutants in African waters is largely unknown (Sorensen et al., 2015). Emerging organic pollutants are called pseudo-persistent organic pollutants as they are less persistent than e.g. PCBs and dioxins and are consistently present in the environment due to a constant release from contaminating wastewater treatment plant effluent and sewer systems (Deblonde et al., 2011; Ebele et al., 2017). Most pharmaceuticals and personal care products are found in surface waters at concentrations of 1.8-40 µg L^{-1} (Zenobio et al., 2015). Some of the emerging pollutants have been linked to chronic toxicity and endocrine disruption (Rosal et al., 2010). Many emerging pollutants are water soluble hence often contaminate groundwater and may exert biological effects on non-target aquatic organisms as well as humans who consume contaminated groundwater or surface water (Besse & Garric, 2008). Emerging contaminants in groundwater are of major concern as they are difficult to eliminate due to low redox conditions underground as well as lack of photodegradation (Peng et al., 2014). There is significant attention to groundwater quality from the South African government as it contributes approximately 13% of the total water use in the country (Groundwater division of GSSA).

Sources and toxicity of organic pollutants

PCDD/Fs and PCBs

PCDD/Fs are complex analytes as they consist of 135 PCDDs and 75 PCDFs (Hung et al., 2017). The chemical profile of PCDD/Fs present in the environment depend on the nature of the sources and anthropogenic activities in urban areas as there are usually wide variations in sources and concentrations, mainly due to fluxes in release and production (Melymuk et al., 2012). Humans are primarily exposed to PCDD/Fs through occupational and accidental exposure as well as consuming contaminated food with a high fatty acid content (Shen et al., 2008). There are vast differences in major contributors to atmospheric PCDD/F between different regions and countries. PCDD/F emission trends for developing and developed countries tend to vary with the primary source of energy, local policies (and regulations) and availability and use of cheap natural resources such as oil and coal. Coal fired power stations are for example an unclean source of power compared to offshore windfarms and hydroelectric power stations, which are more prevalent in developed countries. The reduction of PCDD/F emissions can only be achieved through stringent regulation of industrial activities such as municipal solid waste incineration, medical waste incineration and smelting, which are amongst the major PCDD/F producing activities in a number of regions and countries including the USA and Europe (Quaß et al., 2004; Zhou et al., 2016). Coal fired power plants have been singled out as a major source of PCDD/Fs in some regions (Tu et al., 2011). Natural

sources of PCDD/Fs and PAHs include forest fires and volcanic eruptions (Cheng & Hu, 2010; Shen et al., 2008; Zhang & Tao, 2009). There are large uncertainties in estimation of the contribution of forest fires to the global PCDD/F emissions (Gullett & Touati, 2003). Oil processing and desulphurisation plants have been mentioned as top PCDD/F contributors in some countries such as Kuwait (Martinez-Guijarro et al., 2017). PCDD/F migration is mainly due to long range atmospheric transport (LRAT) in the atmosphere or through river channels, bound to organic matter in sediments (Bae et al., 2002).

The compound 2,3,7,8-TCDD is one of the most potent toxicants known to man (Mandal, 2005). The Aryl hydrocarbon receptor (AhR) mediates the majority of the biochemical, biological, and toxicological responses following 2,3,7,8-TCDD and dioxin-like compound exposure. The AhR signal transduction pathway is initiated when an AhR ligand enters a responsive cell and binds with high affinity to cytosolic AhR (Nie et al., 2001). The AhR-ligand complex undergoes conformational changes before being translocated into the nucleus where the AhR-ligand dimerises with a nuclear protein, the Ah receptor nuclear translocator (Arnt), forming the AhR-ligand-Arnt complex, which is a high affinity DNA binding complex. The AhR-ligand-Arnt complex binds to a specific DNA recognition site termed the dioxin responsive element (DRE) upstream of the cytochrome P450 family 1 subfamily A member 1 (CYP1A1) responsive gene where the 2,3,7,8-TCDD attacks the DNA, causing a variety of effects ranging from DNA damage to genotoxicity, resulting in toxicity and cancers (Mandal, 2005). It also leads to transcription and expression of the CYP1A1 gene, resulting in accumulation of CYP1A1 in the cell (Nie et al., 2001).

PCBs have been produced on an industrial scale since 1930 for use as flame retardants and isolating material in electrical transformers and capacitors as well as for use as lubricants and plasticisers, among other uses (Astoviza et al., 2016; Rushneck et al., 2004). PCB production was restricted before being effectively banned worldwide in the late 1970s (Dias-Ferreira et al., 2016). There are 209 theoretically possible PCB congeners based on the number of chlorine atoms that can be substituted on the biphenyl rings (Antolin-Rodriguez et al., 2016; Sarkar, 2016). There are approximately between 130 and 140 significant PCB congeners in technical mixtures as well as in the global environment, with the remaining theoretically possible PCBs, mainly 2,4,6-substituted congeners, which are energetically unfavourable to be formed, not detected in technical mixtures (Antolin-Rodriguez, 2016; Frame et al., 1996). There are 12 non-*ortho* and mono-*ortho* substituted PCB congeners (dl-PCBs) which show toxicological properties similar to PCDD/Fs due to their specific chlorine substitution in positions that allow a planar conformation (Abella et al., 2016).

PCB contamination was first discovered by the Danish Chemist Sören Jensen in 1966 (Jensen, 1972). Thereafter, PCB contamination was discovered to be widespread worldwide. The consumption of fish from the Great Lakes region of North America at one time was considered one of the most significant human exposure incidences (Wu et al., 2011; Sadraddini et al., 2011). A 30 year assessment (1977-2007) of PCBs in fish caught in the Great Lakes Region in Canada revealed a gradually declining trend (Sadraddini et al., 2011). Commercial PCB mixtures Aroclor 1254 and Aroclor 1260 were found to be prevalent in European soils in the cities of Glasgow (Scotland), Torino (Italy), Aveiro (Portugal), Ljubljana (Slovenia) and Uppsala (Sweden) as well as in Hong Kong, China (Cachada et al., 2009). Aroclor 1016 and Aroclor 1242 have been identified as the major contaminating technical mixes in harbour sediments from Milwaukee, Wisconsin, USA with Aroclor 1254 and 1260 being identified as technical mixes of minor significance (Rachdawong & Christensen, 1997). PCBs may be emitted into the air by volatilisation or onto land by leaking from PCB containing equipment (Hu et al., 2010). Determination of the spatial distribution of PCBs in urban areas is challenging due to the presence of many diffuse and point sources (Hu et al., 2010). Indicator PCBs are useful for environmental monitoring and the congeners analysed are usually present at concentrations high enough to be detected by basic chromatography instrumentation. Six indicator PCBs, nos. 28, 52, 101, 138, 153, 180 or seven, with 118 also included, are commonly used, but are not able to completely characterise the specific contaminating PCB technical mixture(s). Indicator PCBs utilised around the world are listed in Table 1.1.

The Stockholm Convention on POPs encourages authorities to monitor POP concentrations in the environment and ecosystems to follow their reduction and elimination from the environment (Magulova & Priceputu, 2016; Martinez-Guijarro et al., 2017; Pemberthy et al., 2016; Porta et al. 2008; UNEP, 2009). POPs as described by the Stockholm Convention encompass a range of organic compounds with specific properties such as persistence, high toxicity, ability to undergo long-range atmospheric transport and a high bioaccumulation coefficient (Torre et al., 2016; de Vos et al., 2013; Kakareka, 2002; Munoz-Arnaz et al., 2016; Squadrone et al., 2015; Vallack et al., 1998). There are 12 initial chemicals specified in the Stockholm convention as prioritised for global action which include PCDDs, PCDFs, PCBs and nine other organochlorine compounds. These were termed “the dirty dozen” (Harner et al., 2006a; Harner et al., 2006b; Tieyu et al., 2005; Xie et al., 2010). There are not yet any legally binding POP environmental quality standards (EQS) or maximum residue levels for surface water, groundwater, sediments or biota in South Africa which are required for safeguarding a non-toxic environment and ecosystem.

Table 1.1 Selected indicator PCBs used around the world

Author	Indicator PCBs analysed	# of PCBs	Country	Matrix
Willman et al. (1997)	18, 22, 25, 26, 33, 40, 49, 52, 53, 63, 74, 77, 81, 82, 83, 85, 87, 97, 99, 105, 107, 114, 118, 119, 123, 126, 128, 130, 141, 146, 149, 151, 156, 157, 167, 169, 174, 177, 180, 183, 185, 189, 191, 193, 194, 198, 201	47	USA	Sediment, plankton, fish
Rimayi et al. (2017)	28, 31, 44, 47, 49, 52, 56, 66, 74, 85, 87, 97, 99, 101, 105, 110, 118, 128, 137, 138, 141, 149, 151, 153, 156, 170, 180, 187, 194, 202, 206	31	South Africa	Sediment
Rabodonirina et al. (2015)	8, 18, 28, 44, 52, 66, 77, 81, 101, 105, 114, 118, 123, 126, 128, 138, 153, 156, 15 7, 167, 169, 170, 180, 187, 189, 195, 206, 209	28	France-Belgium border	River suspended solid matter (SSM)
Roche et al. (2000)	18, 31, 52, 49, 47, 44, 66, 101, 110, 105, 118, 119, 151, 153, 156, 180, 170, 199, 195, 194, 209	22	France	Fish
Cachada et al., (2012)	1, 5, 18, 28, 31, 44, 52, 66, 87, 101, 110, 118, 138, 141, 151, 170, 180, 183, 187, 206	21	Portugal	Soil
Chamkasem et al. (2016)	1, 5, 18, 31, 44, 52, 66, 87, 101, 110, 137, 141, 151, 153, 170, 180, 183, 187, 206	19	USA	Fish
Ishikawa et al. (2007)	3, 8, 28, 52, 77, 101, 105, 118, 126, 138, 153, 180, 194,206, 209	15	Japan	Indoor air, Flue gases, MSW, ash
Rollin et al. (2009)	28, 52, 99, 101, 105, 118, 138, 149, 153, 156, 170, 180, 183, 187, 194	15	South Africa	Delivering women's blood
Daouk et al. (2011)	28, 52, 101, 105, 118, 132, 138, 149, 153, 156, 170, 180, 194	13	France	PCB fed Zebra fish
Darnerud et al. (2011)	28, 52, 101, 105, 114, 118, 138, 153, 156, 157, 167, 170, 180	13	Sweden	Milk
Focant et al. (2005)	28, 52, 80, 81, 101, 77, 123, 118, 114, 153, 105, 138	12	Belgium	Fish, pork, milk
Dias-Ferreira (2016)	28, 30, 52, 101, 138, 153, 166, 180, 204, 209	10	Portugal	Sediments
Gakuba et al. (2015)	28, 52, 77, 101, 105, 138, 153, 180	8	South Africa	River Sediment
Baars et al. (2004)	28, 52, 101, 118, 138, 153, 180	7	The Netherlands	Foodstuffs
Norli et al. (2011)	28, 52, 101, 118, 138, 153, 180	7	Norway/ Ethiopia	Fish
van Leeuwen et al. (2007)	28, 52, 101, 118, 138, 153, 180	7	The Netherlands	Fish
Whitehead et al. (2013)	105, 118, 138, 153, 170, 180	6	USA	Dust from homes
Toan & Quy (2015)	28, 52, 101, 138, 153, 180	6	Vietnam	Soil and sediment
Pieters & Focant (2014)	28, 52, 101, 138, 153, 180	6	Belgium/S. Africa	Serum
de Vos et al. (2011)	81, 77, 126, 169	4	South Africa	Soil

PAHs

PAHs are not included in the list of compounds scheduled for restriction and elimination in the Stockholm Convention because they do not meet the criteria for bioaccumulation. Petrogenic PAH sources include petroleum products such as gasoline, diesel, kerosene, crude oil, lubricating oil and asphalt whilst pyrogenic PAH sources include products of incomplete combustion of organic matter such as coal, petroleum products and wood (Lafontaine et al., 2015; Saha et al., 2009; Shao et al., 2014). The majority of the studies conducted worldwide indicate a stronger presence of pyrogenic PAH sources, with only a few studies reporting a dominance of petrogenic PAHs (Saha et al., 2009). Dominant petrogenic PAH sources of contamination are associated with accidental oil spills such as the 2010 deepwater horizon spill in the Gulf of Mexico. PAHs comprise up to 10% of hydrocarbons in crude oil. Hence they can be used to trace the extent of oil leaks. Oils spills from offshore prospecting areas under the sea have been a common occurrence from the conception of offshore oil prospecting activities (Sammarco et al., 2013). Natural contamination from oil seeps below the sea or ocean can also occur. Low molecular weight (LMW) PAHs from the oil tend to volatilise into the atmosphere whilst medium molecular weight (MMW) PAHs remain at the surface of the water and high molecular weight (HMW) PAHs are deposited into the sediments (Sammarco et al., 2013).

HMW PAHs, particularly benzo(a)pyrene (BaP) have been discovered to act as AhR ligands (Pieterse et al., 2013). PAHs are themselves chemically inert as they require metabolic activation by mammalian CYP1A1, CYP1A2, and CYP1B1 enzymes to more reactive biologically active epoxide intermediates in order for them to exhibit carcinogenicity, and as such are termed procarcinogens (Miller & Ramos, 2001). (+)- and (-)-B[a]P-7,8-diol-9,10-epoxides are highly reactive towards DNA and thus they were concluded to be the ultimate carcinogenic metabolites of BaP (Shimada & Fujii-Kuriyama, 2004). Of the over 10 000 PAHs occurring in the environment, the U.S Environmental Protection Agency (USEPA) selected sixteen PAHs of environmental concern for priority monitoring in the environment based on toxicity, frequency of occurrence in the environment and potential for human exposure (Bojes & Pope, 2007). The toxicity of other PAHs has been estimated by calculating their BaP equivalents, after establishing individual PAH toxic equivalent factors (TEF) relative to BaP carcinogenic potency. The PAH TEFs for 16 EPA priority PAHs considered by several authors are naphthalene (0.001), acenaphthene (0.001), acenaphthylene (0.001), anthracene (0.01), phenanthrene (0.001), fluorine (0.001), fluoranthene (0.001), benzo(a)anthracene (0.1), chrysene (0.01), Pyrene (0.001), benzo(a)pyrene (1), benzo(b)fluotanthene (0.1), benzo(k)fluoranthene (0.1),

dibenz(a,h)anthracene (1), benzo(g,h,i)perylene (0.01) and indeno[1,2,3-cd]pyrene (0.1) (Nisbet & LaGoy, 1992; Tsai et al., 2004).

Atrazine

Atrazine has been reported to cause developmental toxicity, reproductive toxicity, immunotoxicity, genotoxicity, neurotoxicity and gonadotoxicity in different animals including fish, amphibians and humans (Langerveld et al., 2009; Morgan et al., 2017; Solomon et al., 2008). Atrazine toxicity in humans has not been well characterised as there are limited numbers of occupational and accidental atrazine exposure incidents studied (Coban & Filipov, 2007). There is however, no consistency in the toxicological effects of environmentally relevant atrazine concentrations on animals (Rohr & McCoy 2010). Developmental toxicity of atrazine has been shown to vary according to age at exposure, particularly in organisms which undergo metamorphosis such as frogs for which the toxicological effects vary with the life cycle stage at which atrazine exposure occurs. Frog (*Rhinella arenarum*) larvae were discovered to be more sensitive to atrazine toxicity than the embryo. Hence exposure of larvae to concentrations of 10 mg L^{-1} atrazine led to significantly higher metamorphosis failure and mortalities than exposure of embryos (Brodeur et al., 2009). There have been numerous reports attributing the global frog population decline to atrazine toxicity (Shipitalo & Owens, 2003; Siddiqua et al., 2010).

The International Agency for Research on Cancer classified atrazine as a possible human carcinogen as atrazine has been found to induce significant genotoxicity e.g in fish (*Prochilodus lineatus, Carassius auratus* and *Channa punctatus*) hepatocytes, erythrocytes and gill cells tested using the sensitive Comets assay (Cavas, 2011; Santos & Martinez, 2012; Nwani et al., 2011). Studies on genotoxicity of atrazine have been described by Tennant et al. (2001) as “rife with conflicting data”. The wide array of conflicting results may also be attributed to different toxicity endpoints, different animal models, atrazine exposures at different life stages as well as non-monotonic dose responses due to different exposure concentrations applied in different studies (Rohr, 2017). Genotoxicity tests by Freeman & Rayburn (2004) did not induce any significant genotoxicity in *Xenopus laevis* tadpoles using a less sensitive flow cytometric analysis. Only a single and very high atrazine exposure concentration of 800 µg L^{-1} was utilised in the study, thus may be prone to non-monotonic dose effects.

In a study by Rowe et al. (2006), atrazine was discovered to cause T cell and B cell proliferation in prenatal/lactational exposed mice, a condition that may potentially give rise to autoimmune disease, and in contrast a study by Soltanian (2016) showed that atrazine caused immunosuppression in exposed slider turtles as evidenced by reduced leucocyte numbers as well as reduced phagocytic activity. A large number of studies show sex and age dependant immunotoxicity on exposure to atrazine, hence exposure to a specific age or sex is more inclined to produce a biased non-monotonic dose response (Rowe et al., 2008). Atrazine has been shown to induce gonadotoxicity by inducing demasculisation (feminisation) of male fish, amphibians and mammals, manifested by reduced testosterone and sperm production as well as gonadal dysfunction (Hayes, et al., 2010; Hayes, et al., 2011; Solomon et al., 2008). Atrazine has been discovered to cause neurotoxicity in several animals including mice, fish, mussels and quail (Xia et al., 2017). The neurotoxic effects of atrazine in humans have been described in detail by Ma et al. (2015). Atrazine reduces dopamine levels in the midbrain, affects the absorption of synaptic vesicles and synaptic bodies and interferes with dopamine storage and uptake in synaptic vesicles, which leads to neurodegenerative disorders (Ma et al., 2015). Concerns about atrazine toxicity are mainly based on its widespread distribution in the environment and effects on non target animals as well as aquatic macrophytes which provide food, cover and oxygen to aquatic animals.

Pharmaceuticals and contaminants of emerging concern

Antiretroviral (ARV) drugs such as efavirenz, nevirapine, emtricitabine (mikromol) and lamivudine, the antidepressant dug venlafaxine, the muscle relaxant methocarbamol, the cardiac stimulant etilefrine, and the epilepsy treatment drug carbamazepine are some of the human pharmaceuticals often discovered in urban waters worldwide (Guo et al., 2016; Jain et al., 2013). ARV drugs are used predominantly for treatment of HIV infection, prevention of mother to child transmission of HIV, as well as for pre-exposure prophylaxis to prevent the spread of HIV. The Jukskei River and Hartbeespoort Dam are effluent dominated waters with visible signs of sewage pollution. When unmetabolised human pharmaceuticals are excreted, they can find their way into rivers and lakes through poorly treated wastewater treatment plant (WWTP) effluent and sewage overflow contamination. They may exert biological effects on non-target organisms with effects such as hepatotoxicity and poor predator evasive behaviour (Klatte et al., 2017). This can lead to decline in aquatic organism populations, thereby creating a gap in the food chain. Risk classification of emerging pollutants and active pharmaceutical ingredients (APIs) in the environment has not been well elucidated (Comber et al., 2018).

Long range atmospheric transport of organic pollutants

PCDD/Fs, PCBs and PAHs are capable of LRAT and inter-continental atmospheric migration (Lafontaine et al., 2015; Morales et al., 2014). They can evaporate at elevated temperatures in tropical and sub-tropical regions and deposit at low temperatures in high latitudes due to global cold trapping and fractionation (Khairy et al., 2016; Wania & Mackay, 1996). Due to the volatile nature of POPs and their propensity for LRAT, Antarctica is of particular interest as it is in close proximity to South Africa. POPs research in Antarctica is scanty, hence the influence of POPs released from South Africa on Antarctica is largely unknown (Pozo et al., 2017). Studies of the global atmospheric gas phase composition of PCDD/Fs, dl-PCBs and PAHs in the open oceans including the Indian, Atlantic and Pacific Oceans revealed high concentrations of PAHs, particularly near Asian coastal areas (Genualdi et al., 2009; Lafontaine et al., 2015). Traces of PCDD/Fs and PCBs were also detected, with PCBs detected at higher concentrations than PCDD/Fs and PCB 118 being detected in the highest quantities (Morales et al., 2014). Dl-PCBs are dominant in the gas phase whilst PCDD/Fs are dominant in the aerosol phase (Morales et al., 2014). Regions and countries that have a history of PCB production include USA, China and Europe (Wu et al., 2011). Urban areas have been described as both a source and a sink for PCBs (Wu et al., 2011).

The atmosphere is a major pathway for transport of PAHs as they have a long record of anthropogenic release and transport across long ranges (Bae et al., 2002; Melymuk et al., 2012). However, it has been estimated that 97.2% of PAHs generated over mainland China stay within the borders of mainland China (Lang et al., 2008). Pyrogenic LMW PAHs are predominant in the gas phase whilst MMW and HMW PAHs are sorbed to air bound particles (Bae et al., 2002). PAH point sources may vary substantially over a small stretch of land, depending on the land use activities in the local areas. For example it has been found that in northern China, coke processing is the major contributor in the province Shanxi, biomass burning is the major contributor in Hebei and Shandong whereas coal combustion is the major contributor in the Beijing-Tianjin area. China has been described as having the greatest PAH emissions in the world, contributing 114 giga grams (Gg) per year of 16 Priority USEPA PAHs (16 PAH) in 2004, accounting for 22% of the worldwide PAH emissions (Lang et al., 2008). The global 16 PAH emission was estimated to be 500 Gg in 2004 and in excess of 500 Gg in 2007 (Lin et al., 2015; Zhang & Tao, 2009).

Motor vehicle emissions, coal fired power stations and domestic heating are major contributors to the release of PAHs in many urban areas around the world (Melymuk et al., 2012). After release, PAH deposition occurs either by wet deposition through precipitation or dry deposition (Motelay-Massei et al., 2006). It has been proposed that global warming will lead to a moderate change in environmental transport and behaviour of organic pollutants in some regions of the world (Hansen et al., 2015).

Volatilisation has been identified as the major pathway for triazine compounds into the atmosphere (Thurman & Cormwell, 2000). However, triazine compounds have very low partitioning rates into air due to their low K_{ow} coefficients, hence triazine contaminant transport is mainly water driven (Stroebe et al., 2004). A study by Goolsby et al. (1997) revealed that volatilisation loss of atrazine applied on fields is only 2% and deposition through precipitation can be up to 0.6% of the triazine herbicides sprayed into the field. Triazine herbicides can travel short distances through particle transport when particles sprayed onto the field are carried by air for short distances. Triazine compounds are also dispersed into the environment through physical drift, when compounds sorbed onto particles such as soil are transported by water or wind (Thurman & Cormwell, 2000). Atrazine physical drift concentrations of up to 186 pg L^{-1} were detected 400 m downwind of a sprayed field, sampled using high volume air sampling on polyurethane foam adsorbents in South Africa (Nsibande et al., 2015).

Analysis

Sample extraction involves the isolation of analytes of interest from the sample matrix. The extraction methods of choice depend on the nature of the sample as well as the properties of the analytes of interest. Pressurized liquid extraction (accelerated solvent extraction) is a fast and efficient method for extraction of organic pollutants from sediment samples (Giergielewicz-Mozajska et al., 2001). EPA method 3445A has been developed as the reference pressurized liquid extraction method. A dried and ground sediment sample is loaded into an extraction cell and the surrogate and internal standards added to the sample before extracting the sample using high temperatures and pressure. Commonly used solvents are hexane and acetone for non polar analytes (PCDD/F, PCB and PAH) and more polar solvent combinations of acetone and dichloromethane are often used for extraction of more polar analytes (pharmaceuticals and triazine compounds) (Giergielewicz-Mozajska et al., 2001). A cellulose disk placed at the outlet end of the extraction cell ensures that the sample extract is free from particulate matter from the sample.

Extraction of non polar as well as moderately polar analytes (triazine compounds and pharmaceuticals) from water samples can be efficiently performed using solid phase extraction (SPE), which has many advantages over liquid-liquid extraction such as prevention of formation of emulsions, use of significantly less solvents and higher recoveries (Andrade-Eiroa et al., 2016). Extraction of biological samples/tissue, including fish muscle and frog liver can suitably be conducted using a modified Quick Easy Cheap Efficient Rugged Safe (QuEChERS) method (Vudathala et al., 2010). The QuEChERS method consists of a first sample extraction step, typically using acetonitrile and salts to induce phase separation and extraction of the analytes of interest from the ground and homogenised sample. The second step is a dispersive SPE (dSPE) cleanup typically using the sorbents C18, primary secondary amine (PSA) and $MgSO_4$ (Anastassiades & Lehotay, 2003). Addition of graphitised carbon black and diatomaceous earth into the dSPE tube before cleanup is essential for producing clean extracts that can be injected into the gas chromatograph or liquid chromatograph without further sample cleanup (Vudathala et al., 2010). Extraction of steroid hormones and triazine metabolites from serum can be achieved by precipitating serum proteins using saturated ammonium sulphate before conducting a dSPE cleanup to obtain clean sample extracts (Giton et al., 2012).

For PCDD/F analysis, a H_2SO_4 digestion is essential to digest and remove co-extracted interfering compounds (e.g pesticides, PAHs, fatty acids and fat) from the sample extract. Further cleanup of PCDD/F sample extracts for gas chromatography (GC) and Dioxin Responsive Luciferase reporter gene (DR-Luc) bioassay using gel permeation chromatography is critical to remove acid resistant humic acids and sulphur which are interfering compounds in comprehensive two-dimensional gas chromatography-micro-electron capture detection (GCxGC-µECD) analysis and cytotoxic to murine hepatoma cells in the DR-Luc bioassay (Houtman et al., 2007). PCDD/F analysis creates an analytical challenge as there are many overlapping isomers. Fractionation of PCDD/Fs on graphitised carbon SPE tubes is critical to separate and remove coextracted acid resistant compounds such as PCBs and PBDEs to eliminate interferences in the GCxGC-µECD chromatogram. The µECD detector is suitable for analysis of PCDD/Fs as it provides good sensitivity for trace-level organochlorine pollutants as well as high scanning rates (>20 Hz) required to accurately characterise the narrow GCxGC peaks (Danielsson et al., 2005; Muscalu & Reiner, 2011).

GCxGC was invented in the 1980s (Muscalu & Reiner, 2011). It is a powerful separation technique where a sample is injected onto a first column and separated e.g by boiling point before entering the second column with a different polarity, through a modulator. The analytes are characterised with two retention times, one for each column. None of the common ‘5-type’ columns (5% phenyl, 95%

dimethylsiloxane, including HP-5, DB-5, ZB-5, VF-5, Rxi-5Sil, BPX 5 and CPSil8 CB) are able to separate all seventeen (17) 2,3,7,8-substituted dioxins and furans from the 118 other non-2,3,7,8-substituted PCDD/F isomers. Hence the use of GCxGC coupled with the use of a specialised dioxin column is crucial in PCDD/F analysis (Do et al., 2013). PCBs and PAHs can be extracted from the same sample and separated by fractionating the sample extract into PCB and PAH fractions utilising silica gel extraction according to the method described by Jang & Li, (2001). Congener-specific analysis of PCBs can be conducted on a single 60 m aromatic selective GC column e.g CP-SIL 8 CB (5% phenyl 95% methylsiloxane) and PAH analysis can be conducted on common '5-type' columns e.g SGE BPX 5 column (5% phenyl, 95% dimethylsiloxane). Triazine herbicides can be analysed either by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-tandem mass spectrometry LC-MS/MS. Triazine metabolites which are not GC amenable can be analysed by LC-MS/MS and emerging pollutants can be analysed by LC-MS/MS owing to their polar characteristics and occurrence in the environment at very low concentrations.

As it may not be practically possible to chemically measure each and every pollutant in contaminated water and sediments, the use of effect based tools such as bioassays and biomarkers for monitoring toxicity provides an integrative method of assessing toxicity present in samples as well as chemicals (Wernersson et al., 2015). The use of bioassays to measure the toxicity of environmental samples on a cellular level, coupled with the use of biomarkers to measure biological responses both at individual and cellular level provides useful information on toxicity (Wernersson et al., 2015). The DR-Luc bioassay responds to dioxins and dioxin-like compounds, utilising the Aryl hydrocarbon Receptor (AhR) signal transduction pathway to induce the firefly (*Photinus pyralis*) reporter gene stably transfected into the H4IIE murine hepatoma cell, resulting in production of the firefly luciferase enzyme (Vanderperren et al., 2004). Luciferase activity is then detected using a luminometer after addition of the luciferin substrate (Vanderperren et al., 2004).

Compared to the globally accepted reference GC-HRMS method, which utilises a selective magnetic sector analyser, the DR-Luc bioassay is more practical for large scale assessment studies which require a rapid sample throughput (Denison et al., 2004; Rivera-Austrui et al., 2017; McCulloch et al., 2017). The DR-Luc bioassay is a relatively inexpensive method for measuring toxic equivalency by comparing the sample's response to a dose response curve of 2,3,7,8-TCDD (Behnisch et al., 2001; Croes et al., 2013; Danielsson et al., 2005; Schroijen et al., 2004; van Wouve et al., 2004; Wernersson et al., 2015). Bioassay information provides a link between chemical exposure and biological responses or toxicity (Brack et al., 2017). The DR-Luc bioassay may for example be used as screening

tool for dl-compounds (Wernersson et al., 2015). The DR-luc bioassay is regarded as highly sensitive, though it is prone to interference from both agonistic and synergistic ligands as it detects all AhR ligands (Wernersson et al., 2015; Schroijen et al., 2004). The interferences can, however, be significantly reduced or eliminated by a proper sample cleanup (Schroijen et al., 2004).

Gonadotoxicity of testes exposed to gonadotoxins such as atrazine can be assessed by histological examination of tissue cells by light microscopy with differential staining (hematoxylin and eosin (H&E) and Van Gieson staining techniques). H&E stain is used to stain nuclei and cytoplasm and Van Gieson stain is used to stain collagen and connective tissue. Transmission electron microscopy (TEM) can be utilised to perform ultrastructural histological analysis of cellular organelles, after tissue processing (fixing, embedding and ultramicrotomy) and double staining with uranyl acetate and lead citrate. These analytical techniques, when utilised in tandem, can be used to sufficiently estimate ecological risk assessment of a contaminated environment or ecosystem.

Aim of the thesis

The establishment of operational monitoring of organic pollutants depends on the establishment of rugged analytical methods and availability of suitable analytical instrumentation and competent analytical personnel. The aim of this research was to:

i) provide a framework for determination of selected complex toxic organic pollutants in the classes of polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, polychlorinated biphenyls and PAHs in sediments from South African urban waters.
ii) provide a framework for determination of selected emerging pollutants in the class of triazine herbicides, triazine herbicide metabolites and pharmaceuticals from water and sediments in South African urban waters for establishment of broader investigative and long-term monitoring.
iii) provide new information on the distribution and potential sources of organic pollutants in groundwater, surface water, sediment and biota at hotspots that require immediate attention of authorities.
iv) determine the impact of anthropogenic processes such as the manufacture and use of pharmaceuticals, agricultural use of herbicides and burning of fossil fuels for energy production on the environment.
v) To determine seasonal emerging pollutant contamination trends in the Hartbeespoort Dam.

vi) highlight the utility of screening tools (bioassays and biomonitoring) and enhance the analytical and technical capability in South Africa for establishing routine analysis and monitoring of ubiquitous organic pollutants through development of rugged and reproducible analytical methods.

Outline

The main objective of this research was to develop suitable and rugged methods as well as apply existing methods for the analysis of various ubiquitous organic pollutants in South African urban waters and the ecosystem. This was in order to characterise contaminating organic pollutants as well as their distribution and enable source characterisation as well as temporal and spatial monitoring. The main research area is located in the Gauteng Province which is the most industrialised and populated region of South Africa. Ten hotspots monitored lie within the Witwatersrand catchment area (shown in red outline in Figure 1.1). The Witwatersrand is a 56 km long escarpment that stretches from east Johannesburg to west of Johannesburg. Five hotspots are located in the north of the Witwatersrand in the Jukskei River which drains water from the Witwatersrand in a northern direction. Another five hotspots are situated in the south of the Witwatersrand (Figure 1.1), in the Klip and Vaal Rivers which drain water from the Witwatersrand in a southern direction. The Jukskei River drains the northern Witwatersrand into the Crocodile River which in turn drains water into the Hartbeespoort Dam, contributing 90% of the water in the Hartbeespoort Dam (Amdany et al., 2014). The uMngeni River estuary which experienced an episodic pollution event in May 2016 is situated in the eastern coast of South Africa (Blue star in Figure 1.1 insert).

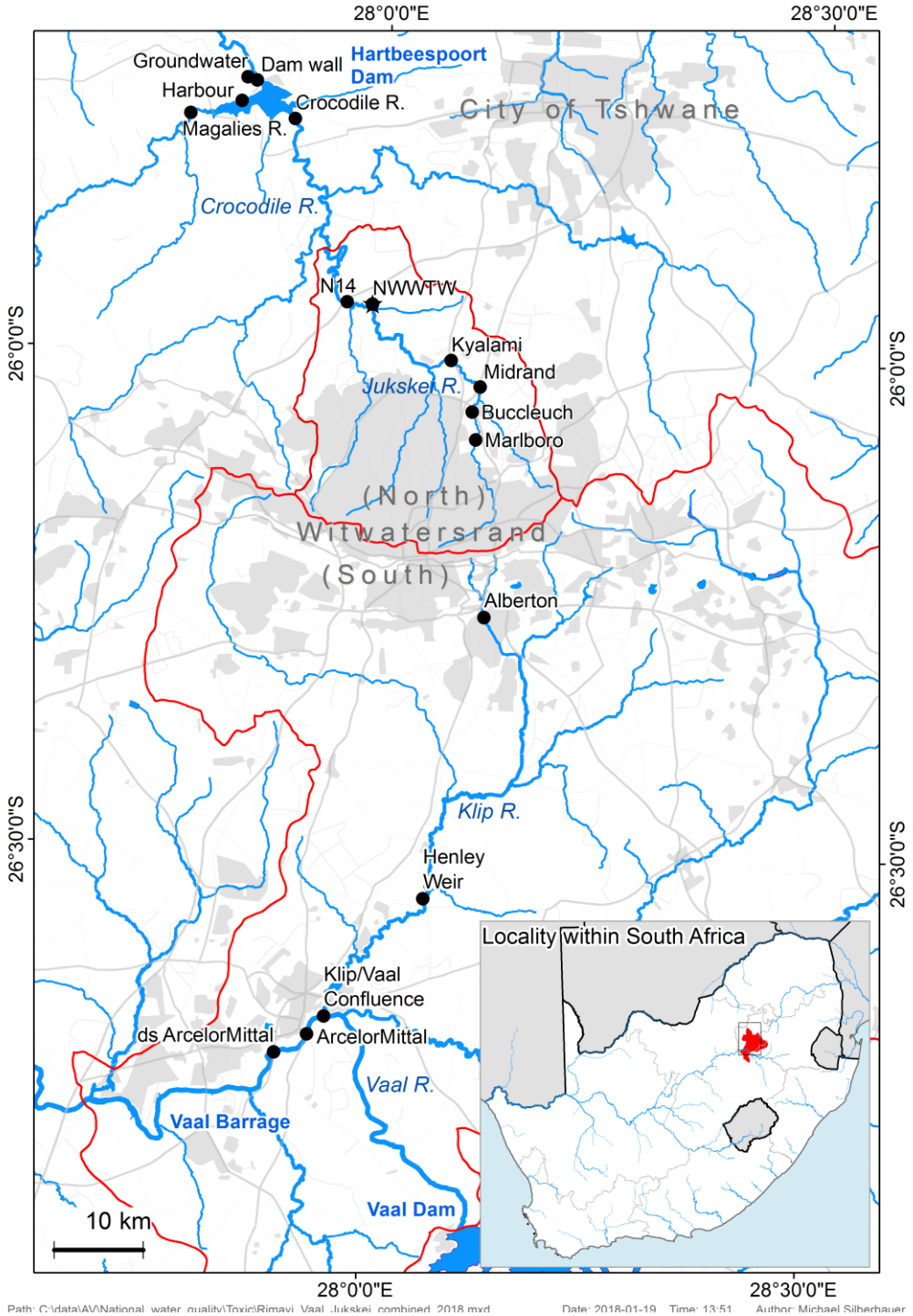

Figure 1.1 Main study area- The Hartbeespoort Dam catchment comprising of the Crocodile and Magalies Rivers as the major rivers. The Witwatersrand catchment (within the red border lines) consists of the Jukskei River catchment in the north of the Witwatersrand and the Klip and Vaal Rivers in the south of the Witwatersrand. Black dots indicate sampling points and blue star indicates the uMngeni River Estuary. Figure artwork was provided by Dr. Michael Silberbauer.

Bioassays were used to screen for dioxin-like compound activity prior to comprehensive two dimensional chromatography to detect and quantify 2,3,7,8-substituted PCDD/Fs (Chapter 2). To meet the obligations of the Stockholm Convention on implementing environmentally sound PCB waste management practices, source characterisation of PCBs in sediments was conducted with a congener-specific method (Chapter 3). Simultaneous extraction and cleanup of PAHs and alkyl PAHs was carried out for adequately determining the distribution of the most ubiquitous industrial contaminants in the South African environment (Chapter 3). Other contaminants of agricultural origin, triazine herbicides and their metabolites were analysed seasonally in the Hartbeespoort Dam catchment water (Chapter 4). Hartbeespoort Dam groundwater contamination by triazine compounds, which is a major concern in South Africa was also assessed seasonally, along with the distribution of chloro-s-triazines along the Jukskei River (in the winter season) which is the major source of water in the Hartbeespoort Dam.

The effects of environmentally relevant sub-chronic atrazine concentrations on African clawed frogs (*Xenopus laevis*) were assessed by exposing tadpoles and adult frogs in a quality controlled laboratory (Chapter 5). The data generated, along with seasonal analysis (Chapter 4) provides a substantial body of evidence that can assist South African authorities to re-assess South Africa's position on the pseudo-persistent atrazine concentrations detected in the environment. The effect of rapid urbanisation in the largest South African city of Johannesburg on surface water quality was assessed by analysis of emerging contaminants in the Jukskei River which passes through Johannesburg (Chapter 6). An episodic contamination event on 18 May 2016 in the uMngeni River estuary was also investigated in Chapter 6 with the analysis of emerging pollutants in water and sediment samples. Contamination of the urban rivers, particularly from raw human waste as evidenced by significant quantities of pharmaceuticals detected, is a major problem that needs urgent intervention. Chemical analysis as well as *in-vivo* biological tests on toxicological effects of atrazine (on frogs and tadpoles) and *in-vitro* (DR-Luc) bioassays on dioxin-like compounds were meant to provide an overview of the extent of pollution and its effects on biological organisms and systems. It is expected that this study will provide relevant information to water resource managers and policy makers in the water and environment sector. A schematic diagram of the conceptual framework of the study for characterisation of persistent and pseudo-persistent organic pollutants for generation of data for establishment of investigative and operational monitoring is shown in Figure 1.2.

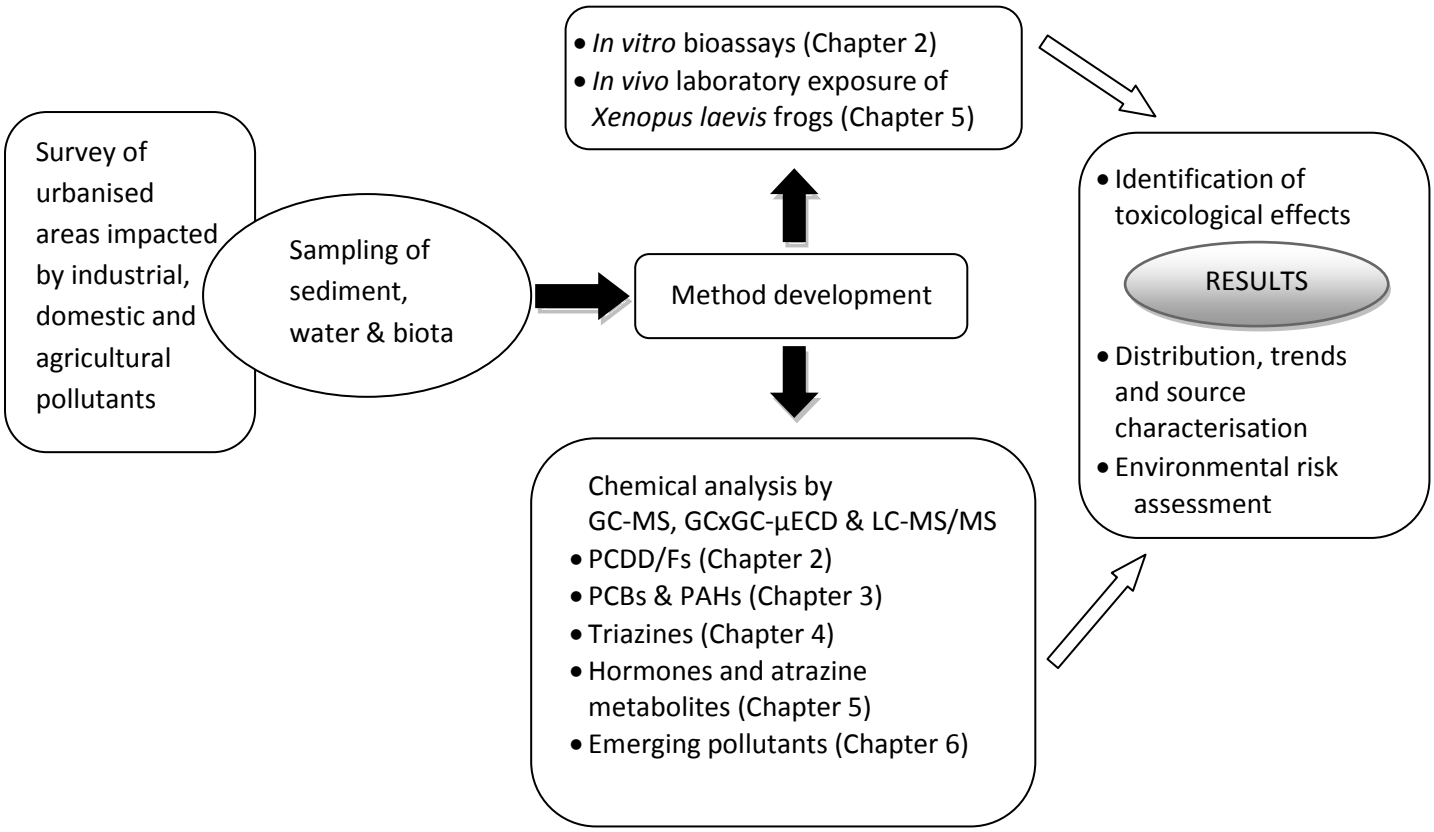

Figure 1.2 Flow chart showing conceptual framework of the study

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Chapter 2

Distribution of 2,3,7,8-substituted poly chlorinated dibenzo-*p*-dioxin and polychlorinated dibenzofuran distribution in the Jukskei and Klip/Vaal catchment areas in South Africa.

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Abstract

Comprehensive two dimensional gas chromatography (GCxGC)-μECD analysis was used to determine the distribution of 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxin (PCDD) and polychlorinated dibenzofuran (PCDF) distribution in the Jukskei and Klip/Vaal catchment areas from ten sites previously identified as persistent organic pollutant hotspots in major rivers in the Gauteng province of South Africa. Five sediment samples from the Jukskei River catchment area and five sediment samples from the Kilp/Vaal River catchment area were collected for analysis. The extracts were screened for dioxin-like activity using the DR-Luc bioassay prior to GCxGC-μECD analysis. All sediment samples tested positive for dioxin-like activity with total activity ranging from 16 to 37 pg toxic equivalents (TEQ) g^{-1} dry weight (dw) for the Jukskei River catchment and 1.5 to 22 pg TEQ g^{-1} dw for the Klip/Vaal River catchment, indicating that the Jukskei River catchment area had higher concentrations of total dioxin-like compounds. Confirmatory tests for the presence of the most potent seven PCDDs and ten PCDFs conducted using GCxGC-μECD revealed presence of 11 PCDD/Fs and 6 PCDD/Fs in the Jukskei and Klip/Vaal River catchments respectively. Total organic carbon (TOC) and particle size distribution analysis were conducted to understand the distribution of PCDD/Fs within the Jukskei and Klip/Vaal catchments.

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are a group of ubiquitous persistent organic pollutants (POPs) primarily of anthropogenic origin. PCDD/Fs are produced unintentionally through processes that include municipal solid waste incineration, metallurgical processing, coal fired power generation, chlorine based bleaching, pulp and paper manufacturing, manufacture of some pesticides such as trichlorophenols and pentachlorophenol, as well as natural events such as volcanic activity (de Mul et al., 2008; Hurst et al., 2004; Kim et al., 2005; Schecter et al., 2006). Together with a group of 12 non-*ortho* and mono-*ortho* polychlorinated biphenyls (PCBs), they are termed dioxin-like (dl) compounds (Hurst et al., 2004). These compounds tend to accumulate in organic rich sediment and soil and are widely spread in the environment as a result of long range transport from their point of production/release (Croes et al., 2011; Du et al., 2011). Many of the dl-compounds remain in sediment, but via the equilibrium between sediment, water and fish lipids, some will ultimately enter the food chain from where they may biomagnify (Hoh, et al., 2007; Hurst et al., 2004), causing species- and tissue-specific toxic effects on the immune, nervous, reproductive, and endocrine systems in fish, humans, birds and mammals (Addeck et al., 2014; Denison et al., 2004).

Environmental spatial studies on the presence of PCDD/Fs in South African rivers are extremely rare, with once-off studies having been conducted by the Department of Water and Sanitation from sites in rivers in Gauteng, North West and Mpumalanga Provinces (Jooste et al., 2008) and three others by Vosloo and Bouwman (2005), Nieuwoudt et al. (2009) and Roos et al. (2011), concluding that detectable levels of PCDD/Fs are present in South Africa. The present study contributes with novel data on PCDD/Fs in the Jukskei River as well as in a previously studied catchment of the Klip and Vaal Rivers in the Gauteng province, two areas previously indicated as hotspots for POP contamination (Jooste et al., 2008).

PCDD/F concentrations in aquatic sediments are normally low but are potent at those low levels (Hurst et al., 2004). Therefore PCDDs and PCDFs need to be measured at low concentrations (pg-fg range). As they are present in complex matrices, they need very efficient sample pre-treatment and cleanup and that makes their analysis very challenging (Focant et al., 2005; Danielsson et al., 2005). Due to its high mass accuracy, congener specificity, selectivity and sensitivity the globally accepted reference method for estimation of individual dl-congeners is gas chromatography combined with high resolution mass spectrometry (GC-HRMS) (Danielsson et al., 2005; Schroijen, et al., 2004). However, this technique is laborious, time consuming and instrumentation is very expensive. Hence, it is difficult to swiftly analyse large numbers of samples (Denison et al., 2004; Du et al., 2011). Most analytical laboratories in South Africa have no access to GC-HRMS (de Vos et al., 2013).

An alternative to GC-HRMS is the application of *in vitro* bioassays, which can measure the total dl-potency of a sample, including 17 PCDD/Fs, 12 dl-PCBs, their brominated and mixed chloro/bromo substituted analogues as well as polybrominated diphenyl ethers (Dabrowska et al., 2010). Dl-compounds are present in environmental samples as complex mixtures in varying concentrations. Hence, the total toxicity of a particular sample is the result of the sum of the concentrations of the individual congeners multiplied by their corresponding toxic equivalency factor (TEF) values, and is expressed as toxic equivalency quotient (TEQ) (van den Berg et al., 1998). The dioxin-receptor luciferase (DR-Luc) bioassay, also called DR-CALUX® is the most widely used bioassay and has been described as the best method for screening PCDD/Fs and dioxin-like compounds (Hoogenboom et al., 2006; Pieke et al., 2013;) as it offers lower detection limits and much faster analysis time than other bioassays such as ethoxyresosufin-O-deethylase (EROD) assay (Murk et al., 1996. Zhang et al., 2002). It is based on manipulation of the AhR signal transduction pathway, with the agonist binding to AhR and ultimately mimicking cytochrome P450 monooxygenase (CYP1A1) gene expression (Dabrowska et al., 2010; Denison et al., 2004; van Wouwe et al., 2004). Bioassays are extremely

useful, particularly when dealing with incidents where rapid screening is required to eliminate non-positives prior to conducting confirmatory chemical analysis (Behnish et al., 2001).

An alternative chemical analytical approach to measure PCDD/Fs in complex matrices is the use of comprehensive two-dimensional GC (GCxGC) with micro-electron capture detection (µECD) instrumentation. This two-dimensional analytical technique provides a formidable sensitivity, although with a narrow linear calibration range (Danielsson et al., 2005). GCxGC analysis allows separation of the dl-compounds from the co-eluting matrix components (Hoh et al., 2007). However, due to the presence of high matrix interference, an extensive cleanup and/or fractionation step is also required, which makes GCxGC-µECD also a time and labour-intensive method (Adahchour et al., 2008; Molina et al., 2000). Nevertheless, the two-dimensional separation technique provides extensive information about the composition of the analysed sample.

The aim of this study was to determine the spatial distribution of PCDDs and PCDFs in hotspots along two major rivers passing through industrial areas in Gauteng Province, the most industrialised region in sub-Saharan Africa. The DR-Luc bioassay was selected as a screening method to include all acid stable dl-compounds to evaluate the full dl-potency of the sediment extracts. GCxGC-µECD analysis confirmed the presence and distribution of PCDD/Fs in the Jukskei and Klip/Vaal catchments. In addition, total organic carbon and particle size distribution were measured to investigate if they influenced the distribution of the PCDD/Fs.

Materials and methods

Sampling and sample site description

In order to ensure representivity, sampling was conducted by random grab sampling either over a 5 m radius in the river bed or over a 5 m length along the river bank where the river bed had a depth >1 m, particularly in the Vaal River. The details of the sampling sites are given in Table 2.1 and are illustrated in Figure 2.1 with the map of the Jukskei and Klip/Vaal Rivers. The Jukskei, Klip and Vaal Rivers are sometimes prone to seasonal flooding into the flood plain in the summer season from February to March.

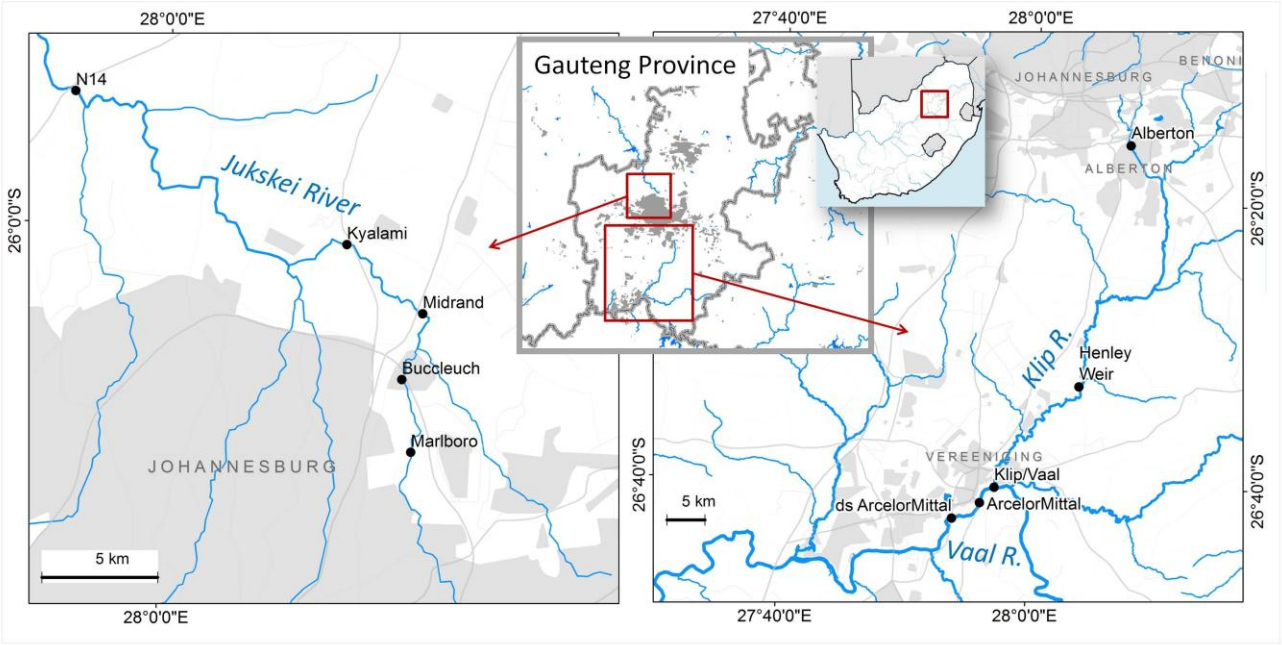

Figure 2.1 Sampling sites in the Jukskei and Klip/Vaal catchments: Gauteng Province, South Africa

Jukskei catchment

The Jukskei River catchment area in the Gauteng Province, South Africa, covers an area of 800 km^2 and is situated in the most industrialised region in southern Africa (Pitman, 1978). The Jukskei River flows in a north-western direction and passes within 7 km of the coal fired Kelvin power station, which has been operational since 1957. Many of the numerous small hospital waste incinerators within the Jukskei River catchment area are outdated and fall far short of modern emission standards, with PCDD/F emissions 2 to 40 times higher than the South African regulated limits (Rogers and Brent, 2006). A situation caused in part by the widely considered poor old legislative instruments, particularly the Atmospheric Pollution Prevention Act (Act 45 of 1965) which lead to high urban industrial pollution in South Africa from 1960 to the late 1990s (Naiker et al., 2012). The sampling site names and coordinates for the Jukskei River catchment sampling sites are listed in Table 2.1.

Klip/Vaal Catchment

The Klip/Vaal catchment lies mainly in the Gauteng Province, South Africa and stretches from the southern Johannesburg town of Alberton to Sasolburg flowing in a southern direction. It is 61 km long between selected sampling points and is in the "steel capital" of South Africa owing to the wide array of iron processing, steel mills and speciality steel production plants in the region. It is therefore considered an identified air pollution hotspot (Naiker et al., 2012). The Klip/Vaal catchment is situated in the midst of heavy industrial activity, a coal mine (Anglo American Thermal Coal) and a large number of potential PCDD/F emitters including ArcelorMittal (the largest inland iron smelting company in sub-Saharan Africa), a number of cement manufacturing plants (e.g. Afrisam), dozens of steel processing plants, a coal fired power station (Lethabo), an oil refinery company (Natref), a pulp and paper plant (Nampak), and a Fischer-Tropsch liquid petroleum production company (Sasol) (Nieuwoudt, et al., 2009). The Alberton site is situated in the central business district of the industrial town of Alberton which is located adjacent to old gold and other mineral mining sites. The Alberton site is located upstream of the Klip/Vaal catchment and has been identified as a dl-compound hotspot by Jooste et al. (2008) owing to the numerous mining equipment manufacturing industrial activities in the town. The names and coordinates of the Klip/Vaal River sampling sites are listed in Table 2.1.

Table 2.1 Jukskei and Klip/Vaal River catchment sampling sites and GPS coordinates

Sampling site name	Catchment	Latitude (South)	Longitude (East)
Marlboro	Jukskei	-26.08494	28.10881
Buccleuch	Jukskei	-26.05700	28.10400
Midrand	Jukskei	-26.03139	28.11222
Kyalami	Jukskei	-26.00560	28.07836
N14	Jukskei	-25.94933	27.95878
Alberton	Klip	-26.26393	28.12418
Henley Weir	Klip	-26.54939	28.06443
Klip/Vaal	Klip	-26.67055	27.95527
ArcelorMittal	Vaal	-26.68916	27.93638
*ds ArcelorMittal	Vaal	-26.70829	27.89960

*ds = downstream

Materials

The DR-Luc bioassay requires H4IIE line murine hepatoma cells stably transformed with pGudluc1.1 plasmid (UC Davis, California, USA), minimum essential medium alpha (MEM) (Life Technologies, Bleiswijk, The Netherlands) supplemented with 10% foetal calf serum (FCS), (Life Technologies, Bleiswijk, The Netherlands), penicillin/streptomycin, dimethylsulphoxide, luciferin, phosphate buffered saline (PBS) solution, trypsin, lysis buffer solution, glow mix solution (20mM trycin, 1.07 mM magnesium hydroxide carbonate pentahydrate, 2.67 mM magnesium sulfate, 0.1 mM ethelenedicaminetetraacetic acid, 33.3 mM dithiothreitol, 270 µM co-enzym A, 470 µM luciferin, Sigma-Aldrich, Missouri, USA), 2,3,7,8-TCDD standard (Wellington laboratories, Guelph, Ontario, Canada), 48 well tissue culture plates and 98 well tissue culture plates.

For GCxGC analysis, a mixture of 7 PCDDs and 10 PCDFs (Cambridge Isotope laboratory, Andover, USA) with a purity of ≥98% was used. Acetone, hexane, dichloromethane (DCM), toluene and iso-octane were purchased from J. T. Baker (Deventer, The Netherlands) with a purity of >99.7%. Analytical grade sulphuric acid (97%) (Merck, Darmstadt, Germany) and 3 mL, 250 mg sorbent bed ENVI-Carb SPE cartridges (Sigma-Aldrich, Bellefonte, USA) were used for clean up.

Sample preparation and extraction

Aquatic sediment samples were collected in August 2013 (Spring season) from five sites in the Jukskei River catchment and five sites in the Klip/Vaal River catchment area (Figure 2.1). Jooste et al. (2008) identified the selected sites as hotspots for POPs. The samples were frozen and transported to The Netherlands for sample extraction according to US-EPA method 3545A. The samples were freeze dried, granulated using a porcelain mortar and pestle, sieved through a 2.5 mm mesh sieve and extracted using Accelerated Solvent Extraction (ASE), (Dionex ASE 350) with two cycles using solvents hexane: acetone (3:1 v/v) and extraction conditions: temperature 100°C, pressure 10.3 MPa, oven heat time: 5 min, static time: 5 min

DR-Luc bioassay analysis

The DR-Luc bioassay has been described in detail by Dabrowska et al. (2010) and Olivares et al. (2013). ASE extracts were cleaned up using a silica/sulphuric acid column packed from the bottom with 5 g 33% sulphuric acid silica, 5 g 20% sulphuric acid silica and a 1 cm sodium sulphate layer at the top. The supernatant was evaporated to near dryness using a gentle stream of nitrogen and 20 µL DMSO was added. 100 µL H4IIE.Luc cell suspension in α-MEM was seeded into 96 well plates with

the outer wells filled with 200 µL PBS solution and incubated for 24 hours at 37 ^{o}C in a 5% CO_2 environment. After 24 h incubation the cell layer was 80-90% confluent, and 100 µL of fresh culture medium containing the 2,3,7,8-TCDD calibration standards (concentrations 0, 0.3, 1, 3, 10, 30, 100 and 300 pM 2,3,7,8-TCDD) and sample extracts in maximal 0.4% DMSO was added to each well, all in triplicate.

After a further 24 h incubation, each well was checked for cytotoxicity by observing the shape of the cells under a microscope. Medium from each well was discarded and 50 mL lysis buffer (10 mM Tris, 2 mM dithiothreitol (DTT), 2 mM 1,2,-dia-minocyclohexane-N,N,N0,N0-tetra-acetic acid, 10% glycerol, 1% Triton-X100, pH 7.8) was added to each well and plates were shaken at 700 rpm at a temperature of ≤25°C on a plate shaker for 10 min. For luciferase measurement, the microtiter plate was inserted into the luminometer. 100 µL glow mix containing the substrate luciferin for emission of bioluminescence was added to each sample and the measurement reported in relative light units (RLU). Graphpad Prism 5 software was used to compute sigmoid shaped dose-response calibration curves using responses from the luminometer for quantification of samples.

Sample cleanup and fractionation for chemical analysis

ASE extracts were evaporated to near dryness with nitrogen before adding 1 mL hexane. 1 mL sulphuric acid was added to the extract and the mixture was vortexed before being left for 5 min to settle. The organic upper layer was removed, washed with Milli-Q water and evaporated with nitrogen gas. The solvent was changed to DCM before fractionation using gel permeation chromatography (GPC) according to the method specified by Weiss et al. (2009). The GPC was equipped with two polystyrene-divinylbenzene columns in series (10 µm, 50 Å, 600 x 25 mm, Polymer Laboratories) to collect the fraction containing PCDD/Fs and PCBs which eluted between 16.5 and 24 min and discard the sulphur containing fraction which eluted between 25 and 29 min.

The isolation and cleanup of PCDD/Fs was done using the Supelco ENVI-Carb solid phase extraction cartridge using the techniques specified by Danielsson et al. (2005) and Molina et al. (2000). 15 mL hexane was used to elute and remove di-, tri- and tetra-*ortho* PCBs, followed by 20 mL hexane: toluene (99:1 v/v) to remove mono-*ortho* PCBs, then 20 mL toluene to remove the more toxic non-*ortho* PCBs. The cartridge was inverted to elute and collect the PCDD/F fraction using 80 mL toluene in four portions of 20 mL. The extracts were evaporated to approximately 1 mL using a Buchi Syncore (Flawil, Switzerland) evaporation system and pooled together before adding 100 µL internal

standards (^{13} C-labelled mirex, 100 mg L^{-1}) and further reducing to approximately 15 µL, with gravimetric volume correction.

Chemical analysis

A GCxGC system with a µECD detector (Agilent Technologies 7890, Palo Alto, USA), with a data acquisition rate of 50 Hz, utilising a flow switch modulator was used with 99.999% helium as carrier gas and 99.999% nitrogen as make up gas with a flow of 120 mL min^{-1}. The first dimension column was an SGE BPX-5 (5% phenyl, 95% methyl polysiloxane, SGE Analytical Science, Melbourne, Australia, dimensions: 15 m x 0.22 mm x 0.25 µm) with a flow rate of 4 mL min^{-1}. The second dimension column was an HP-INNOWax (polyethylene glycol, Agilent Technologies J&W, Amstelveen, The Netherlands, dimensions: 3 m x 0.25 mm x 0.1 µm) with a flow rate of 30 mL min^{-1}. The GC inlet with a 1 µL injection volume was programmed at a temperature of 280 $^{\circ}$C in pulsed splitless mode, pulsed at 310 kPa for 1.5 min, split 50 mL min^{-1} at 1.4 min. GC oven temperature programme was: 90 $^{\circ}$C (3 min), 30 $^{\circ}$C min^{-1} to 170 $^{\circ}$C (5 min), 5 $^{\circ}$C min^{-1} to 270 (45 min). Modulator settings were set up with a modulation delay of 5 min, a modulation period of 4 sec and sample time 3.9 sec. The µECD detector temperature was 270 $^{\circ}$C.

GCxGC analysis was carried out using GC image software (Zoex, Lincoln, USA). Calibration standards were made up to concentrations 62.5, 25, 12.5, 6.25 and 3.313 ng mL^{-1}. A volume of 500 µL standards were blown down using a gentle stream of nitrogen to 50 µL to give final gravimetrically corrected concentrations of 535, 200, 150, 120 and 35 ng mL^{-1}. Internal standard calibration curves were made up with ^{13}C-mirex as an internal standard, using Microsoft Excel with linear regression (R^2) models >0.99 for all curves.

Particle size distribution

Air dried and homogenized 5 g sediment samples were further dried in an oven for 3 hours at 105 $^{\circ}$C. The sediment samples were cooled in a desiccator before re-weighing then further dried at 1 h intervals in the oven at 105 $^{\circ}$C until there was no further weight loss. The dried sediments were sieved with mesh sizes 1000, 500 and 125 µm. The sieved portions were collected and weighed.

Total organic carbon

Total organic carbon (TOC) analysis was conducted using modified Walkley-Black method as it offers a distinct advantage of measuring the oxidisable organic carbon in the sediment samples with a high degree of digestion and oxidation of organic matter through the application of external heating

during digestion, without the oxidation of inorganic carbon (Abraham, 2013; Gelman et al., 2012; Schumacher, 2002). 8 mL of 1 N $K_2Cr_2O_7$ (Minema, Johannesburg, South Africa) was added to 1 g air dried and sieved sediment sample. The mixture was vortexed in a test tube before adding 2 mL concentrated H_2SO_4 and transferring test tube to a block digester to oxidise the organic carbon in the sediment. The samples were digested for 30 minutes at 150 $^{\circ}$C. After cooling, the digestate was transferred to a conical flask, and rinsed with 25 mL deionised water before adding 2 mL H_3PO_4 to eliminate interfering Fe^{3+} ions. 8 drops of 0.2% barium diphenylamine-4-sulfonate (Sigma-Aldrich, Missouri, USA) colour indicator solution was added before back titrating the residual $K_2Cr_2O_7$ to a green endpoint with 1 M ferrous ammonium sulphate ($Fe(NH_4)_2(SO_4)_2*6H_2O$) solution (BDH Merck Limited, Poole, England).

Results and discussion

DR-Luc analysis of sediment samples from five sites from the Jukskei River and five sites from the Klip/Vaal catchment area were analysed in triplicate over three weeks to ensure accurate quality controlled results were produced. The averages of the triplicate analyses are given in Table 2.2. GCxGC-µECD instrumental limits of quantification (LOQs) for PCDD/Fs varied between the lowest value of 0.13 pg g^{-1} for 2,3,7,8-tetrachlorinated dibenzofuran to 5.2 pg g^{-1} for OCDD (Table 2.2). The average LOQ value (based on 10x signal to noise ratio) for DR-Luc analyses was 0.72 pg TEQ g^{-1} (Table 2.2). No PCDD/Fs were detected in the solvent blanks.

Table 2.2 Concentrations (pg g-1 dry weight) of PCDDs and PCDFs in sediment from the Jukskei and Klip/Vaal Rivers determined by GCxGC-µECD and DR-Luc bioassay TEQs (EC50) in corresponding samples

				Jukskei River catchment					Klip/Vaal River catchment				
PCDD/F	2005 WHO-TEF	GCxGC-µECD Recovery % (n=2)	GCxGC-µECD LOQ	Marlboro	Buccleuch	Midrand	Kyalami	N14	Alberton	Henley weir	Klip/Vaal	Arcelor-Mittal	ds Arcelor-Mittal
2,3,7,8-TCDD	1	99	0.2	1.6	1.2	2.5	1.3	n.d	5.7	n.d	0.8	n.d	n.d
1,2,3,7,8-PeCDD	1	161	0.7	2.9	2.3	n.d	2.8	<0.7	n.d	3.6	3.1	<0.7	4.8
1,2,3,4,7,8-HxCDD	0.1	75	2.5	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
1,2,3,6,7,8-HxCDD	0.1	113	2.5	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
1,2,3,7,8,9-HxCDD	0.1	77	3.1	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
1,2,3,4,6,7,8-HpCDD	0.1	69	1.0	n.d	<1.00	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
OCDD	0.0003	16	5.2	<5.2	<5.2	n.d	<5.2	n.d	n.d	<5.2	n.d	n.d	n.d
2,3,7,8-TCDF	0.1	120	0.1	n.d	<0.1	n.d	n.d	0.8	1.6	n.d	<0.1	1.0	2.1
1,2,3,7,8-PeCDF	0.03	175	1.1	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
2,3,4,7,8-PeCDF	0.3	88	1.3	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
1,2,3,4,7,8-HxCDF	0.1	85	3.4	n.d	<3.4	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
1,2,3,6,7,8-HxCDF	0.1	91	3.4	<3.4	<3.4	<3.4	n.d	n.d	n.d	n.d	n.d	n.d	n.d
1,2,3,7,8,9-HxCDF	0.1	69	3.2	n.d	<3.2	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
2,3,4,6,7,8-HxCDF	0.1	63	2.0	n.d	n.d	n.d	n.d	3.6	5.2	n.d	n.d	n.d	n.d
1,2,3,4,6,7,8-HpCDF	0.01	85	1.9	n.d	<1.9	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
1,2,3,4,7,8,9-HpCDF	0.01	34	0.7	n.d	<0.7	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
OCDF	0.0003	38	5.2	<5.2	<5.2	<5.2	n.d	n.d	<5.2	<5.2	n.d	n.d	n.d
GCxGC-µECD PCDD/F pg TEQ g^{-1}				4.5	3.5	2.5	4.1	4.4	12.5	3.6	3.9	1.0	6.9
DR-Luc pg TEQ g^{-1} (EC50)				24.0	28.6	18.2	15.7	37.4	21.6	3.9	4.3	1.5	7.9
TOC (%)				0.64	0.64	0.58	0.58	0.60	0.57	0.52	0.63	0.35	0.22

< = below specified LOQ

n.d =not detected

2005 WHO TEF's (van den Berg et al., 2006)

The dl responses measured by the DR-Luc in the Juksei River sediments are 4-9-fold higher than the calculated dl-responses based on GCXGC-µECD (Figure 2.2). This is may be explained by the possible presence of other dl-compounds such as PCBs, PBDD/Fs and their brominated and mixed chloro/bromo analogues, which were not included in the target chemical analysis. Also, some of the PCDD/Fs that were detected below the LOQ of the GCxGC method would otherwise have contributed to the concentrations measured by GCxGC, but this would not have made up for the differences found. The results show that the N14 site, which is downstream of the Jukskei River catchment, had the highest dl-potency in the Jukskei river catchment. In contrast with the Jukskei sediment results, the dl-potency values found by the DR-Luc in the Klip/Vaal catchment did not differ substantially from those calculated by the GCxGC data (Figure 2.3). This suggests that PCDD/Fs have a significantly higher contribution to the DR-Luc TEQ in the Klip/Vaal catchment, accounting for up to 67-92% of the DR-Luc TEQ for the 4 sites downstream of the Alberton site. Sediments from Alberton point, which is upstream of the Klip/Vaal River catchment, had the highest dl-potency in the Klip/Vaal River catchment. Additional target analysis is needed to determine the cause of the difference in dl-potency at Alberton and the Jukskei catchment area.

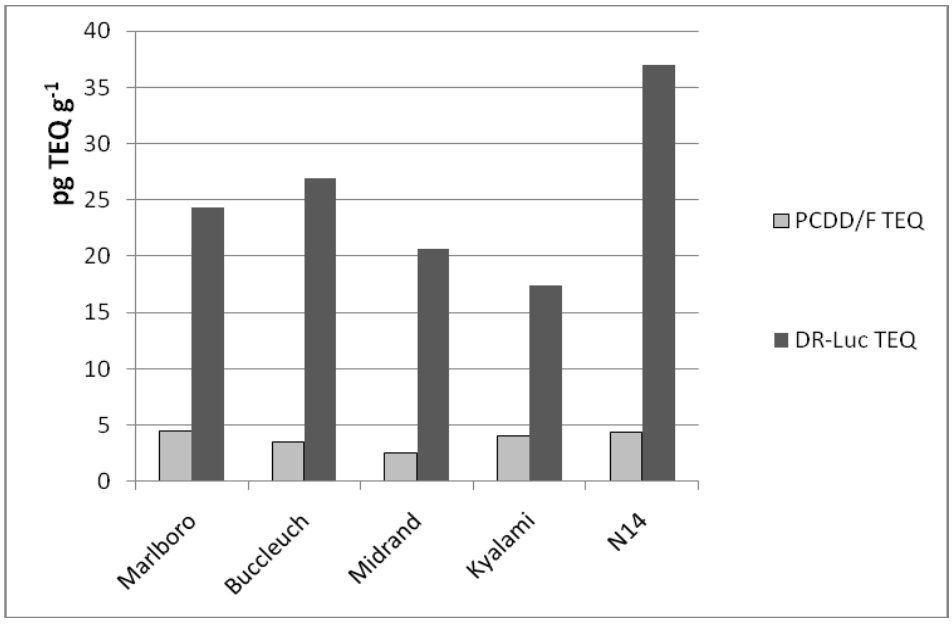

Figure 2.2 DR-Luc TEQ and PCDD/F TEQ (pg TEQ g-1dw) for the Jukskei River catchment sediment samples

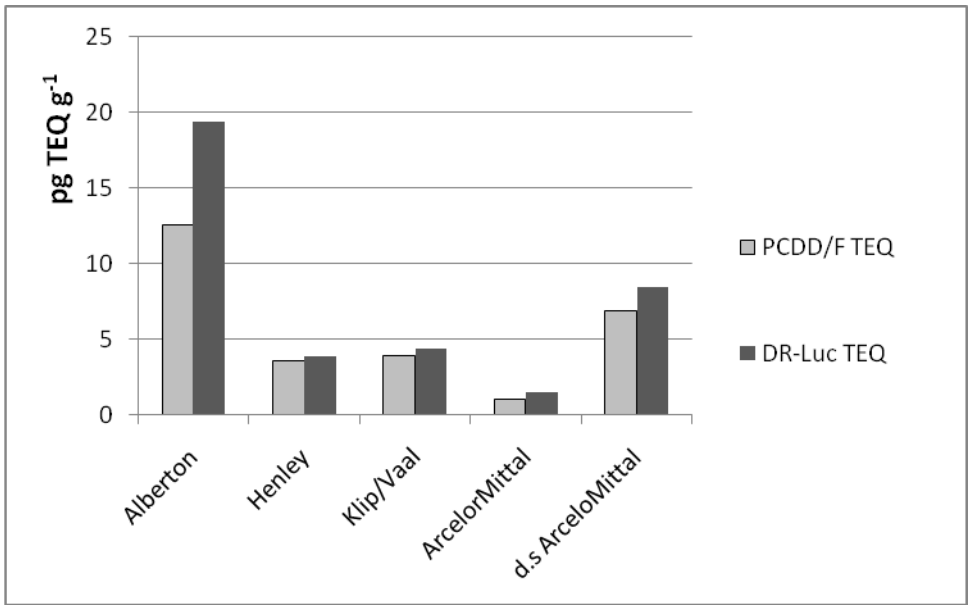

Figure 2.3 DR-Luc TEQ and PCDD/F TEQ (pg TEQ g-1dw) for the Klip/Vaal River catchment sediment samples

TOC and sediment particle size distribution

TOC levels were very low, with all samples recording values <1% for both the Jukskei and Klip/Vaal River catchments (Figure 2.4). In such low concentrations, uncertainties in TOC normalisation are generally substantial and their value for normalisation becomes insignificant as there is in fact too little carbon available for adsorption and the equilibrium between organic carbon and water is disturbed (Koelmans et al., 1997). Particle size distribution has an effect on the distribution of POPs in sediments, which in fact similar to the effect of the organic carbon content. Larger, coarse grained sediments typically contain less organic carbon than finer grained sediments such as silt clays (Poppe et al., 2000) and organic carbon fractions may be so small that sorption dominates partitioning (Koelmans et al., 1997). The sediment samples were sieved into three different sizes, that is 500-1000 µm which consisted of coarse sand, 125-500 µm which consisted of fine sand and <125 µm which consisted of silt and clay. The particle size distribution in both the Jukskei and Klip/Vaal River sediments is largely heterogeneous for the different sampling sites (Figure 2.4). The ds ArcelorMittal site notably had significant quantities of black coloured coal ash dust mainly 125-500 µm particle size.

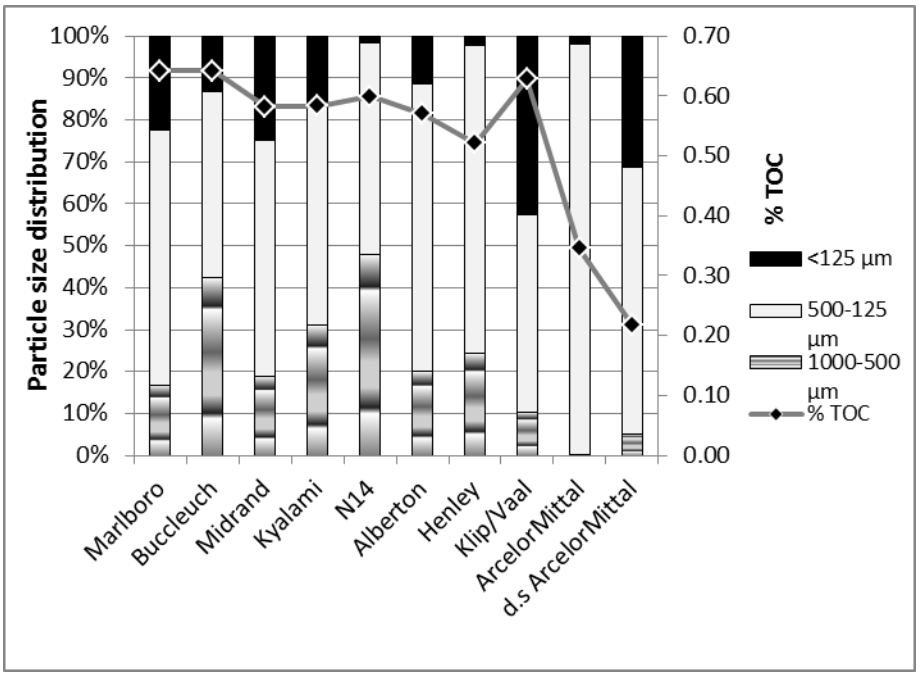

Figure 2.4 Comparison of particle size distribution (%) and TOC (%) of the Jukskei River and Klip/Vaal River catchment sediment samples

The Klip/Vaal catchment sediment samples, with the exception of the ds ArcelorMittal sediments had an inverse relationship between TOC content and sediment particle size, as found by Poppe et al. (2000) in Long Island Sound. The differences in TOC and proportion of fine particles in the Jukskei River catchment samples are small. However, The N14 seems to show a deviating and inconsistent TOC/particle size ratio. The high quantity of sandy particles in the Klip/Vaal sediment may explain why both GCXGC-µECD PCDD/F and DR- Luc TEQs values for the ArcelorMittal site are low, even though it is located next to a large steel manufacturing plant.

PCDD/F and DR-Luc TEQ distribution

GCxGC-µECD analysis shows that 1,2,3,7,8-PeCDD and 2,3,7,8-TCDD were the most abundant PCDDs quantified above the LOQ. 1,2,3,7,8-PeCDD was present at eight sites and 2,3,7,8-TCDD at six sites. As a result of high detection limits of OCDF and OCDD coupled with poor recoveries (38% for OCDF and 16% for OCDD), quantification results were not reported. This is consistent with trace level recoveries obtained by Hoh et al. (2008) for cod liver samples spiked at 25 pg g^{-1} concentration determined to be below the limit of quantification (LOQ). Low instrument responses and high LOQs for OCDF and OCDD are consistent with previous studies by Korytar et al., (2004) and Hoh et al. (2008). The highest

concentration of 2,3,7,8-TCDD in the Jukskei River was at Midrand, 2.5 pg g^{-1}, compared with 1.2 pg g^{-1} at the nearby upstream point of Buccleuch and 1.6 pg g^{-1} at Marlboro. These three sites are located in the most industrialised region of Gauteng. The highest concentration of 2,3,7,8-TCDD in the Klip/Vaal catchment, 5.7 pg g^{-1}, was at the Alberton site on Klip River. DR-Luc results show that the Alberton site had the highest TEQ value in the Klip/Vaal catchment, 22 pg TEQ g^{-1}. The results are in accordance with the study of Jooste et al. (2008), where the Alberton sediment had the highest TEQ concentration of 0.8 pg TEQ g^{-1}, with a similar trend of decreasing concentrations downstream. Other PCDD/Fs were sparsely distributed within the Jukskei and Klip/Vaal River catchment, with 11 of the 17 PCDD/Fs tested being detected in Jukskei River catchment and only 6 of the 17 PCDD/Fs detected in the Klip/Vaal catchment.

DR-Luc TEQ results for the Jukskei River sediments were comparable with DR-CALUX TEQs from sediments from various sites in United Kingdom estuaries, which averaged 23.8 pg TEQ g^{-1} for 35 sites tested (Hurst et al., 2004), with DR-Luc TEQ results from the Klip/Vaal River in this study being only slightly lower. Historical comparison of the sediments sampled on the same sites in the Klip/Vaal catchment in February 2006 in the study by Jooste et al., 2008 and this study indicates that the concentration of PCDD/Fs has increased approximately 12-fold in five years. The increase was expected due to the fact that before 1 April 2015, air pollution control devices were not compulsory in South Africa hence hot combustion gases could be emitted directly into the atmosphere (Brent and Rogers, 2002). On 22 November 2013 new minimum Emission Standards for activities listed in section 21 of the National Environment Management: Air Quality Act (Act 39 of 2004), identified as major PCDD/F emitters were promulgated for regulating municipal solid waste incineration, hospital waste incineration, sewage and sludge incinerators, crematoriums, pulp and paper mills, metal smelting, cement kilns and coal fired power stations. The lack of air pollution monitoring devices in South Africa made it virtually impossible to accurately estimate the major sources of PCDD/Fs to the environment.

Due to the lack of similar studies on surface sediments in South Africa, comparisons can only be done with other geographical regions world-wide. In Africa, only Lake Victoria which is surrounded by Uganda, Rwanda, Kenya and Tanzania has been analysed. The PCDD/F concentrations detected in this study were, higher than those detected in Lake Victoria (Ssebugere et al., 2013). The Jukskei and Klip/Vaal River PCDD/F concentrations are generally lower than those reported for the period 1993 to 2003 in the Baltic sea region sediments (Verta et al., 2007), and those found in the lakes, shores and tributaries of the Great Lakes region in North America by up to 3 or 4 orders of magnitude (Shen et al., 2009).

Generally, the Jukskei and Klip River sediments have lower PCDD/F TEQs than the upper Detroit and lower Rougue Rivers in the USA and concentration ranges are comparable to those found in the East River in China (Table 2.3).

Table 2.3 Comparison of PCDD/F TEQ determination in sediments from other parts of the world

Site	Year sampled	PCDD/F TEQ concentration ranges	PCDD/F congener LOQ range pg g^{-1}
[a]Jukskei River, South Africa	2013	2.5-4.5 (WHO_{2005})	0.13-5.2
[a]Kilp/Vaal River, South Africa	2013	1-12.5 (WHO_{2005})	0.13-5.2
[b]Lake Victoria, Tanzania/Uganda/Kenya	2011	0.07-5.53 (WHO98)	0.01-0.33
[c]Upper Detroit River, USA	1998	3.14-62.1 (WHO_{98})	Not specified
[c]Lower Rougue River, USA	1998	12.3-25.8 (WHO_{98})	Not specified
[d]East River, China	2007	2.1-8.1 (WHO_{98}); 2.4-8.1 (WHO_{2005})	0.05-0.8

[a]This study; [b]Ssebugere et al., (2013); [c]Kannan et al., (2001); [d]Ren et al., (2009)

Conclusion and recommendations

This study is the first to report the dioxin-like potency for sediments in the Jukskei River catchment in South Africa. The detection of 11 of the 17 PCDD/Fs analysed in the Klip/Vaal catchment explain up to 92% of the measured dl-potency. A direct comparison of the dl-potency calculated from GCxGC-µECD measured compounds and the dl-potency acquired from the bioassay DR-Luc was not possible because other persistent dl-compounds (e.g. PCBs and PAHs) were not chemically analysed in this study. However, this study demonstrates the feasibility to measure the total dl-potency for the purpose of screening for hotspots. Due to the very low TOC values found, the influence of TOC and particle size distribution on distribution and abundance of PCDD/F congeners could not sufficiently account for the trends identified. A better understanding of the complex ecological risk of the contaminated sediment samples may be achieved by improved structural and qualitative characterisation of the organic forms in sediments, particularly to determine forms of organic carbon present and the geochemical composition of the sediment particles. We recommend setting up of a screening approach using bioassays with an incidental check by GCxGC-MS for mapping the dioxin-like activity including dl-PCBs of river catchment areas in South Africa to identify hot-spots and priority sites for future remediation activities.

Acknowledgements

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Chapter 2 – Supporting Information

Figures

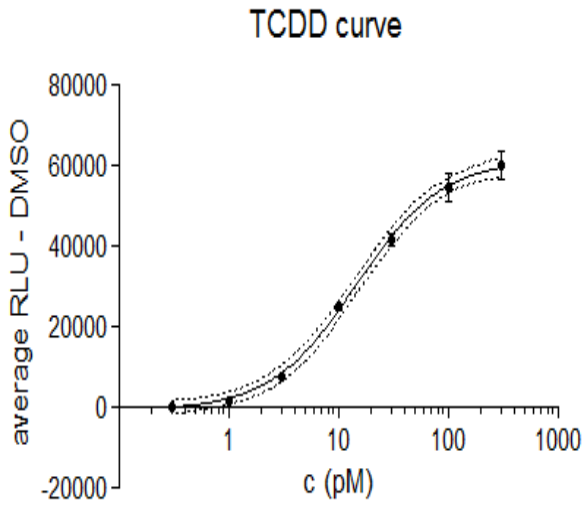

Figure S2.1 Dose-response curve for showing RLU versus 2,3,7,8-TCDD concentration (n=3)

Chapter 3

Source characterisation and distribution of selected PCBs, PAHs and alkyl PAHs in sediments from the Klip and Jukskei Rivers, South Africa

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Abstract

A study of the distribution of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) utilising 16 priority PAHs, benzo(e)pyrene, perylene, 19 alkylated PAHs and 31 *ortho* substituted PCBs in South Africa is presented. It was aimed to: (a) deduce characteristic contamination patterns for both PCBs and PAHs and (b) provide the first comprehensive dataset for establishment of source characterisation of PCBs and PAHs. This is in line with new South African legislation on mandatory monitoring of PCB and PAH emissions. Bar charts, principal component analysis (PCA) and biplots were utilised to identify signature contamination patterns and distribution of PCBs and PAHs within the Jukskei and Klip Rivers. Sediments from the Jukskei and Klip River catchments both showed distinct contamination signatures for hexa to nonachlorinated PCBs, characteristic of contamination by Aroclor 1254 and 1260 technical mixtures. PCB signature patterns in order of abundance were 138> 180> 206> 153> 187> 149 and 138> 153> 180> 149> 187> 110> 170 for the Jukskei and Klip River sediments, respectively. The upstream Alberton point had the highest Σ31 PCB and Σ (parent+alkyl) PAH concentrations in the Klip River of 61 µg kg^{-1} dry weight (dw) and 6000 µg kg^{-1} dw respectively. In the Jukskei River, the upstream Marlboro point had the highest Σ31 PCB of 19 µg kg^{-1} dw and the N14 site recorded the highest Σ (parent+alkyl) PAH concentrations and 2750 µg kg^{-1} dw. PAH concentrations in both the Jukskei and Klip River were significantly higher than the PCB concentrations. Fluoranthene, phenanthrene and pyrene were found in the highest concentrations in both the Jukskei and Klip River sediments. Both the Jukskei and Klip River sediments showed trends of a mixed pyrogenic-petrogenic PAH source contamination.

Introduction

Before the worldwide ban on the production and use of polychlorinated biphenyls (PCBs), they had been used in a variety of applications such as plasticisers, cooling agents, heat resistant hydraulic fluids and dielectric fluids for capacitors and transformers (O'Donovan et al. 2011; Eisler and Belisle 1996; El-Shahawi et al. 2010; Vallack et al. 1998). Unlike PCBs which are man-made, polycyclic aromatic hydrocarbons (PAHs) are naturally present in fossil fuels, but like PCBs, their presence in the environment is mainly due to anthropogenic activities such as burning fossil fuels and wood (Arruti et al. 2012). PAHs are some of South Africa's major environmental contaminants, mainly due to the country's

heavy reliance on coal for electricity supply (Okedeyi et al. 2013; Menyah and Wolde-Rufael 2010). Due to the complex and heterogeneous nature of surface waters and especially sediment matrices, it can be very difficult to understand the spatial distribution of PCBs and PAHs beyond the locally contaminated sites (Opel et al. 2011). It is known that, due to their high log K_{ow} values, PCBs and PAHs in the environment tend to adsorb and accumulate in organic matter in sediment. This is critical in understanding their behaviour in environmental systems, particularly regarding their partitioning between water and sediment (Koelmans et al. 1997; Meylan et al. 1996).

The need for environmental risk assessment studies on PCBs and PAHs is derived from their adverse toxic effects on both aquatic and terrestrial organisms (e.g mutagenic, oncogenic, teratogenic and carcinogenic properties), bioaccumulation and environmental persistence (Ghaeni et al. 2015; Olenycz et al. 2015; Quinn et al. 2009; Monikh et al. 2014; Stogiannidis and Laane 2015; Wang et al. 2015; Wei et al. 2015; Yunker and Macdonald 2003). PAHs and PCBs may enter human bodies via contaminated food or water (Perelló et al. 2015; Martorell et al. 2010; Gioia et al. 2014) and in addition PAHs can also enter human bodies as a result of inhaling contaminated air from burning fossil fuels (Kim et al. 2013; Zhou and Zhao 2012). South Africa has an overdependence on coal with as much as 93% of South Africa's electricity being generated by coal fired power stations (Menyah and Wolde-Rufael 2010). The South African Department of Environmental Affairs has published stricter regulations on compulsory monitoring PCB and PAH emission of air quality by all entities holding atmospheric emission licenses as from 1 April 2015 DEA, (2012).

Environmental PAH and PCB congener compositions are dependent on the source of contamination as well as biological and photolytic degradation (Brown and Wagner 1990; McFarland and Clarke 1989). Source characterisation of both PAHs and PCBs is challenging due to the presence of both pyrogenic and petrogenic PAHs, weathering of PCBs, as well as the earlier use of different technical PCB mixtures (Aroclors) which have been widely used in the South African energy sector (Gandhi et al. 2014; Stogiannidis and Laane 2015). A study by Gandhi et al. (2014) on the evaluation of the efficiency of PCB indicator schemes for monitoring environmental PCB contamination, along with a similar study by Daouk et al. (2011) indicates that the study of only the six common indicator PCBs (28, 52, 101, 138, 158, 180) would be insufficient to effectively deduce PCB patterns within catchments as these congeners only partly cover the PCB patterns present in the environment (Frame et al. 1996; Gandhi et al. 2014; Daouk et al. 2011; Seeger et al. 1997). Analysis of a larger set of PCB congeners provides a

better understanding of site-specific PCB contamination trends and patterns. However, it is widely recognised that a full congener-specific analysis of all 209 PCBs is not necessary for all tiers of risk assessment (Henry and DeVito 2003).

It is estimated that a third of the approximately 1.3 million tonnes of PCBs produced globally are still circulating in the environment and it is known that power stations and equipment containing PCBs in South Africa often experience spills and leaks (Vallack et al. 1998; Gioia et al. 2011; Jiang et al. 2015; Moodley and Carsky 2013). The lack of a PCB inventory in South Africa, coupled with the lack of basic information about PCB distribution in the environment makes the country's 2025 and 2028 goals, respectively to eliminate PCB containing equipment and implement environmentally sound PCB waste management practices difficult to achieve (Moodley and Carsky 2013; National Department of Environmental Affairs 2012, 2014).

This study was undertaken to determine the distribution of 16 priority PAHs, benzo(e)pyrene, perylene, 19 alkylated PAHs and 31 *ortho* substituted indicator PCBs in sediments from 2 major rivers in the Gauteng province of South Africa impacted by high anthropogenic activity. The aim of this study was to characterise the contaminating technical PCB mixtures and pyrogenic/petrogenic PAHs in sediment, provide the first comprehensive baseline study of PCB levels and provide new data on distribution of alkylated PAHs which have never been analysed in South Africa before.

Materials and methods

Study area and sampling

The study area consisted of the Jukskei River and Klip River in the Gauteng province of South Africa which drain water from the Johannesburg highlands called the Witwatersrand (Figure 3.1). The Jukskei River flows in a north-westerly direction, passing through heavy industrialised areas of northern Johannesburg. The Klip River flows from Alberton, south of Johannesburg, which is also home to heavy industrial activities, in a southwards direction before joining the Vaal River. The Gauteng Province soil deposits consist mainly of weathered clastic sedimentary mineral and carbonate-rich rock (Eriksson et al. 1995). Sediment samples were taken twice, on the 3rd and on the 18th of August 2013 using random surface grab (top 0-5cm) sampling across a 5 meter diameter in the middle of the flowing river bed. Equal portions of the samples from the 2 sampling dates were mixed together to make one

representative sample. The sampling site names and coordinates are listed in Table 3.1. The samples were frozen and transported to The Netherlands (IVM, VU University) for extraction according to US Environmental Protection Agency (EPA) method 3545A for both PAH and PCB analysis.

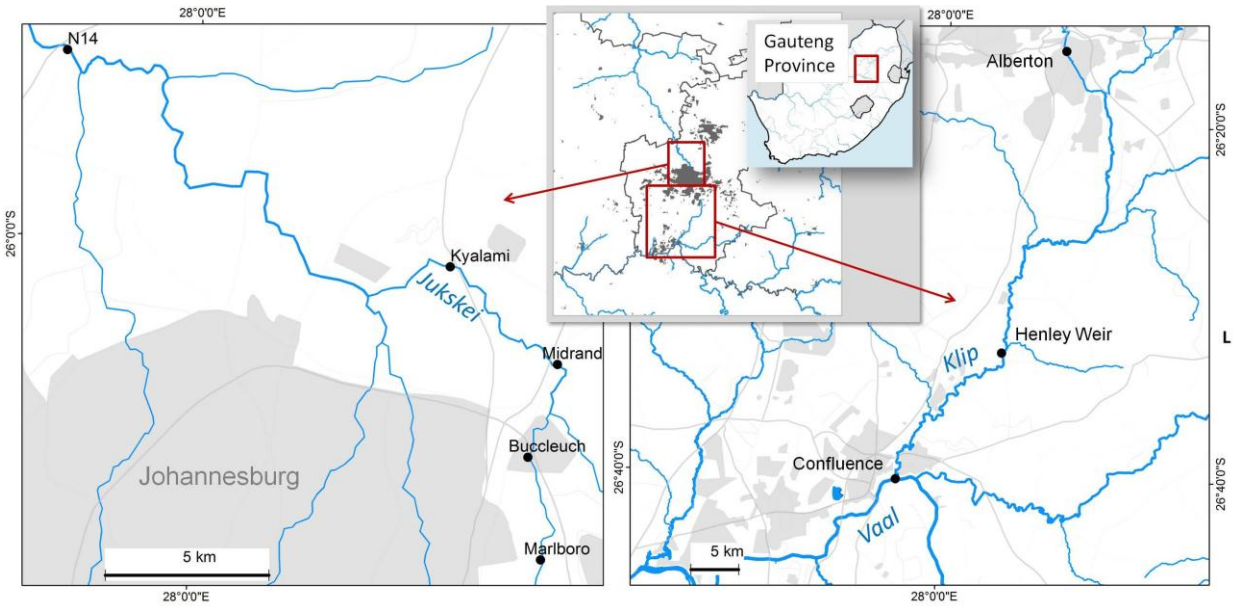


Figure 3.1 Sampling sites in the Klip and Jukskei River catchments: Gauteng Province, South Africa

Table 3.1 Sampling sites and GPS coordinates

River	Sampling site	Latitude (south)	Longitude (East)
Klip	Alberton	-26.26393	28.12418
Klip	Henley	-26.54939	28.06443
Klip	Confluence	-26.67055	27.95527
Jukskei	Marlboro	-26.08494	28.10881
Jukskei	Buccleuch	-26.057	28.104
Jukskei	Midrand	-26.03139	28.11222
Jukskei	Kyalami	-26.0056	28.07836
Jukskei	N14	-25.94933	27.95878

Chemicals

Analytical standards for PCBs 28, 31, 44, 47, 49, 52, 56, 66, 74, 85, 87, 97, 99, 101, 105, 110, 118, 128, 137, 138, 141, 149, 151, 153, 156, 170, 180, 187, 194, 202, 206 (Numbers. according to Ballschmiter et al. 1992 (Dr. Ehrenstorfer, Augsburg, Germany)) and a 100 ng mL^{-1} ^{13}C labelled internal standard 12 PCB mix (Wellington Laboratories, Ontario, Canada) were used as calibration and internal standards respectively. For PAH and alkyl PAH analysis, a NIST standard reference material (SRM)® 2260a (Gaithersburg, MD, USA) parent PAH calibration mixture and a NIST SRM 1491a (Gaithersburg, MD, USA) alkyl substituted PAH calibration mixture were used, respectively. A deuterated isotope labelled PAH mix CIL-ES-2528 (Cambridge Isotope Laboratories, Andover, USA) with deuterated compounds D8 acenaphthylene, D10 acenaphthene, D10 fluorene, D10 phenanthrene, D10 anthracene, D10 fluorene, D10 pyrene, D12 benz(a)anthracene, D12 Chrysene, D12 benzo(b)fluoranthene and D12 benzo(k)fluoranthene was used as an internal standard for both PAH and alkyl PAH analysis. All analytical and internal standards had a purity >98%. Acetone, hexane, and isooctane (J. T. Baker, Deventer, The Netherlands) with a purity >99.7%, silica gel and alumina (Sigma-Aldrich, Bellefonte, USA) were used for sample cleanup.

Sample extraction

The sediment samples were freeze dried, granulated using a porcelain mortar and pestle and sieved through a 2.5 mm mesh sieve. 100 µL of a 50 ng mL^{-1} mixture of ^{13}C-labelled PCBs and 70 µL of a 100 ng mL^{-1} mixture of deuterated PAH internal standards were added to 5 g dried and sieved sediment samples before extraction using accelerated solvent extraction (ASE, Dionex ASE 350) with two cycles using solvents hexane: acetone (3:1, v/v). Extraction conditions were: temperature 100°C, pressure 10.3 mPa, oven heat time: 5 min, static time: 5 min. The extract was evaporated using a gentle stream of nitrogen to near dryness before reconstitution in 0.5 mL hexane.

Cleanup and fractionation

The 0.5 mL sediment extracts in hexane were cleaned up by loading on a glass chromatography column filled with 15 g of 8% deactivated alumina (92% alumina: 8% deionised Milli-Q water, w/w) and eluting with 170 mL pentane to remove lipids and humic acids. The extract was reduced to 0.5 mL using a Kuderna Danisch apparatus with isooctane as the keeper solvent. Fractionation of PCBs and PAHs was

achieved by loading the 0.5 mL extracts through 1.6 g silica gel deactivated with 1.5% Milli-Q water (98.5% silica: 1.5% deionised Milli-Q water w/w) in a glass column and eluting with 11 mL hexane to elute and collect PCBs followed by 10 mL hexane: diethyl ether (85:15, v/v) to elute and collect PAHs. All extracts were reduced to near dryness using a gentle stream of nitrogen before reconstituting with 0.5 mL isooctane.

Chemical analysis

Gas chromatography-mass spectrometry (GC-MS, GC 6890A and MS 5975, Agilent Technologies, Amstelveen, The Netherlands) with helium (99.999% purity) carrier gas was used for PCB, PAH and alkyl PAH analysis. The MS detector was operated in selective ion monitoring mode at 70eV with electron impact ionisation. For PCB analysis, an initial oven temperature of 90°C (3 min) was used followed by 30°C min^{-1} to 200°C (10 min), 5°C min^{-1} to 265°C (3.5 min), 3°C min^{-1} to 275°C with a 1 µL splitless injection. A CP-SIL 8 CB (5% phenyl, 95% methyl polysiloxane, Agilent Technologies J&W, Amstelveen, The Netherlands) column (60 m X 0.25 mm X 0.25 µm) was used with an average gas velocity of 26 cm sec^{-1}. Total runtime was 62.43 min. Quantification was achieved using an 8-point internal standard calibration curve. For PAH and alkyl PAH analysis an initial oven temperature of 80°C (1 min), 30°C min^{-1} to 150°C, 5 ^{o}C min^{-1} to 220°C, 2°C min^{-1} to 290°C, 10°C min^{-1} to 320°C (5 min) with a 1 µL splitless injection was used. Total runtime was 52.43 min. An SGE BPX 5 (5% phenyl, 95% methyl polysiloxane, SGE Analytical Science, Melbourne, Australia, dimensions: 25 m X 0.22 mm X 0.25 µm) column was used with an average velocity of 50 cm sec^{-1}. LOD and LOQ were set at 3 and 10 times the signal-to-noise ratio, respectively. LOQs for PAHs and alkyl PAHs varied between 0.03 and 0.12 µg kg^{-1} and LOQs for PCBs varied between 0.12 and 0.89 ng kg^{-1}.

Total organic carbon and particle size distribution

Total organic carbon (TOC) of the samples was measured using the modified Walkley-Black method as described by Rimayi et al. (2016) and the particle size distribution was measured by weighing sieved sediment fractions with mesh sizes 1000-500 µm, 500-125 µm (silt) and <125 µm (clay).

Statistical analysis

Statistical analysis of the results was performed using STATISTICA 8 (StatSoft Inc. Tulsa, OK, USA). A combination of principal component analysis (PCA) and biplots was used to fully characterise the data.

The quality of the data was verified by an R^2X (cumulative) test. The analytes were plotted in the principal component coordinates with analytes having similar distribution in the catchments located in close proximity.

Evaluation of method performance

The GC-MS method was validated for accuracy, repeatability and reproducibility by extracting and analysing 5 sediment PCB certified reference material (BCR®535, Sigma-Aldrich, Bellefonte, USA) and 5 sediment PAH certified reference material (BCR®536, Sigma-Aldrich, Bellefonte, USA) quality control samples along with the samples. Repeatability was <5.6% relative standard deviation (RSD) for all analytes and reproducibility for samples ran on different days was <6.8% RSD for all analytes. Milli-Q water used for preparation of reagents was produced by a Millipore Advantage A10 and had a TOC <3 µg L^{-1}.

Results and discussion

ΣPCB concentrations in Jukskei and Klip River sediment

The total concentrations of the 31 PCB congeners (ΣPCB) analysed in the river sediments are shown in Figure 3.2, from the Marlboro site downstream to N14 for the Jukskei River and from the Alberton site downstream to Confluence for the Klip River. In the Jukskei River, the upstream point of Marlboro recorded the highest ΣPCB concentration and the following site, i.e. Buccleuch recorded the lowest ΣPCB concentration, 19 µg kg^{-1} and 2.9 µg kg^{-1} respectively. There was a linear increase in ΣPCB from the Buccleuch point to the furthermost downstream point, N14. The Alberton and Confluence points in the Klip River recorded the highest ΣPCB concentrations of the two river catchments, i.e. 61 µg kg^{-1} and 27 µg kg^{-1}, respectively. The relatively high PCB levels in the Alberton, Confluence and Marlboro sites may be linked to uncontrolled PCB leakages on land as indicated in the NERSA report (NERSA 2015), followed by stormwater runoff particularly from industrial discharges originating from surrounding industrial areas adjacent to the Jukskei and Klip Rivers. The PCB distribution patterns do not correlate with TOC and sediment sample composition (Supplementary Information (SI) Table S3.1). This is most likely due to the low TOC contents and the small mutual differences in TOC between the sampling sites.

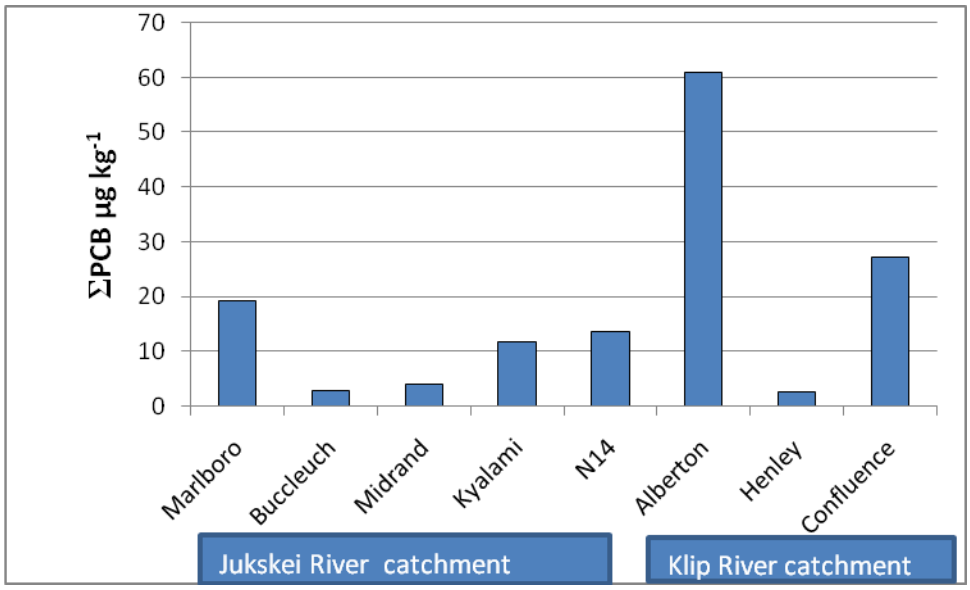


Figure 3.2 ΣPCBs concentrations in the Jukskei and Klip River sediments

Limited information is available on the occurrence of PCBs in African river sediments, however available studies show that PCB concentrations in the African coastal environment have been steadily rising in the past decade, despite the fact that PCBs were never produced on the continent (Gioia et al. 2014). Since 97% of the globally produced PCBs were used in the northern hemisphere, it is expected that the southern hemisphere would be at a lower risk of PCB contamination (Gioia et al. 2011). As with the Jukskei and Klip River sediments in this study, the dominant PCB congeners detected in uMngeni River sediments (located on the east South African coast) were PCBs 180, 138 and 153 (Gakuba et al. 2015). TOC was not measured in the uMngeni River sediment study, however PCB levels in the uMngeni River sediment were up to 10 times higher than PCBs levels detected in the Jukskei and Klip Rivers (Gakuba et al. 2015). A table of ΣPCB concentrations analysed in river sediments around the world has been compiled by Lv et al. (2015). The levels presented in this study are within the same range as reported world-wide, though a relative comparison with this study is not practical as it could yield an unbalanced comparison due to the different number of PCB congeners included in the analysis. Recent studies on river sediment profiles in France (Deule, Sensee and Scarpe Rivers) and China (Yangtze River) show an abundance of lower chlorinated PCBs, tri to pentachlorinated biphenyls, with PCBs 138, 153 and 180 being measured at lower concentrations (Net et al. 2015; Yang et al. 2009). West African coast and USA (Delaware River) sediments show a prominent presence of higher chlorinated PCBs as with the Jukskei and Klip River sediments (Gioia et al. 2014; Praipipat et al. 2013).

PCB congener profiles and trends in the Klip and Jukskei Rivers

To date, there has never been an attempt to characterise the source of the ubiquitously distributed PCBs in South African river sediments. This may be due to the small dataset of indicator PCBs usually analysed in the limited number of studies available which fail to provide formidable contamination trends and patterns of individual PCBs (Hu et al. 2011). There are no known point sources of PCBs in the Gauteng province. The prominence of higher chlorinated PCBs in both the Jukskei and Klip River sediments indicates that possible contaminants could be Aroclor 1254 (similar composition to Kanechlor 500), Aroclor 1260 (similar composition to Chlorpen A-60 and Phenochlor DP-6) or Aroclor 1262. The latter having higher chlorinated hexa to nona CBs which are mostly not detected in the majority of other technical mixtures (ASTDR 2000). Aroclors 1242, 1254 and 1260 have been widely used in electrical transformers by Eskom, the energy utility in South Africa which imported the majority of the PCB containing equipment in South Africa, some of which are still in use and continually pose a contamination risk (Gray 2004). It has been established that it is difficult to ascertain the contaminating technical mixtures in environmental extracts with a high degree of certainty, particularly in aged sediments where PCB profiles in sample extracts have different patterns from contaminating technical mixtures (Beyer and Biziuk 2009).

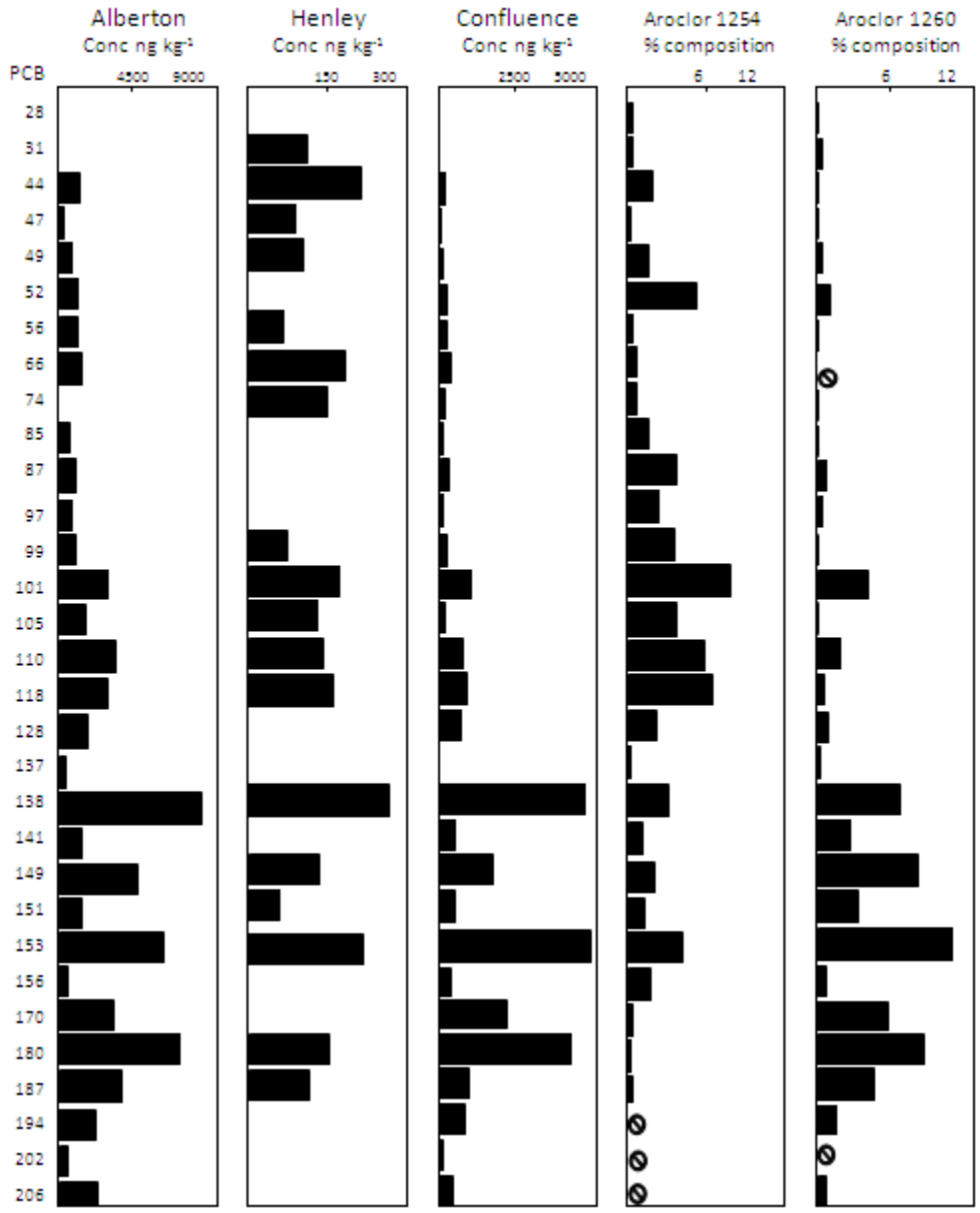
In Figure 3.3, Aroclor 1254 and 1260 profiles are compared to the PCB patterns in the Klip and Jukskei River (Figure 3.4) sediments. The Jukskei and Kilp River sediments show characteristics of contamination by Aroclor 1254 which is dominated by mid chlorinated PCBs (tetra to hexachlorinated PCBs) and Aroclor 1260 which is dominated by higher chlorinated PCBs (hexa to nonachlorinated PCBs, Figure 3.3). Aroclor 1254 and Aroclor 1260 show similar congener patterns for higher chlorinated PCBs 138 to 170, though in different proportions relative to other PCB congeners in their respective mixtures. The majority of tri and tetrachlorinated biphenyls which account for >75% of Aroclor 1242 congeners recorded low concentrations (<300 ng kg^{-1}) in both the Klip and Jukskei Rivers (Albro and Parker 1979). The absence of the most abundant Aroclor 1242 PCBs such as PCB 28 in the river sediments, which has a molecular percentage of 13% in Aroclor 1242 indicates that Aroclor 1254 and 1260 are the major contaminants in the Jukskei and Klip River sediments (Albro and Parker 1979).

A recent 2014/15 report compiled by the National Energy Regulator of South Africa lists leaking transformer oil as the major non-compliance in electricity substations in Gauteng and other provinces in

South Africa (NERSA, 2015). These findings concur with observations that the PCB profiles in the majority of the Klip and Jukskei River sediments (Figures 3.3 & 3.4) indicate the presence of relatively unweathered Aroclor 1254 and Aroclor 1260 PCBs. The relatively low abundance of lower chlorinated PCBs also indicates that the Jukskei and Klip River sediments contain limited quantities of photolytically and anaerobically transformed PCBs (Abramowicz 1994; Abramowicz 1995; Jones et al. 2003). The fate of Aroclor 1242 cannot be established with a high degree of certainty as lower chlorinated PCBs are generally more soluble in water where they are more prone to degradation (Borja et al. 2005; Miao et al. 1999). However, considering the low abundance of major Aroclor 1242 congeners in sediments and the continuous presence of leaking PCB containing equipment in the Gauteng province, this suggests that the use of Aroclor 1242 containing equipment has not been prevalent. The Buccleuch site in the Jukskei River as well as the Henley site in the Klip River show exceptionally different PCB contamination patterns from other points in their respective rivers, indicating the presence of site specific PCB contamination trends.

In the Jukskei River, the upstream sampling point, Marlboro which is closest to central Johannesburg, recorded the highest single PCB congener concentrations of 2660 and 2540 ng kg^{-1} for PCBs 180 and 138 respectively. Generally, the Jukskei River sediments had PCB concentrations <2000 ng kg^{-1} except for PCBs 180, 138, and 153 at the Marlboro point. The Jukskei River was dominated by PCBs 138, 149, 153, 180, 187 and 206 for all points except for the Buccleuch site which shows a low abundance of most PCBs except PCB 206. The strong presence of PCB 206 in the Buccleuch site is not fully understood, however PCB 206 is present in Aroclor 1260 and has the second highest log K_{ow} value (9.14) of all PCBs after PCB 209 (Eisler and Belisle 1996). Hence it is found at high concentrations (>1000 ng kg^{-1}) in most Jukskei sediments due to its high adsorption capacity to organic matter and sediment (Passatore et al. 2014). The data suggests contamination from Aroclor mixtures 1254 and 1260 for the Marlboro, Midrand, Kyalami and N14 sites whose lower chlorinated PCBs from trichlorobiphenyls to pentachlorobiphenyls show variable PCB congener distribution patterns. Distinctively conspicuous Aroclor 1260 signature patterns were observed for hexachlorobiphenyls to nonachlorobiphenyls, PCBs 138 to 206 for all Jukskei sites except the Buccleuch point.

Single PCB congeners present at the highest concentrations in the Klip River sediment were generally in the order: 138> 153> 180> 149> 187> 110> 170> 101> 118> 206> 194. The Alberton and Confluence points had 11 and 3 PCB congeners with concentrations >2000 ng kg^{-1}, respectively. Distinct PCB congener patterns can be observed for PCBs 138 to 187 which are characteristic of both Aroclors 1254 and 1260, however PCB 99 to 128 patterns affirm the dominance of Aroclor 1254. The Alberton site which is located adjacent to the Alberton industrial area recorded the highest PCB congener concentration of 8110 ng kg^{-1} for PCB 138. The Henley point which had the lowest proportion of clay particles (<125 µm, Supporting Information (SI) Table S3.1) had the lowest overall PCB concentrations with the highest concentration being recorded for PCB 138 having a concentration of only 270 ng kg^{-1}. The Henley point shows similar lower chlorinated (tri to tetrachlorinated) PCB concentration levels to other points in the Klip River (Figure 3.3). However, higher chlorinated penta to nonachlorinated PCBs for the Henley point show significantly lower concentration levels than other points in the Klip River. Compared to other points in the Klip River, the Henley point shows a stronger influence of Aroclor 1242 as a result of lower contamination levels from Aroclor 1254 and Aroclor 1260.



⊘ = no data
Aroclor 1254 and 1260 patterns (Eisler and Belisle, 1996).

Figure 3.3 PCBs in the Klip River sediments, Aroclor 1254 and Aroclor 1260 (ng kg-1 dry weight)

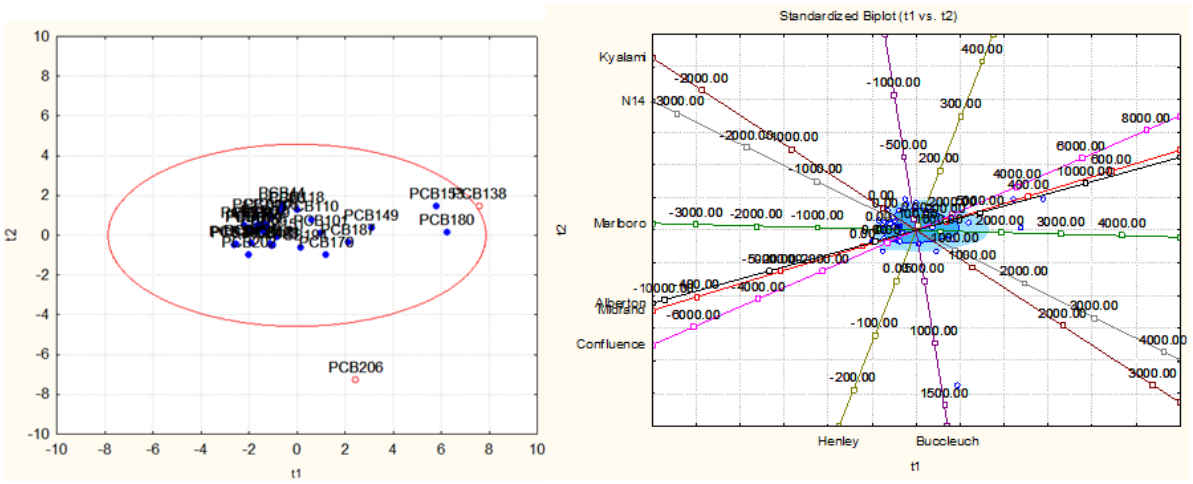


Figure 3.4 PCBs in the Jukskei River sediments (ng kg-1 dry weight)

The data collected shows that with the exception of the lowly contaminated sites of Buccluech and Henley, all sampling points in both the Jukskei and Klip River sediments had similar PCB patterns with dominating hexa to nonachlorinated congeners. The identification of contaminating PCB technical mixtures in the majority of both the Klip and Jukskei River sediments was possible as there was no evidence of extensive PCB weathering. The presence of relatively unweathered PCBs in both the Jukskei

and Klip Rivers concurs with recently published reports showing significant leakages from electricity substations.

PCA scores in Figure 3.5 correspond to Figures 3.3 & 3.4. The PCA analysis identifies PCBs 138 and 206 as outliers as they fall outside the eclipse of normality. PCBs 153, 138, 180 and 206 are clustered away from the origin as they recorded significantly higher concentrations than other congeners in the 2 catchments. PCBs 153 and 138 are clustered around the Alberton, Midrand and Confluence sites and PCB 180 is clustered between the Alberton and Marlboro sites. PCB 206 is a strong outlier, particularly for the Buccleuch site where it recorded a significantly higher concentration compared to other congeners (Figure 3.4). The majority of the other PCBs are clustered around the origin, indicating that they are evenly distributed throughout the catchment. This distribution indicates that contamination is likely due to diffusely distributed leaking PCB electrical equipment rather than a point source. The biplot data corresponds well with the Figure 3.2, showing that the Alberton and Confluence sites contribute the greatest PCB loads in the Klip River and the Marlboro and N14 sites contribute the greatest PCB loads in the Jukskei River. As PCB data in South Africa is scanty, PCB point sources cannot be established with a high level of certainty.



Component 1 R^2X = 0.18; Component 2 R^2X = 0.24

Figure 3.5 PCA and biplot analysis of PCBs in different sites in the Jukskei and Klip River sites

PAH levels and trends in the Klip and Jukskei Rivers

The Alberton site in the Klip River recorded the highest ΣPAH (incl. alkyl PAH) concentration of 6000 µg kg^{-1} which drastically decreased downstream to 56 µg kg^{-1} at the Henley site before increasing slightly further downstream to 187 µg kg^{-1} at the Confluence site (Figure 3.6). The ΣPAH concentrations in the Jukskei River decreased from a high of 1520 µg kg^{-1} at the Marlboro to 640 µg kg^{-1} at the Buccleuch site before gradually increasing linearly further downstream to 2750 µg kg^{-1} at the N14 site (Figure 3.6). As with the Jukskei River, the most dominant PAHs at the Alberton site are fluoranthene, phenanthrene and pyrene whereas retene is the most dominant PAH at the Henley and Confluence sites (Figure 3.8).

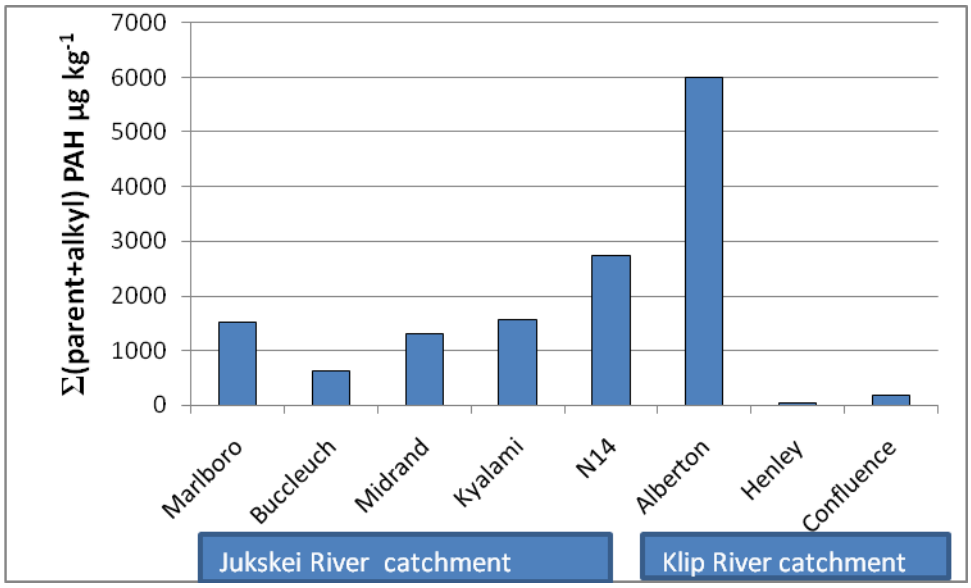


Figure 3.6 Σ (parent+alkyl) PAHs concentrations in the Jukskei and Klip River sediments

Average Σ16 PAH concentrations of 1130 and 1060 µg kg^{-1} in the Klip and Jukskei River sediments, respectively were up to 20 times lower than average Σ16 PAH concentrations detected in northern France river sediments of 20000 µg kg^{-1} (Net et al. 2015) but similar to average Σ16 PAH concentrations detected in the Malacca River sediment in Malaysia (1022 µg kg^{-1}) 4 times lower that those detected in the Prai River sediments in Malaysia (4357 µg kg^{-1}) (Net et al. 2015; Keshavarzifard et al. 2014). Recent studies in the Bharalu River sediment in India and the Pearl River sediment in China which are located downstream of highly industrialised areas with heavy vehicular traffic recorded higher average Σ16 PAH

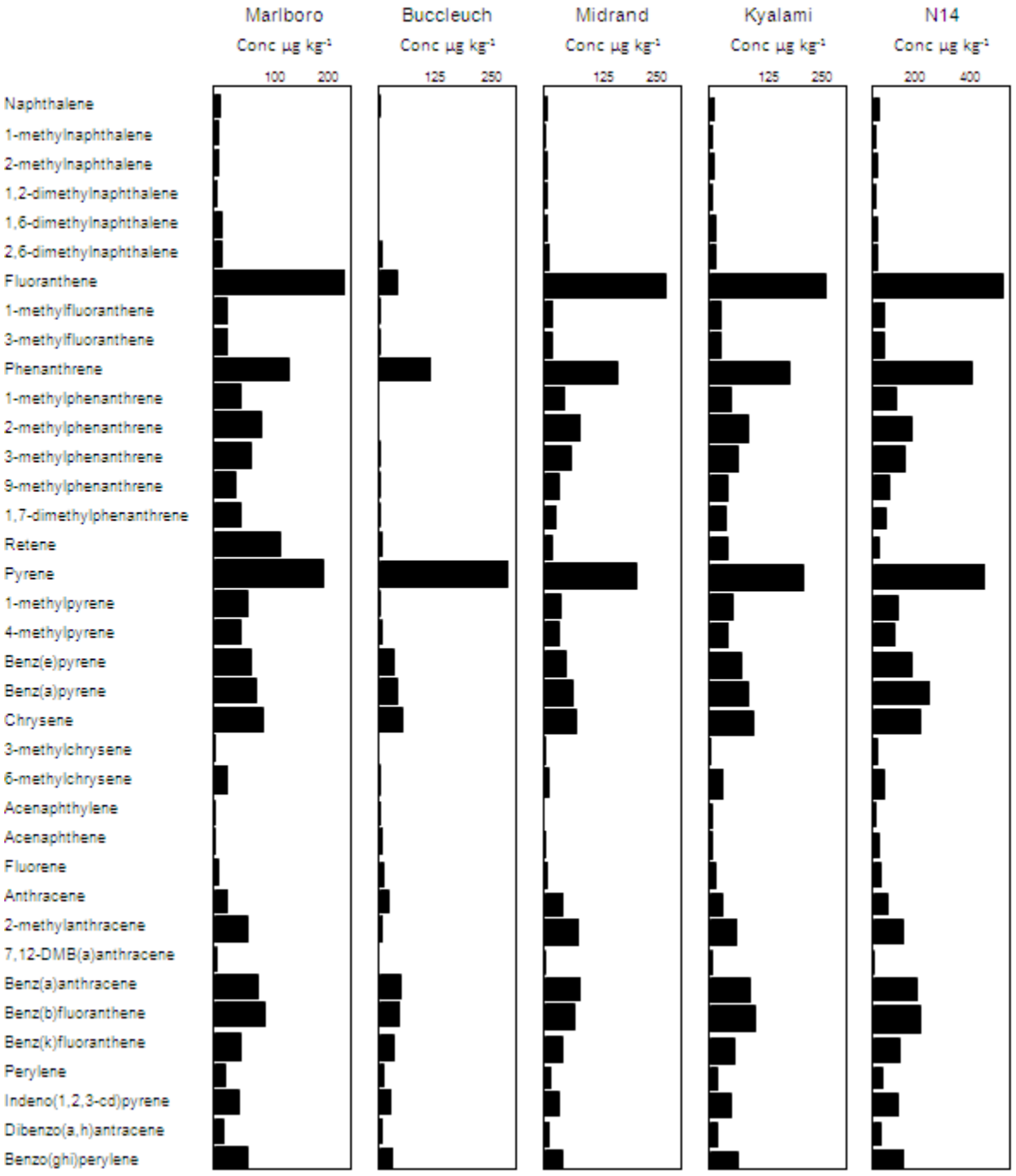
concentrations of 4970 µg kg^{-1} and 5570 µg kg^{-1}, respectively than the Jukskei and Klip River averages (Hussain et al. 2015; Feng et al. 2012).

Discrimination of pyrogenic and petrogenic PAH sources requires an intricate analysis of PAH ratios from source emission inventories (Stogiannidis and Laane 2015; Dvorská et al. 2011). However, the PAH ratios in environmental samples can be disproportionately influenced by photolytic and biological weathering. Interpretation of PAH profiles is not always simple as combustion temperatures, combustion efficiencies as well as quality and type of fuel combusted may vary. It may for example be difficult to distinguish between weathered coal sources from petroleum sources (Yunker and Macdonald 2003; Howsam et al. 1998). Criteria (listed in SI Tables S3.2 & S3.3) for source identification of PAHs of pyrogenic origin include; (a) presence of high molecular weight PAHs, (b) parent PAH: alkyl PAH ratios with maxima at parent PAHs, (c) 1-methylphenanthrene: phenanthrene (1-MP/P) ratio <1, particularly when 0.4<1-MP/P<0.7 and (d) fluoranthene: pyrene ratios with F/(F+Py)>0.5 e) benzo(e)pyrene: benzo(a)pyrene (BeP/BaP) ratio with BeP/BaP <1 (Stogiannidis and Laane 2015).

Petrogenic source indicators include a phenanthrene concentration greater than an anthracene concentration and a benz(a)anthracene: chrysene ratio (BaA/C) above 0.5 (Stogiannidis and Laane 2015). Mixed pyrogenic and petrogenic sources can be defined by a phenanthrene: anthracene (P/A) ratio between 10 and 5. An abundance of fluoranthene (F), pyrene (Py) and phenanthrene (P) indicates a coal or wood PAH source. The methylphenanthrenic index (MPI 3) which can be used to determine a coal source of the same thermal maturity and origin is calculated by the formula: (2-methylphenanthrene + 3-methylphenanthrene)/ (9-methylphenanthrene + 1-methylphenanthrene).

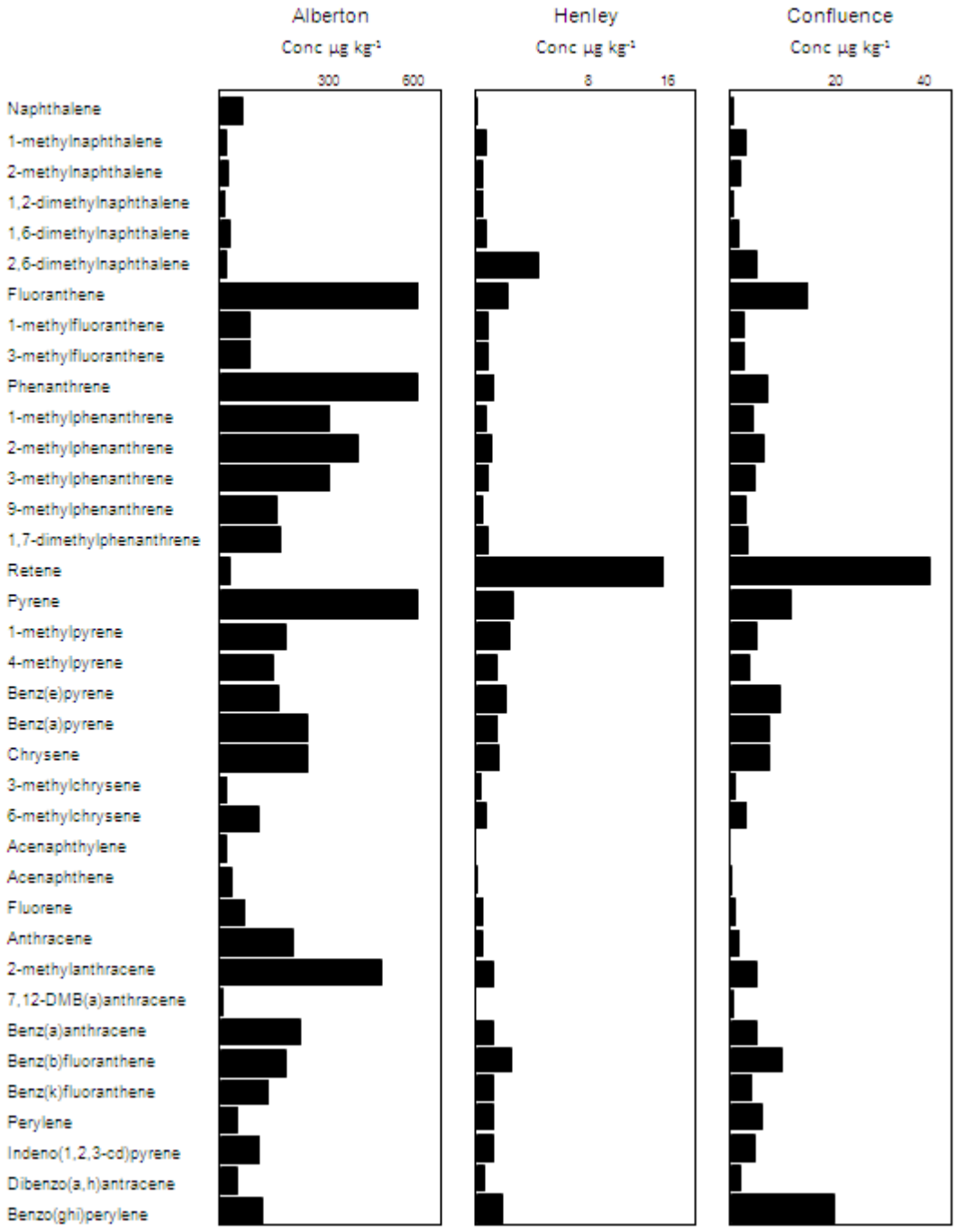
Other published criteria such as benzo(e)pyrene: benzo(a)pyrene ratios may be deemed less reliable due to overlap between pyrogenic and petrogenic sources and others are subject to high uncertainties due to significant differential weathering rates between the PAHs under comparison (Dvorská et al. 2011; Stogiannidis and Laane 2015). Approximately 3% of the total coal consumption in South Africa is used for household heating and cooking, which in general is the largest contribution to total air pollution, up to 65% in urban areas due to low combustion efficiencies. The remaining 97% coal consumption is primarily used by power stations which make use of mainly low-grade coal which is often mixed with coal of a higher coal rank (Balmer 2007; Jeffrey 2005).

The prominence of parent PAHs compared to alkyl PAHs in both the Jukskei and Klip Rivers (Figures 3.7 & 8) shows the dominance of pyrogenic sources as alkyl PAHs from combustion sources are thermodynamically unstable, which leads to a lower abundance at higher temperatures (Saha et al. 2012). A higher abundance of 2-methylanthracene (2-MA) over anthracene in all the Klip and Jukskei River sediments except at the Buccleuch site in the Jukskei River (SI Tables S3.2 & S3.3), shows the influence of a petrogenic source (Stogiannidis and Laane 2015). Phenanthrene and pyrene alkyl derivatives were the most abundant alkyl PAHs in both the Jukskei and Klip Rivers.



7,12-DMB(a)anthracene = 7,12-dimethylbenz(a)anthracene

Figure 3.7 PAHs in the Jukskei sediments (µg kg-1 dry weight)



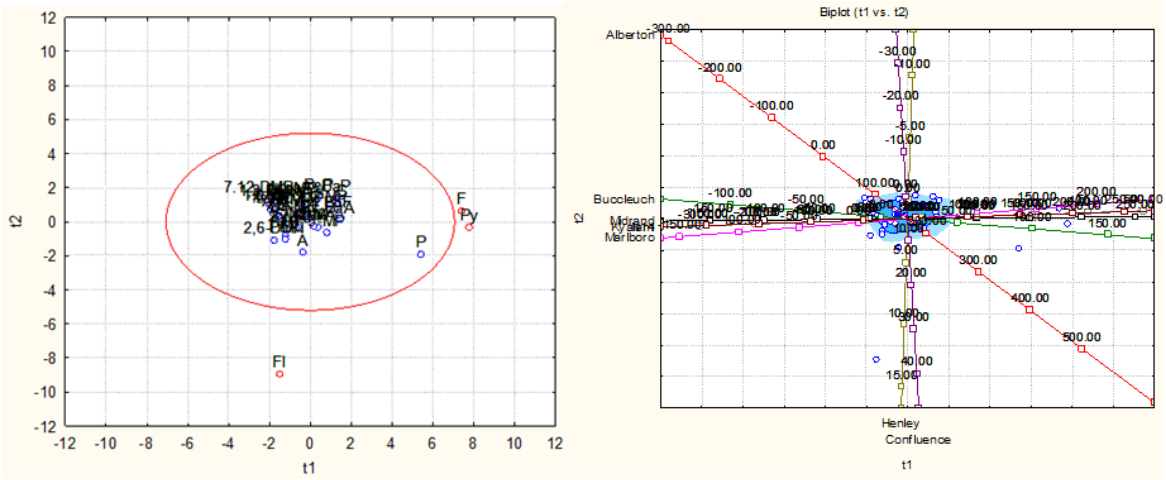
7,12-DMB(a)anthracene = 7,12-dimethylbenz(a)anthracene

Figure 3.8 PAHs in the Klip River sediments (µg kg-1 dry weight)

The commonly used P>A index is prone to overlap and shows a petrogenic source for the Jukskei River whilst the A/(A+P) ratio indicates the presence of a pyrogenic source. The P/A index therefore confirms the presence of a mixed petrogenic-pyrogenic source with values falling within 10>P/A>5 for all the Jukskei River sediments except the Midrand site, which shows a stronger influence of a pyrogenic source with a P/A ratio of 4.4 (SI Table S3.2). All sampling sites in the Jukskei River, with the exception of the Buccleuch site had an MPI 3 index of approximately 1.8 (SI Table S3.2) and show a higher dominance of pyrogenic sources. The deviating MPI 3 index of 0.8 at Buccluech may be influenced by the high volumes of vehicular traffic at the nearby Buccluech traffic interchange.

The P>A index commonly used shows a petrogenic source for the Klip River sediments but in contrast the A/(A+P) ratio shows a pyrogenic source for all the 3 Klip sites. The third P:A index, shows a P/A<5 in the Klip River, confirming a pyrogenic source. All the sites in the Klip River had an MPI 3 index of approximately 1.5 (SI Table S3.3), indicating a coal source of the same thermal maturity and origin. The data points out to an overall mixed pyrogenic-petrogenic source in the Klip River with a stronger influence of pyrogenic sources.

PCA scores in Figure 3.9 correspond to Figures 3.7 & 3.8. The PCA analysis indicates that fluoranthene, pyrene and phenanthrene are outliers, though phenanthrene is within the eclipse of normality. The 3 compounds are the most abundant PAHs in the Jukskei River and are clustered primarily around all the Jukskei River sites. Fluorene (Fl) is identified as a strong outlier due to its disproportionate distribution in the Klip River, recording a high of 72 μg kg^{-1} in the Alberton site and concentrations <0.8 μg kg^{-1} in the downstream sites of Henley and Confluence where fluorene is clustered. The majority of the parent and alkyl PAHs are clustered around the origin, indicating that they are evenly distributed throughout the Jukskei and Klip Rivers



Component 1 $R^2X = 0.12$; Component 2 $R^2X = 0.19$

Figure 3.9 PCA and biplot analysis of PAHs and alkyl PAHs in different sites in the Jukskei and Klip River sites

Conclusions

The Jukskei and Klip River sediments show trends of contamination by relatively unweathered Aroclor 1254 and 1260 with similar PCB congener signatures for higher chlorinated PCBs 138 to 170 in all sites except the lowly contaminated sites of Buccluech and Henley. PCBs 138, 153 and 180 were the most abundant PCBs in the majority of the sites studied. All the Kilp and Jukskei River points show trends of a mixed pyrogenic-petrogenic PAH contamination source with a stronger influence of a pyrogenic source. Fluoranthene, pyrene and phenanthrene were the most abundant PAHs in both the Jukskei and Klip River sediments, with alkyl PAHs recording lower concentrations than parent PAHs with the exception of 2-methylanthracene. There was evidence of site specific contamination trends as the second upstream Buccleuch site in the Jukskei River as well as the second upstream Henley site in the Klip River recorded the lowest ΣPCB and ΣPAH concentrations. TOC and particle size distribution could not adequately account for the spatial trends observed because the TOC levels were very low and varied only marginally between the sampling sites. PCB congener ranges detected in most of the studied sites were comparable to those detected in industrialised countries around the world. Σ16 PAH concentrations in both the Jukskei and Klip Rivers were significantly lower than those recorded in big cities in China and India.

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Chapter 3 – Supporting Information

Tables:

Table S3.1 TOC and sediment composition of sediments

Sampling site	% Sand	% Silt	% Clay	% TOC
Alberton	19	67	11	0.57
Henley	24	73	2	0.52
Confluence	10	45	41	0.63
Marlboro	16	60	22	0.64
Buccleuch	41	42	13	0.64
Midrand	19	55	24	0.58
Kyalami	29	50	16	0.58
N14	47	50	2	0.6

The Jukskei and Klip River sediments show similarly low TOC levels, recording values of <1%. This indicates that there is no significant difference in the organic pollutant partitioning between sampling sites. The proportion of clay particles varied from 2% to 41% for the Klip River sediments and 2 to 22% for the Jukskei River (Table S3.1).

Table S3.2 Jukskei River PAH source identification

Source Index	Marlboro	Buccleuch	Midrand	Kyalami	N14	Comment
Maxima at parent PAH vs alkyl PAH	✓(2-MA)	✓	✓(2-MA)	✓(2-MA)	✓(2-MA)	Pyrogenic source
1MP/P <1	✓	✓	✓	✓	✓	Pyrogenic source
F: Py ratio F/(F+Py) >0.5	✓	✓	✓	✓	✓	Pyrogenic source
BeP/BaP <1	✓	✓	✓	✓	✓	Pyrogenic source
P>A (commonly used)	✓	✓	✓	✓	✓	Petrogenic source
A/(A+P)>0.1	✓	✓	✓	✓	✓	Pyrogenic source
10>P/A>5	✓	✓	X (4.4)	✓	✓	mixed source
BaA/C >0.5	✓	✓	✓	✓	✓	Either petroleum or coal combustion
Abundance of F, Py, & P	✓	✓	✓	✓	✓	Coal and wood source
Methylphenanthrenic ratio (MPI 3)	1.8	(0.8)	1.8	1.8	1.7	Coal source of the same thermal maturity and origin

Table S3.3 Klip River PAH source identification

Source Index	Alberton	Henley	Confluence	Comment
Maxima at parent PAH vs alkyl PAH	✓ (2-MA)	✓ (2-MA)	✓ (2-MA)	Pyrogenic source
1MP/P <1)	✓	✓	✓	Pyrogenic source
F: Py ratio (F/F+Py) >0.5	X (0.5)	X (0.47)	✓	Pyrogenic source
BeP/BaP<1	✓	✓	✓	Pyrogenic source
P>A (commonly used)	✓	✓	✓	Petrogenic source
A/(A+P)>0.1	✓	✓	✓	Pyrogenic source
P/A<5	✓	✓	✓	Pyrogenic source
BaA/C >0.5	✓	✓	✓	Either petroleum or coal combustion
Abundance of F, Py, & P	✓	✓	✓	Coal and wood source
Methylphenanthrenic ratio (MPI 3)	1.5	1.6	1.5	Coal source of the same thermal maturity and origin

Chapter 4

Seasonal variation of chloro-s-triazines in the Hartbeespoort Dam catchment, South Africa

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Abstract

Seasonal variation of eight chloro-s-triazine herbicides and seven major atrazine and terbuthylazine degradation products was monitored in the Hartbeespoort Dam catchment using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS/MS). Lake, river and groundwater were sampled from the Hartbeespoort Dam catchment over four seasons and the downstream Jukskei River was monitored during the winter season. Triazine herbicide concentrations in the Hartbeespoort Dam were in the order atrazine>simazine>propazine>ametryn>prometryn throughout the four seasons sampled. Triazine herbicide concentrations in the Hartbeespoort Dam surface water were highest in summer and gradually decreased in successive seasons of autumn, winter and spring. Terbuthylazine was the only triazine herbicide detected at all sampling sites in the Jukskei River, though atrazine recorded much higher concentrations for the N14 and Kyalami sites, with concentrations of 923 and 210 ng L^{-1} respectively, compared to 134 and 74 ng L^{-1} for terbuthylazine. Analytical results in conjunction with river flow data indicate that the Jukskei and Crocodile Rivers contribute the greatest triazine herbicide loads into the Hartbeespoort Dam. No triazine herbicides were detected in the fish muscle tested, showing that bioaccumulation of triazine herbicides is negligible. Atrazine and terbuthylazine metabolites were detected in the fish muscle with deethylatrazine (DEA) being detected in both catfish and carp muscle at low concentrations of 0.2 and 0.3 ng g^{-1}, respectively. Desethylterbuthylazine (DET) was detected only in catfish at a concentration of 0.3 ng g^{-1}. With atrazine herbicide groundwater concentrations being >130 ng L^{-1} for all seasons except spring and groundwater ∑triazine herbicide concentrations ranging between 527 and 367 ng L^{-1}, triazine compounds in the Hartbeespoort Dam catchment may pose a risk to humans and wildlife in light findings of endocrine and immune disrupting atrazine effects by various researchers.

Introduction

The presence of chloro-s-triazines in the environment is predominantly due to herbicide application, particularly from atrazine, simazine, terbuthylazine, ametryn, gesatamin, propazine and prometon (Wackett et al., 2002; Zhang et al., 2016). South Africa is historically the 9th biggest corn producer in the world and atrazine is often used as the herbicide of choice, with 88% of atrazine in the country being applied on corn, though in combination with other triazine herbicides (Dabrowski, 2015; Du Preez et al.,

2005). After application, triazine herbicides can seep through soil to contaminate groundwater due to their moderately hydrophilic nature, low K_{ow} and weak soil adsorption (Du Preez et al., 2005; Graymore et al., 2001; Solomon et al., 2008). Atrazine is the most widely used chloro-s-triazine globally and has been detected in various water bodies and groundwater throughout South Africa since the 1980's (Ansara-Ross et al., 2012; Takáts et al., 2001). Its use is banned in Europe since 2004 due to its relative persistence and risk of water contamination, though the use of the other triazine herbicides is still permitted (Ackerman, 2007; Hang et al., 2005; Herranz et al., 2008; Lin et al., 2011; Omotayo et al., 2011; Wang and Xie, 2012). Atrazine is however still used extensively in China, USA, India, South America, Africa and Australia (Ansara-Ross et al., 2012; Liu et al., 2016; Siddiqua et al., 2010). The maximum allowable limit of atrazine in drinking water in the USA and India is 3 µg L^{-1} (Singh and Cameotra, 2014) whilst the European maximum permissible level of atrazine in drinking water is 0.1 µg L^{-1} (El Sebaï et al., 2011; Herranz et al., 2008; Omotayo et al., 2011).

Triazine herbicide mode of action proceeds by inhibiting photosynthetic electron transport and blocking CO_2 fixation, causing accumulation of CO_2 in the plant, leading to plant death (Wackett et al., 2002; Wiegand et al., 2001). Atrazine is a herbicide of major significance in the Hartbeespoort Dam catchment as it is historically found in the highest concentrations in surface water (Ansara-Ross et al., 2012). In fish, atrazine has been shown to have effects ranging from inhibition of acetylcholinesterase, leading to decreased reflexes, increased respiration (Wiegand et al., 2001) and in addition, it has been discovered to cause a range of adverse effects on the reproductive and immune systems of oral and intravenously exposed rats and frogs (Ross and Filipov, 2006). The effects of ecologically relevant atrazine concentrations on biological species is highly controversial and the scientific community remains divided as there have been inconsistent conclusions drawn by various scientists in different studies, based on different endpoints including growth and gonadal abnormalities (Rohr and McCoy, 2010).

Atrazine has been proven to be an endocrine disruptor in many species (Freeman et al., 2011; Rohr and McCoy, 2010). However, numerous studies of effects of atrazine on the reproductive, immune, nervous and immune systems on humans and animals have produced contradicting results with lack of consistency (Rohr and McCoy, 2010; Solomon et al., 2008; Wirbisky and Freeman, 2017). Atrazine has been observed to cause gene alterations that lead to endocrine disruption in zebrafish, inhibition of ovary growth in crabs, altering sex ratios in crayfish and frogs as well as demusculinising male gonads in

fish, amphibians, reptiles and mammals (Hayes et al., 2011; Loughilin et al., 2016; Silveyra et al., 2017; Wirbisky and Freeman, 2017).

Atrazine and terbuthylazine degradation

Metabolism of atrazine and terbuthylazine is species-specific (Catenacci et al., 1993). In the majority of animals fed with radiolabelled atrazine, the vast majority of atrazine was eliminated unmetabolised, indicating a low bioaccumulation (Solomon et al., 2008). The degradation of triazine herbicides proceeds by microbial, photolytic or non-enzymatic chemical action such as the benzoxazinone catalysed reaction (Lin et al., 2008; Wiegand et al., 2001). This results in dechlorination, deamination or dealkylation, forming a variety of degradation products (Belfroid et al., 1998; Graymore et al., 2001; Loos and Niessner, 1999; Wang and Xie, 2012). The degradation of atrazine in the environment is mainly due to microbial action (Wackett et al., 2002). Atrazine can be metabolized by plants through N-dealkylation primarily by cytochrome P-450 followed by a phase II conjugation reaction to glutathione by glutathione-S-transferase (Ross and Filipov, 2006; Wiegand et al., 2001). Atrazine metabolites have been detected in carp liver of fish exposed to different concentrations of atrazine ranging from 4.28 to 428 µg L^{-1} (Xing et al., 2014) and *in vitro* atrazine exposure tests have shown the presence of monodealkylated deethylatrazine and desisopropylatrazine. Desisopropylatrazine can also be formed by degradation of both atrazine and simazine whilst deethylatrazine can be formed by degradation of atrazine and propazine (Jiang et al., 2005).

Terbuthylazine inhibits photosynthesis by altering chloroplast membrane proteins. Its degradation proceeds primarily either by microbial N-dealkylation of one of the s-triazine side chains to form desethylterbuthylazine (DET) or photolytic hydroxylation and dechlorination of the C2 chlorine to form hydroxyterbuthylazine and desethylhydroxyterbuthylazine (Bottoni et al., 2013; Sanlaville et al., 1996; Velisek et al., 2016). Terbuthylazine is slightly toxic to fish and can also be degraded to desisopropylatrazine and atrazine desethyldesisopropyl which are secondary degradation products arising from terbuthylazine metabolism (Du Preez et al., 2005). This study aims to assess the degree of triazine pollution in the Hartbeespoort Dam catchment and determine the points with the most significant influence triazine pollution for ecological risk profiling. The study design is only indicative of the seasonal variation of triazine herbicides as well as atrazine and terbuthylazine degradation products

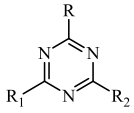
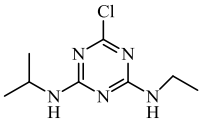
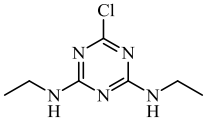
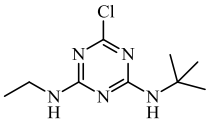
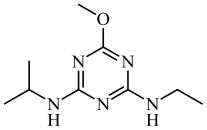
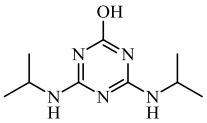
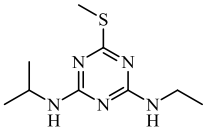
in the Hartbeespoort Dam catchment area. Though triazine herbicides are still used in South Africa, very little studies have looked at their fate in the environment and biota, particularly triazine metabolites.

Materials and Methods

Chemicals and reagents

All analytical standards had a purity ≥96% (Tables 4.1 & 4.2) and all solvents had a purity >99.5%. Ultrapure Milli-Q water used in all preparation work was produced by a Millipore Advantage system (Merck, Johannesburg, South Africa) with a total organic carbon <3 mg L^{-1}.

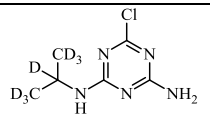
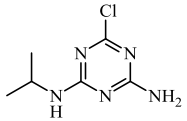
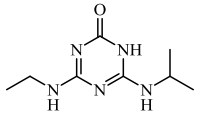
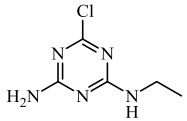
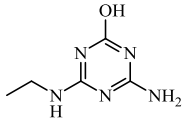
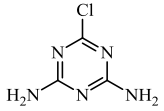
Table 4.1 Triazine herbicides analysed by GC-MS

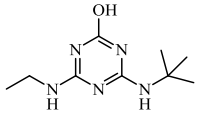
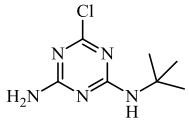
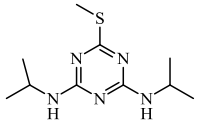
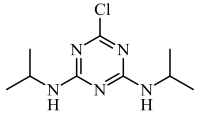
Compound		Supplier	Purity (%)	T	Q1	Q2	Q3
Triazine functional group							
Atrazine	 CAS # 1912-24-9	Accustandard	100	200	215	202	173
Simazine	 CAS # 122-34-9	Accustandard	99.6	201	186	158	173
Terbuthylazine	 CAS # 5915-41-3	Accustandard	100	214	173	216	229
Gesatamin	 CAS # 1610-17-9	Accustandard	99.8	196	211	169	58
Prometon	 CAS # 1610-18-0	Accustandard	100	210	168	225	58
Ametryne	 CAS # 834-12-8	Accustandard	100	227	212	170	98

T=Target ion

Q=Qualifier ion

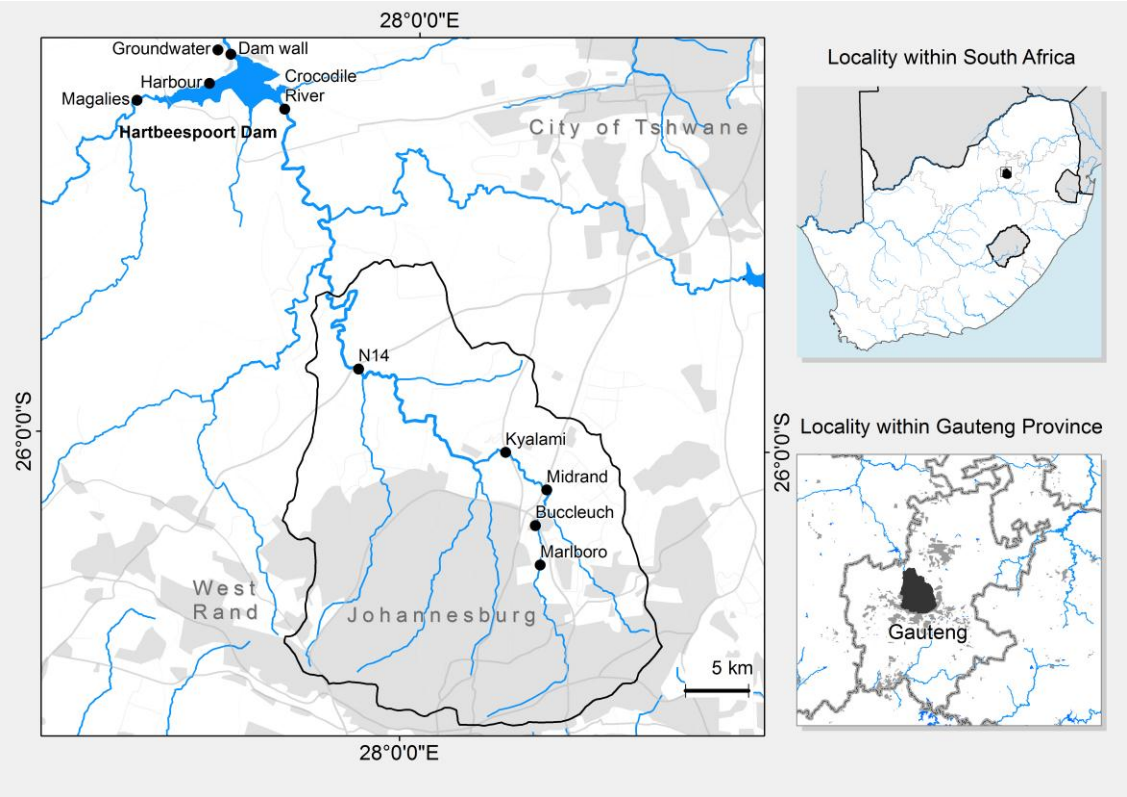
Table 4.2 Triazine degradation products and herbicides analysed by LC-MS/MS

Compound		Supplier	Formation mechanism	Purity (%)	$[M+H]^+$	Product ion (Confirmation ions)
D7 deethylatrazine	 CAS # 1216649-31-8	Toronto Research Chemicals	Internal Standard	96	195.6	147.5 (68.2, 62.2)
Deethylatrazine (DEA)	 CAS # 6190-65-4	Dr Ehrenstorfer	Cytochrome P450 dealkylation (liver microsome metabolism) and microbial dehalogenation of atrazine (Graymore et al., 2001; Papadopoulos et al., 2012; Singh and Cameotra, 2014).	98	188.1	146.4 (68.2, 62.2)
Hydroxyatrazine (HA)	 CAS # 2163-68-0	Dr Ehrenstorfer	Chemical action (Graymore et al., 2001; Palm and Zetzsch, 1996) and microbial (enzymatic) dechlorination of atrazine (Lin et al., 2008; Seffernick et al., 2007; Wackett et al., 2002).	98	198.1	156.4 (86.2, 69.2)
Desisopropylatrazine (DIA)	 CAS #1007-28-9	Toronto Research Chemicals	Cytochrome P450 dealkylation (liver microsome metabolism), microbial dehalogenation of atrazine (Graymore et al., 2001; Papadopoulos et al., 2012) and terbuthylazine dealkylation (Du Preez et al., 2005).	99	174.1	68.2 (79.2, 62.2)
Atrazine desisopropyl-2-hydroxyl (AD-2OH)	 CAS # 7313-54-4	Dr Ehrenstorfer	Microbial dechlorination and dealkylation of atrazine (Singh and Cameotra, 2014).	98	156.1	69.2 (114.3, 86.3)
Atrazine desethyl-desisopropyl (ADD) (Didealykylatrazine)		Dr Ehrenstorfer	Microbial dehalogenation of atrazine (Graymore et al., 2001; Singh and Cameotra, 2014) and terbuthylazine metabolism (Du Preez et al., 2005)	96	146	68.2 (104.2, 62.2)

Hydroxyterbuthylazine (HT)	CAS #3397-62-4  CAS # 66753-07-9	Dr Ehrenstorfer	Chemical and microbial dechlorination of terbuthylazine with concomitant hydroxylation (Palm and Zetzsch, 1996; Papadopoulos et al., 2012)	98	212.1	156.4 (69.2, 86.2)
Desethylterbuthylazine (DET)	 CAS # 30125-63-4	Dr Ehrenstorfer	Cytochrome P450 dealkylation of terbuthylazine (liver microsome metabolism) (Du Preez et al., 2005; Papadopoulos et al., 2012)	98	202.1	146.4 (68.2, 62.2)
Prometryn	 CAS # 7287-19-6	Dr Ehrenstorfer	Herbicide	99	241	68.2 (200.5, 74.2)
Propazine	 CAS # 139-40-2	Dr Ehrenstorfer	Herbicide	99	214	68.2 (79.2, 62.2)

Study area and sampling

Five sampling sites were located primarily around the major inlets and outlet of the Hartbeespoort Dam (Figure 4.1) for determination of the point with the most influence on the Hartbeespoort Dam pollution. Another 5 points were located upstream of the Hartbeespoort Dam, in the Jukskei River where atrazine-use maps, produced by Dabrowski (2015), show significant atrazine use. The GPS coordinates of the Hartbeespoort Dam catchment sampling points are listed in supplementary information (SI) Table S4.1. The Jukskei River sampling points were sampled in the winter season. They are located downstream of heavily anthropogenically impacted areas further downstream in Johannesburg where the N14, Kyalami, Midrand, Buccleuch and Marlboro sites are located. The Marlboro, Buccleuch and Midrand sites are located primarily in areas with significant built construction. Sources of triazine herbicides in the Jukskei River catchment include golf courses located within 7 km upstream of the Marlboro site and peri-urban agricultural activities. The Magalies River point is located downstream of rich farming land further upstream where herbicides are extensively used (Dabrowski, 2015). The Harbour point is situated at the Department of Water and Sanitation offices and is also where boats are launched daily into the Hartbeespoort Dam for dam remediation projects. The groundwater site is situated in close proximity to the Dam wall point (1.1 km away) and the water is used for drinking purposes by the surrounding community and government offices. Sampling was conducted between November 2014 and September 2015, covering the four seasons of the year (Table 4.3). Sediment samples were sampled using a Van Veen grab sampler, filling a 500 mL glass jar. Water samples were sampled by sub-surface grab sampling using a bailer for the Hartbeespoort Dam 5 and 30 metre depths, filling a 4 litre amber glass bottle. All samples were taken in a random manner in a 2 metre radius. The sampling plan was designed to coincide with peak herbicide application.



The GPS coordinates of the sampling sites (as marked by the dots) can be found in the SI (Table S4.1). The Jukskei River catchment is shown in black border

Figure 4.1 Sampling area- Hartbeespoort Dam catchment

Seasons and climate

The Hartbeespoort Dam is characterized by a subtropical climate with summer rainfall and dry winters (Hely-Hutchinson and Schumann, 1998). According to the South African weather service, South African climate can be divided into 4 seasons (Table 4.3).

Table 4.3 Sampling seasons and dates

Calendar dates	Season	Sampling dates
1 September to 30 November	Spring	14 November 2014
1 December to 28/29 February	Summer	13 February 2015
1 March to 31 May	Autumn	15 May 2015
1 June to 31 August	Winter	14 August 2015

(South African Weather Service a)

The 2014/2015 sampling period was characterized by moderately less than normal rainfall in the Hartbeespoort Dam catchment area, though the average rainfall was still within the 300-500 mm, 7-year historical level (South African Weather Service b). Of particular significance is the average annual rainfall for the Gauteng region which maintained the historical levels of 200-2000 mm, whose run-off contributes 90% of the water in the Hartbeespoort Dam from flow through the Crocodile River (Amdany et al., 2014; South African Weather Service b). Three main strategic sampling points have been identified as overtly influential to humans and wildlife. These are (i) the Crocodile River (and its upstream Jukskei sampling points) due to its significant contribution to the Hartbeespoort Dam by volume (ii) the Dam wall point, due to its offloading of pollutants out of the dam and (iii) the groundwater point due to it being a source of drinking water for humans.

Sample preparation and analysis

Sample preparation

Water samples

Bond Elut Plexa solid-phase extraction (SPE) cartridges (200 mg Styrene divinyl benzyl, Agilent Technologies, Chemetrix, Johannesburg, South Africa) were conditioned sequentially with 6 mL methanol (Sigma-Aldrich, Germany), 6 mL dichloromethane (DCM, (Sigma-Aldrich, Germany)) and 6 mL Milli-Q water before loading 1 L water samples onto them at a flow rate of 10 mL min^{-1}. The cartridges were dried under gentle vacuum before transporting to The Netherlands under frozen conditions for extraction and analysis. The SPE method was validated against precision and reproducibility using D7 deethylatrazine as an internal standard. For GC analysis, the cartridges were eluted with 6 x 500 µL DCM portions before evaporating under a gentle stream of nitrogen and reconstituting with 1 mL toluene. For LC-MS/MS analysis the cartridges were eluted with 3 x 500 µL DCM followed by 3 x 500 µL methanol portions. Extra sample cleanup was performed by dispersive SPE (dSPE) after concentrating the DCM/methanol extract to near dryness with a gentle stream of nitrogen and changing the solvent to acetonitrile (Sigma-Aldrich, Missouri, USA). The acetonitrile extracts were added to a 10 mL Supel™ QUE PSA/C18(EN) (Deisenhofen, Germany) cleanup tube before adding 6 mL acetonitrile and vortexing for 30 sec at 35 Hz, centrifuging for 8 min at 3800g (4000 rpm) and transferring the acetonitrile extract to a new test tube. The extract was evaporated to near dryness under a gentle stream of nitrogen and reconstituted in 200 µL of 10% methanol (Milli-Q water: Methanol, 90:10 v/v).

Fish samples

Three 5 year old catfish (*Clarias gariepinus*) and three 5 year old carp (*Cyprinus carpio)* were caught on 14 August 2015 using mobile nets and transported to the laboratory where internal organs were removed and separated prior to frozen storage for 2 days at -20 ^{o}C before analysis. Gravimetric measurements of the fish are shown in SI Table S4.2. The freezing process increases membrane permeability and causes phase separation of membrane components (Wolkers et al., 2007). The age of the catfish was estimated using weight-length ratios and the age of the carp was estimated by using a combination of weight-length ratios and scale annuls, using scales taken from between the lateral line and pectoral fin. Five year old fish were selected as their long life could have potentially lead to significant levels of exposure and bioaccumulation of pollutants. Fish muscle (15 g) from each of the 3 fish was pooled together and homogenized using a blender to create a representative sample. Fish muscle samples (28 g fresh weight [fw]) were weighed into two equal portions of 14 g into two 50 mL fluoroethylenepropylene centrifuge tubes. A 40 µL internal standard with a concentration of 1 mg L^{-1} D7 deethylatrazine was added to each centrifuge tube. Acetonitrile (8 mL) and 0.2 g NaCl (Merck, Darmstadt, Germany) were added before manually shaking vigorously for 15 sec, vortexing for 15 sec at 35 Hz and allowing the mixture to equilibrate for 12 hours. The samples were centrifuged for 15 min at 3800g (4000 rpm) and the upper organic layer was removed before adding another 8 mL acetonitrile, vortexing for 15 sec at 35 Hz and centrifuging for 15 min at 3800g (4000 rpm). The second organic layer was combined with the first organic layer in a clean centrifuge tube.

A modified Quick Easy Cheap Efficient Rugged Safe (QuEChERS) method was developed based on prior work conducted by Vudathala et al., (2010). A QuEChERS kit (Agela Technologies, Delaware, USA) containing 50 mg primary secondary amine (PSA), 50 mg C_{18}, 150 mg $MgSO_4$ was added to the fish extracts, together with an additional 200 mg $MgSO_4$ (Sigma-Aldrich, Missouri, USA), 200 mg PestiCarb (Agela Technologies, Delaware, USA), 100 mg diatomaceous earth (Sigma-Aldrich, Missouri, USA) and 250 mg basic alumina (Sigma-Aldrich, Missouri, USA) in a centrifuge tube. The sample extracts were vortexed for 15 sec at 35 Hz before being left to settle for 5 min and centrifuged for 8 min at 3800g (4000 rpm). The supernatant was transferred to a clean test tube, combining the two 14 g sample extracts and evaporated in a water bath at 38 ^{o}C to near dryness. Extracts for GC-MS analysis were reconstituted with 1 mL toluene (J. T. Baker, Deventer, The Netherlands) and for LC-MS/MS analysis with 200 µL of 10% methanol.

Chemical analysis

Analysis of atrazine, simazine, terbuthylazine, ametryn, prometon and gesatamin was performed with gas chromatography coupled to a mass-spectrometer (GC-MS, GC 6890N, MS 5975, Agilent Technologies, CA, USA) with oven temperature programming 70 ^{o}C (2 min), 25 ^{o}C min^{-1} to 150 ^{o}C, 3 ^{o}C min^{-1} to 200 ^{o}C, 8 ^{o}C min^{-1} to 280 ^{o}C (17 min) with a 1 µL splitless injection and an injector temperature of 280 ^{o}C. A Zebron ZB-Multiresidue 1 column (30 m x 0.25 mm x 0.25 µm; Phenomenex, USA) was used with constant pressure of 151 kPa.

Analysis of propazine, prometryn, deethylatrazine (DEA), hydroxyatrazine (HA), desisopropylatrazine (DIA), atrazine desisopropyl-2-hydroxyl (AD-2OH), atrazine desethyl-desisopropyl (ADD), hydroxyterbuthylazine (HT) and desethylterbuthylazine (DET) was performed with liquid chromatography coupled to a triple quadrupole mass-spectrometer (LC-MS/MS, 1200 series LC system, 6410 triple quadrupole MS; Agilent Technologies, Amstelveen, The Netherlands). After comparing different C_{18} columns with different ionization modes at different pH, buffer conditions and mobile phase ratios, the method was finally optimized on a Biphenyl 100 Å LC-column (2.6 µm, 100 x 2.1 cm) with gradient mobile phase comprising of 5 mM ammonium formate (pH 4, solvent A) and 1.5% formic acid in methanol (solvent B) with a 10 µL sample injection volume. A 0.3 mL min^{-1} flow rate was used with a gradient of 0% B 0-2 min, 20% B 2 min; 95% B 10 min, 0% B 15.5-30 min. The LC-MS/MS was performed using an electrospray ionization (ESI) source in positive mode, source spray voltage 4 kV, transfer capillary temperature 350 ^{o}C, gas flow 9 mL min^{-1} and nebulizer pressure 40 psi. Transitions are listed in Table 4.2.

Stock solutions for GC-MS analysis were made up to 100 mg L^{-1} in toluene. Stock solutions for LC-MS/MS analysis were made up to 1 mg mL^{-1} in methanol, with the exception of HT, ADD and HA which have very low solubility in methanol hence were made in a lower concentrations of 100 mg L^{-1} in Milli-Q water: methanol (50:50 v/v). A 7 point calibration curve was used for GC-MS analysis, ranging from 0.0156 mg L^{-1}, 0.0313 mg L^{-1}, 0.0625 mg L^{-1}, 0.125 mg L^{-1}, 0.25 mg L^{-1}, 0.5 mg L^{-1} to 1 mg L^{-1}. A 10 point calibration curve was used for LC-MS/MS analysis, ranging from 0.2 ng L^{-1}, 0.4 ng L^{-1}, 0.8 ng L^{-1}, 2 ng L^{-1}, 4 ng L^{-1}, 10 ng L^{-1}, 20 ng L^{-1}, 40 ng L^{-1}, 80 ng L^{-1} to 200 ng L^{-1}. All calibration curves had a regression of 0.998 or better. A combination of standard addition and labelled internal standard calibration was used to calculate recoveries and compensate for matrix effects. Recoveries for atrazine herbicides ranged from 80 to

102% and atrazine degradation products ranged from 69 to 112% (SI Table S4.3). Limit of detection (LOD) and limit of quantification (LOQ) were calculated at 3x and 10x the signal to noise ratio respectively. The analytical method robustness was successfully tested against precision and repeatability using 11 samples. No triazine compounds were detected in the blank samples (n=6) analysed.

Results and discussion

The ∑triazine herbicide abundance concentrations in the Hartbeespoort Dam were in the order atrazine>simazine>terbuthylazine>propazine>ametryne>prometryn (Table 4.4). The results show that ∑triazine concentrations in the Hartbeespoort Dam peak in summer and gradually decrease throughout successive seasons of autumn, winter and spring. This can be explained as the vast majority of the maize crops have emerged by mid-summer and herbicide application to kill weeds has been conducted as pre- or post-emergence application. In the Hartbeespoort Dam, atrazine was found in the highest concentrations in summer with average concentrations around 800 ng L^{-1} where sampling was conducted at the height of the seasonal annual rains. The average atrazine levels declined to around 600 ng L^{-1} in autumn, further declining to around 500 ng L^{-1} in winter and declining again to around 400 ng L^{-1} in spring. Flow data for the Crocodile River (Figure 4.2) obtained from gauging station A2H012 and flow data for the Magalies River (Figure 4.3) obtained from gauging station A2H013 (DWA Hydrology, 2017) shows that the summer season had the highest flows as it is in the peak of the rainy season. The summer season recorded average flows of 21 and 0.54 m^3 s^{-1} in the Crocodile and Magalies Rivers respectively.

The ∑Crocodile River triazine herbicide concentrations were similar to the ∑Dam wall triazine herbicide concentration at 30 m below the water surface (2 to 5 m above the bottom) in autumn (2185 and 2378 ng L^{-1}, respectively) with a relative standard deviation (RSD) of 6% and winter (1863 and 1903 ng L^{-1}, respectively) with a RSD of 1.5%. This shows that the in the cold season, the colder water from the Crocodile River (Figure 4.4) passes along the bed of the dam to the outflow from autumn through to spring as described by Hely-Hutchinson & Schumann, (1998). In the spring and summer seasons where the Crocodile River water was warmer than the Hartbeespoort Dam water (Figure 4.4), the ∑Crocodile River triazine herbicide concentrations showed much higher differences with the ∑Dam wall 30 m

triazine herbicide concentrations. The ∑Crocodile River triazine herbicide and ∑Dam wall 30 m triazine herbicide concentrations were 760 and 1653 ng L^{-1} respectively in spring (RSD of 52%), and 2287 and 5061 ng L^{-1} respectively in summer (RSD of 55%).

With 8 days recording flow rates >40 m^3 s^{-1} in the summer season, the Crocodile River has much higher flows that the Magalies River which recorded flows <3.5 m^3 s^{-1} in all seasons (Figures 4.2 & 4.3). The Magalies River showed similar ∑triazine herbicide concentrations with the Crocodile River, however the much lower Magalies River flows into the Hartbeespoort Dam (Figure 4.3) means it has a less significant effect on the Hartbeespoort Dam water chemistry compared to the Crocodile River (Figure 4.2). The high triazine compound concentrations in summer (Tables 4.4 & 4.5), coupled with increased summer flow rates into the Hartbeespoort Dam shows that the summer season has the largest contribution of triazine herbicides into the Hartbeespoort Dam. The last month of spring experienced high flows into the Hartbeespoort Dam whilst the first month of autumn experienced high flows of water as these mark the beginning and end of the rainy season, respectively. The Crocodile River flow is on average 38 times higher than the Magalies River flow, year on year. This shows that the Crocodile River has a much higher influence on the water quality of the Hartbeespoort Dam. The hydraulic residence time of water in the Hartbeespoort Dam is one year, with water at the bottom having a lower residence time as the outflow port is 11 m above the bottom of the dam (Hely-Hutchinson and Schumann, 1998). The high summer ∑triazine herbicide concentration (5061 ng L^{-1}) at the Dam wall at 30 m depth (Table 4.4) can be explained by the combined effect of thermal stratification with loading of the hypolimnion by triazines from the Crocodile and Magalies Rivers and resuspension of pollutants in the sediments by bottom feeding fish.

The lower Jukskei River catchment sampling sites of Midrand, Buccleuch and Marlboro which are located in highly industrialised areas recorded very low atrazine levels, below the LOD as there are no significant undeveloped land areas available for growing plants. The furthermost downstream point of Marlboro which is located downstream of two golf courses, recorded a significant simazine concentration of 658 ng L^{-1}. However the immediate successive three downstream points had no significant simazine concentrations as they were below the LOD. This may be attributed to the dilution effect as the Jukskei River flow gets significantly stronger downstream of the Marlboro point. The terbuthylazine concentrations through the Jukskei River transect was fairly consistent, approximately 100 ng L^{-1} and propazine concentrations were between 65 and up to 208 ng L^{-1} (Table 4.4).

In the Jukskei River, terbuthylazine and propazine were quantified at all the Jukskei River points. The N14 site recorded high atrazine, and simazine concentrations of 923, and 480 ng L^{-1} and the Marlboro point recorded the highest simazine concentration of 658 ng L^{-1}. The highest Jukskei River atrazine winter concentration of 923 µg L^{-1} was recorded in the N14 site, being higher than the downstream Crocodile River inlet point winter concentration of 523 ng L^{-1} as well as the Magalies River inlet point winter concentration of 503 ng L^{-1}. This indicates that the Jukskei River contributes significantly to Hartbeespoort Dam atrazine herbicides. A comparison of historical data shows a decline in atrazine concentrations over the past three decades as values recorded by Ansra-Ross et al., (2012) show surface water concentrations of 730 to 14,970 ng L^{-1} and groundwater concentrations of 490 to 3,890 ng L^{-1} during the early 1990's in maize growing areas in Gauteng Province. The major cause of the decrease is due to expanded urban development in the province where city developments have taken over previous farming areas especially along the Jukskei River.

Groundwater triazine concentrations were similar throughout the four seasons, recording averages of 156, 155 and 67 ng L^{-1} for atrazine, simazine and terbuthylazine respectively. Groundwater atrazine concentrations did not follow the surface water trends of a gradual decrease from summer season, as the highest concentration of 180 ng L^{-1} was recorded in summer followed by the winter season with a concentration of 152 ng L^{-1}. The average atrazine groundwater concentration for the four seasons of 156 ng L^{-1} was much lower than the atrazine concentrations detected in the Hartbeespoort Dam surface water but higher than the European maximum permissible atrazine level for drinking water of 100 ng L^{-1} and lower than the USA and Indian maximum permissible levels of 3000 ng L^{-1}. There are currently no maximum permissible levels for atrazine in drinking water prescribed in South Africa, though a target range of 0 to 2000 ng L^{-1} is stated in the South African Water Quality Guidelines for domestic water use. The highest atrazine concentration recorded was at the Dam wall site in summer which peaked at 1570 ng L^{-1} sampled at a depth of 30 m which is within the target range.

Table 4.4 Triazine herbicides in the water samples from Hartbeespoort Dam catchment (ng L^{-1})

	Atrazine	Simazine	Terbuthylazine	Ametryn	Prometon	Gesatamin	Propazine	Prometryn	∑Triazine herbicide
LOD 3x	5	5	5	5	5	5	0.1	0.1	
LOQ 10x	17	17	17	15	17	16	0.3	0.2	
Summer									
Crocodile R.	940	580	N.D	340	N.D	N.D	419	7	2287
Dam wall 30m	1570	610	1969	306	N.D	28	574	4	5061
Harbour	1237	654	684	112	N.D	N.D	877	6	3570
Magalies R.	830	630	19	260	N.D	20	486	5	2250
Groundwater	180	30	80	N.D	N.D	N.D	77	N.D	367
Autumn									
Crocodile R.	631	556	547	86	N.D	N.D	362	3	2185
Dam wall 30m	699	598	576	88	N.D	N.D	415	3	2379
Harbour	760	636	602	95	N.D	N.D	790	5	2888
Magalies R.	644	570	504	87	N.D	N.D	359	3	2166
Groundwater	138	217	61	N.D	N.D	N.D	75	N.D	491
Winter									
Crocodile R.	523	471	425	75	N.D	N.D	366	4	1863
Dam wall 30m	576	485	460	78	N.D	N.D	301	3	1903
Harbour	543	480	433	75	N.D	N.D	415	5	1950
Magalies R.	503	438	412	77	344	N.D	231	6	2010
Groundwater	152	221	66	N.D	N.D	N.D	88	N.D	527
Spring									
Crocodile R.	483	N.D	117	N.D	N.D	N.D	150	10	760
Dam Wall 30m	503	503	378	71	N.D	N.D	198	N.D	1653
Harbour	453	453	378	68	N.D	N.D	348	3	1703
Magalies R.	100	99	53	N.D	N.D	N.D	48	1	299
Groundwater	154	154	64	N.D	N.D	N.D	76	N.D	447
Dam wall 5m	450	395	360	67	N.D	N.D	167	2	1442
Jukskei River Winter									
N14	923	480	134	N.D	N.D	N.D	66	<0.2	1602
Kyalami	210	<5	74	N.D	N.D	N.D	208	6	499
Midrand	<5	<5	148	N.D	N.D	N.D	65	1	214
Buccleuch	<5	<5	110	N.D	N.D	N.D	92	1	202
Marlboro	<5	658	130	N.D	N.D	N.D	80	1	869

N.D.= Not detected

< Below LOQ

Data on atrazine and terbuthylazine degradation products in South Africa is scanty and has never been studied in the Hartbeespoort Dam, though DIA, DEA and ADD have been assessed upstream of the Magalies River (Du Preez et al., 2005). DET is the degradation product found in the highest concentrations in the Hartbeespoort Dam sites with concentrations >300 ng L^{-1} for the Crocodile River site in summer, autumn and winter seasons (Table 4.5). Seasonal trends for triazine degradation products follow the triazine herbicide trends with the highest seasonal triazine ∑degradation product concentrations of 2529 ng L^{-1} being recorded in summer and gradually decreasing in successive seasons through to spring which recorded the lowest ∑degradation product concentration of 1220 ng L^{-1} (Table 4.5). Of the triazine degradation products tested in the Jukskei River, DET concentrations were highest, recording concentrations >100 ng L^{-1} at all sites. DEA had the second highest triazine ∑degradation product concentrations in both the Hartbeespoort Dam and Jukskei River points. DET has been described as the main degradation product of terbuthylazine and is usually found in high concentrations in impoundments in close proximity to areas treated with terbuthylazine (Bottoni et al., 2013; Stara et al., 2016) The triazine ∑degradation product concentration was in the order DET>DEA>DIA>HT>HA. ADD and AD-2OH could not be detected in any of the water samples tested. The data reveals a wide variation in ∑degradation product concentration contribution from different sites with the Crocodile River showing the highest ∑degradation product concentration in summer and winter of 667 and 509 ng L^{-1} respectively, whilst the Harbour site had the highest ∑degradation product concentration in the autumn and summer seasons of 578 and 569 ng L^{-1} respectively.

Table 4.5 Atrazine and terbuthylazine degradation products (DP) in water samples from the Hartbeespoort Dam catchment (ng L^{-1})

	DIA	HA	DEA	HT	DET	ADD	AD-2OH	∑DP
LOD 3X	0.2	0.1	0.1	0.1	0.1	0.1	0.1	
LOQ10X	0.5	0.3	0.2	0.3	0.3	0.3	0.2	
Summer								
Crocodile R.	16	4	184	16	447	N.D	N.D	667
Dam wall 30m	17	6	164	20	388	N.D	N.D	594
Harbour	18	4	127	17	402	N.D	N.D	569
Magalies R.	14	4	162	13	277	N.D	N.D	470
Groundwater	6	1	85	1	136	N.D	N.D	229
Autumn								
Crocodile R.	14	3	157	13	313	N.D	N.D	500
Dam wall 30m	14	4	144	18	321	N.D	N.D	501
Harbour	15	3	171	12	377	N.D	N.D	578
Magalies R.	13	3	114	11	247	N.D	N.D	387
Groundwater	5	1	52	1	99	N.D	N.D	157
Winter								
Crocodile R.	22	4	166	13	303	N.D	N.D	509
Dam wall 30m	18	4	147	13	239	N.D	N.D	421
Harbour	19	4	172	14	282	N.D	N.D	491
Magalies R.	10	2	129	7	225	N.D	N.D	373
Groundwater	2	1	33	1	122	N.D	N.D	159
Spring								
Crocodile R.	10	2	106	6	197	N.D	N.D	322
Dam Wall 30m	9	2	105	6	174	N.D	N.D	297
Harbour	6	1	80	5	251	N.D	N.D	343
Magalies R.	2	1	19	2	30	N.D	N.D	53
Groundwater	6	1	68	1	129	N.D	N.D	204
Dam Wall 5m	6	2	91	8	232	167	2	507
Jukskei River Winter								
N14	3	1	45	5	104	N.D	N.D	158
Midrand	8	3	82	11	187	N.D	N.D	291
Kyalami	3	1	46	4	120	N.D	N.D	174
Marlboro	15	5	92	9	149	N.D	N.D	271
Buccleuch	9	2	81	4	133	N.D	N.D	230

ND= Not detected

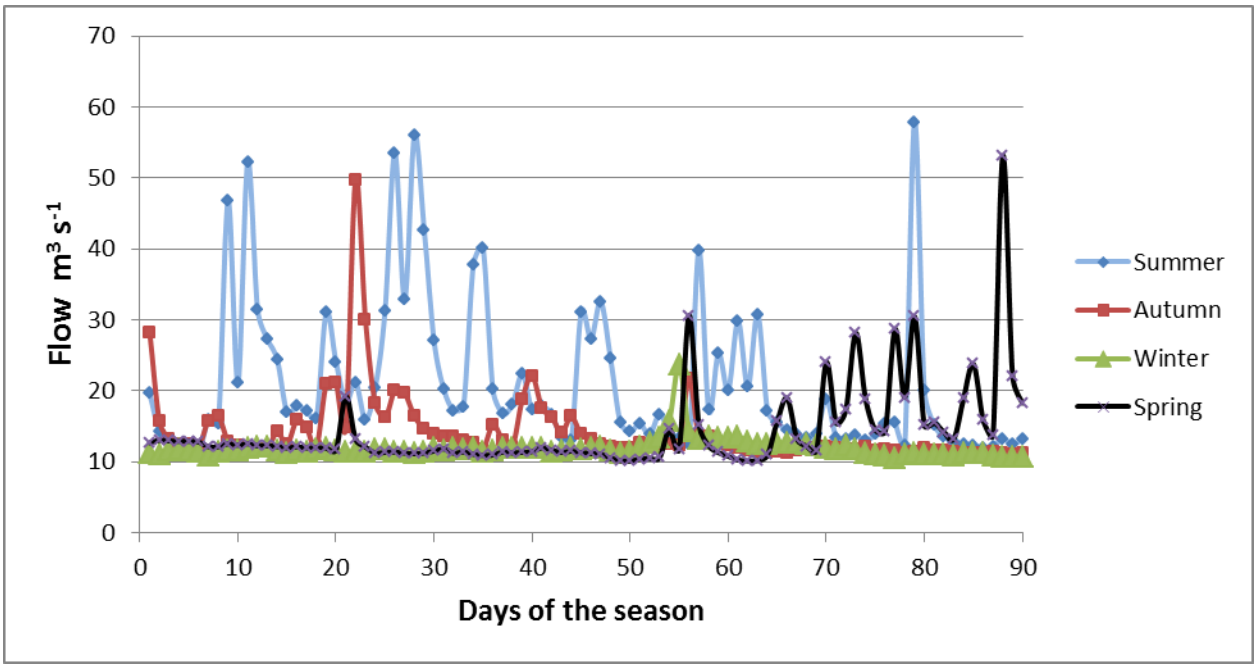


Figure 4.2 Crocodile River flow

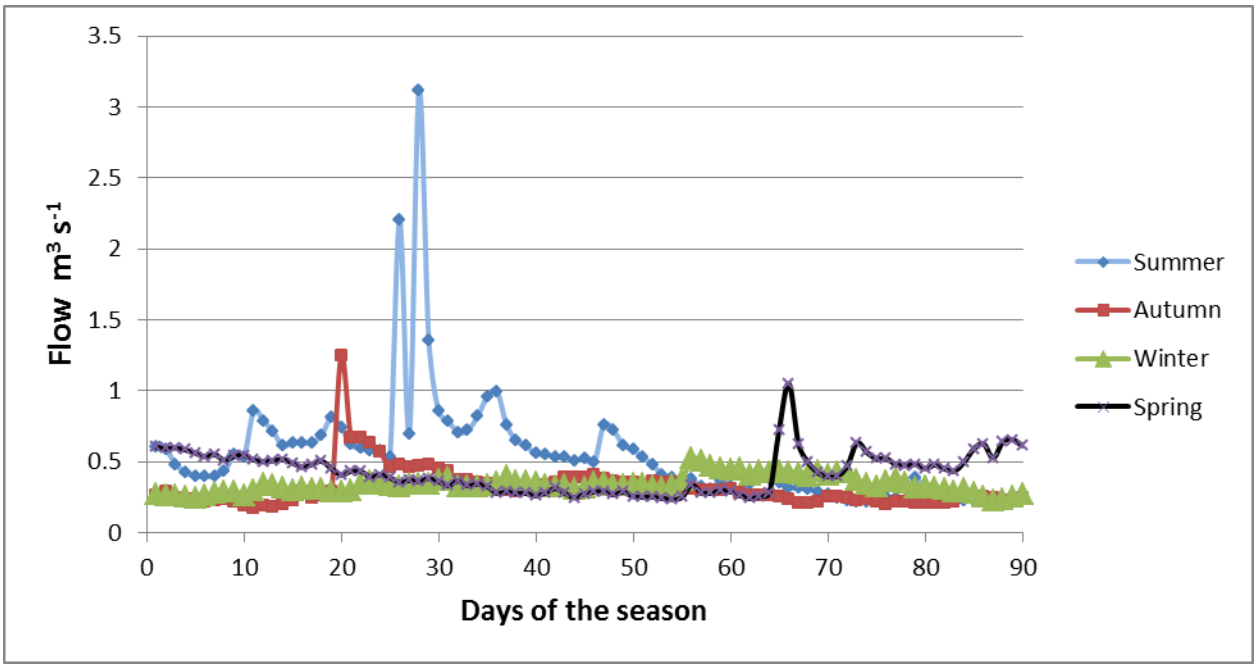


Figure 4.3 Magalies River flow

Mixing and pollutant stratification

Density mixing in the Hartbeespoort Dam has been studied intricately in the past three decades and has been reported to occur as early as in April, and can repeat after further combinations of cold weather, wind and gravity (Hely-Hutchinson and Schumann, 1998; Robarts et al., 1982). This leads to cool surface water that is otherwise warm in other seasons, to move down to the bottom of the dam as it becomes denser, causing an overturn (Robarts et al., 1982). The dissolved oxygen (DO) and temperature data (Figure 4.4) shows that the overturn occurred in autumn as characterized by sudden and concurrent drops in DO and epilimnion water temperature. Consistent Dam Wall point vertical depth temperatures recorded in the months April to June at 0 to 30 meters below the water surface show that overturn had occurred in April and mixing continued from autumn to winter.

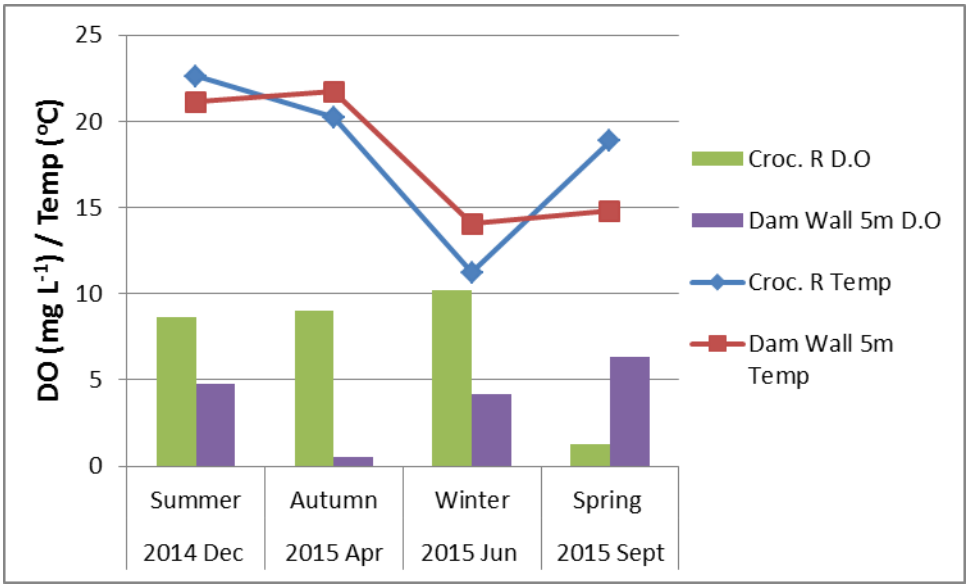


Figure 4.4 Temperature and DO profiles of the Crocodile River and Dam wall epilimnion

Figure 4.4 shows DO and temperature measured using a calibrated YSI multiparameter reading instrument. There are no significant differences in the triazine concentrations between the 30 m and 5 m level below the surface in spring, suggesting that the water was well mixed in spring after the April mixing as described by Robarts et al., (1982). This shows that there is adequate mixing of the water between the epilimnion and hypolimnion. The temperatures recorded for the Crocodile River and Dam Wall 5m below the surface (Figure 4.4) correspond well with historical data. The Crocodile River water

temperature was cooler than the Dam wall epilimnion in autumn and winter, but warmer in spring and in summer. The April overturn is characterized by the epilimnion becoming warmer than the Crocodile River water, as shown by the Dam wall 5 m depth which becomes warmer than the Crocodile River in winter (Figure 4.4). This causes the cooler and denser Crocodile River water to sink down to the epilimnion as re-stratification commences after winter, creating optimum conditions for the Crocodile River water to flow through the dam fairly rapidly as it exits the dam out of the outlet 11 m above the surface of the bed of the dam. This creates a lower residence time for the Crocodile River water. To some extent, this may assist to offload large volumes of pollutants from the Crocodile River out of the dam. The Dam wall DO epilimnion concentration in mid-April 2015 reached 0.5 mg L^{-1} which is consistent with historical measurements and maintained the mixing throughout the autumn-winter season before re-stratifying in spring.

Groundwater interaction

The presence of atrazine in groundwater may point to the presence of a surface water and groundwater hydraulic link. The contamination of groundwater is a major concern as many communities in South Africa rely on groundwater for drinking water purposes, as they have no access to treated water (Dabrowski, 2015). It is difficult to confidently formulate sound hypotheses on surface water-groundwater interactions unless stable isotopes with ^{18}O, ^{2}H, ^{3}H dyes or salts are used (Abiye et al., 2015). Surface water-groundwater interaction in the Hartbeespoort Dam precinct is poorly understood (DWA Groundwater, 2010). The Hartbeespoort Dam has been described by different authors as lying on quartzite rock consisting mainly of shale and homfels with 62% SiO_2, 19% Al_2O_3 and 8% Fe_2O_3 (Abiye et al., 2011; Coetzee, 1993). The Hartbeespoort Dam quartzite is located adjacent to an expanse of a dolomitic water bearing aquifer from the West Rand area up to further north in the City of Tshwane (Abiye et al., 2015; Abiye et al., 2011). There is evidence from isotope studies that some groundwater within the Hartbeespoort Dam catchment has been circulating underground for more than 50 years. Hence it is possible that groundwater contaminants may prevail for decades (Abiye et al., 2011) .

The low permeability of the Hartbeespoort Dam quartzite rock without evidence of significant fractures and fissures (Abiye et al., 2015) makes it difficult to confidently draw interactions between the aquifer water and the Hartbeespoort Dam surface water. The groundwater site is located on a slope with the borehole descending vertically, primarily between sheets of sloping quartzite and hybrid conglomerate

granophyric rock, but appears to be sealed off from interaction with the Hartbeespoort Dam by impervious rock that can be accurately described as quartzite, locally embedded with hornfels and slate (Abiye, 2011; Scott et al., 1977; Schifano 1971, Map). The low concentration of atrazine in groundwater compared to the Hartbeespoort Dam may indicate that seepage of irrigation water from top soil is the main source of the groundwater pollution and that there is little or no groundwater-Hartbeespoort Dam surface water interaction.

Triazines and degradation products in fish muscle

African sharptooth catfish (*Clarias gariepinus*) and common carp (*Cyprinus carpio*) were selected for triazine pollution monitoring as they are major fish species in the Hartbeespoort Dam by biomass. They are also the most consumed fish by humans as they are the major catch from the dam (Koekemoer & Steyn, 2005). Catfish and carp have a high ecological significance as they are edible piscivorous fish which are high up the food chain and have the potential to bioaccumulate organic pollutants to concentrations higher than those found in the rest of the aquatic environment (Squandrone et al., 2013a; Squandrone et al., 2013b). No triazine herbicides were detected in fish muscle by GC-MS or LC-MS/MS analysis (Table 4.6). This indicates that triazine herbicide bioaccumulation is negligible.

Table 4.6 Triazine degradation products in fish muscle (ng g^{-1} fw)

Fish sample	ADD	AD-2OH	DIA	HA	DEA	HT	DET
LOD 3x (ng g^{-1})	0.1	0.1	0.2	0.1	0.1	0.1	0.1
LOQ 10x (ng g^{-1})	0.3	0.2	0.5	0.3	0.2	0.3	0.3
Catfish	N.D	N.D	N.D	N.D	0.2	N.D	0.3
Carp	N.D	N.D	N.D	N.D	0.3	N.D	N.D

N.D.= not detected

Bioturbation of the hypolimnion due to large quantities of bottom feeding carp and catfish fish in the Hartbeespoort Dam is known as the cause of resuspension of pollutants in the dam (Hart and Harding, 2015). Only DET and DEA were detected in catfish muscle with concentrations of 0.3 and 0.2 ng g^{-1} respectively. As with the Hartbeespoort Dam water concentrations, DET was found in the highest concentration in catfish. Only DEA was detected in carp muscle with a concentration of 0.3 ng g^{-1} (Table 4.6). Toxicity data on triazine herbicide metabolites is scanty but is thought to be equivalent to parent triazine herbicides (Ralston-Hooper et al., 2009).

Statistical analysis on factors affecting Hartbeespoort Dam triazine concentrations

The influence of season, sampling point and triazine pollution on the Hartbeespoort Dam triazine pollution was statistically tested using the software package Statistical Package for Statistical Sciences (SPSS) version 16 to determine the factors which have the largest influence on the triazine load in the Hartbeespoort Dam. A test for the homogeneity of variances between the season, sampling point and triazine produced a *p*-value of 0.961 which indicates that equal variances are assumed. A one way ANOVA (Table 4.7) was used to determine statistically significant differences between groups and to prove/disprove that the differences between the triazine concentrations is independent of the season, sampling site and triazine type.

A test for homogeneity of variances between the different seasons indicates that there are no statistically significant differences between the total triazine concentrations between the seasons as the *p*-value is 0.82 (Table 4.7). To test the assumption of homogeneity of variances between the five Hartbeespoort Dam sampling points, the *p*-value of 0.04 (Table 4.7) computed indicates that there are statistically significant differences between triazine concentrations at different sampling points. To test if there are statistically significant differences between the different triazine compounds detected and quantified throughout the seasons, across all Hartbeespoort Dam sampling points, a *p*-value of 0.02 (Table 4.7) was computed. This indicates that there are indeed statistically significant differences between different triazine compounds detected and quantified. The differences between the different triazine concentrations in the Hartbeespoort Dam are therefore statistically independent of the sampling season but dependant on the sampling point location as well as the specific triazine compound.

Table 4.7 One way ANOVA results

		Sum of Squares	df	Mean Square	F	*p*-value
Seasons	Between Groups	250	204	1.22	0.667	0.82
	Within Groups	11	6	1.83		
	Total	261	210			
Sampling points	Between Groups	400	204	1.96	3.921	0.043
	Within Groups	3	6	0.50		
	Total	403	210			
Triazine	Between Groups	3323	204	16.29	5.43	0.019
	Within Groups	18	6	3.00		

$p < 0.05$ is statistically significant

Conclusions

Terbuthylazine had the highest detection rate in the Jukskei River and its degradation product DET was the most abundant triazine degradation product in the Jukskei River catchment as well as the Hartbeespoort Dam surface water and groundwater. The N14 site downstream of the Jukskei River recorded high atrazine concentrations which were higher than concentrations in the majority of the sampling sites in the Hartbeespoort Dam. The significantly higher flow from the Crocodile River into the Hartbeespoort Dam indicates that the Jukskei River has the highest influence on the Hartbeespoort Dam triazine load. In fish muscle, no triazine herbicides were detected. In the Hartbeespoort Dam, the summer season recorded highest ∑triazine herbicide and ∑triazine degradation product concentrations in the water samples with a declining trend in the following successive seasons. Groundwater ∑triazine and ∑triazine degradation product concentrations were fairly consistent throughout the seasons. Groundwater atrazine concentrations recorded values greater than the European maximum permissible level for drinking water of 100 ng L^{-1} in all seasons except spring, which recorded a value of 100 ng L^{-1}. This indicates that there is a significant risk in consumption of the contaminated groundwater. The analytical and geohydrological data show no evidence of groundwater-Hartbeespoort Dam surface water interaction. Thus, the source of triazines to the groundwater needs further investigation.

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Chapter 4 – Supporting Information

Tables:

Table S4.1 Sampling sites and GPS coordinates

Site	Latitude (South)	Longitude (East)
Crocodile River	-25.76596	27.89394
Dam wall	-25.72808	27.84971
Harbour	-25.74929	2783328
Magalies River	-25.76267	27.77582
Groundwater	-25.72528	27.83922
N14	-25.94933	27.95878
Kyalami	-26.0056	28.07836
Midrand	-26.03139	28.11222
Buccleuch	-26.05700	28.10400
Marlboro	-26.08494	28.10881

Table S4.2 Fish gravimetric measurements

5 year old carp (n=3)	Average standard length	64.5 cm
	Average total length	76cm
	Average fork length	70.5 cm
	Average weight	7.2 kg
	% Lipid in muscle	1.4%
	% Moisture in muscle	23%
5 year old catfish(n=3)	Average standard length	57cm
	Average total length	64 cm
	Average weight	5.2 kg
	% Lipid in muscle	1.6%
	% Moisture in muscle	24%

Method validation

Sample extraction was validated using a C18 and a styrenedivinylbenzene (SDB) extraction cartridge The SDB cartridge provided more precise recoveries and better method ruggedness. The each compound was validated using the internal standards ^{13}C3 Melamine and D7 deethylatrazine. D7 deethylatrazine was found to the most stable internal standard for all the analytes. Sample extraction volumes of 200 mL, 500 mL and 1000 mL were tested with a 10, 20 and 50 µL sample injection volume. A 1000 mL sample volume coupled to a 10 µL injection volume was determined to provide sufficient sensitivity. LOD was calculated using a precise signal-to-noise ratio of 1:3 on an average of 11 calibration curves and the LOQ was calculated using a precise signal-to-noise ratio of 1:10 on an average of 11 calibration curves. Linearity was >0.998 for all calibration curves.

Table S4.3 Analytical validation parameters: analytical instrument, retention time, recoveries, repeatability, precision and regression

	Analytical Instrument	RT (min)	Average recovery (%)	Repeatability (RSD%)	Precision (%variation)	Regression coefficient (r^2)	Calibration fit
Atrazine	GC-MS	14.694	80	3.1	5.4	0.999	linear
Simazine	GC-MS	14.633	93	2.6	4.5	0.998	linear
Terbuthylazine	GC-MS	15.166	96	4.5	3.9	0.999	linear
Ametryn	GC-MS	19.082	87	5.3	5.3	0.999	linear
Prometon	GC-MS	14.301	102	2.4	4.6	1	linear
Gesatamin	GC-MS	14.165	84	3.7	3.3	1	linear
Propazine	LC-MS/MS	10.819	89	2.8	3.2	0.998	linear
Prometryn	LC-MS/MS	11.142	73	2.2	4.8	0.999	linear
DEA	LC-MS/MS	8.358	70	3.4	4.2	0.999	linear
HA	LC-MS/MS	7.887	112	2.8	3.4	1	linear
DIA	LC-MS/MS	7.232	78	3.2	2.7	0.998	linear
AD-2OH	LC-MS/MS	2.991	103	2.1	2.6	1	linear
ADD	LC-MS/MS	3.038	99	2.6	3.3	0.999	linear
HT	LC-MS/MS	8.802	78	2.4	4.1	1	linear
DET	LC-MS/MS	9.741	69	3.6	3.6	1	linear

Lipid analysis

Lipid analysis was conducted with acid hydrolysis of protein bound lipids. 2 mL ethanol (Sigma-Aldrich, Missouri, USA) and 6 mL 8 N HCl (Sigma-Aldrich, Missouri, USA) was added to 2.5 g fw diced fish muscle and heated, with shaking at 80 ^{o}C for 40 minutes in a digestion tube. The sample was allowed to cool before adding 6 mL hexane and shaking vigorously for 2 minutes before leaving to settle for 5 minutes. The upper organic layer was removed before repeating the extraction with 3 further 6 mL of hexane portions. The hexane portions were combined in a previously weighed beaker before evaporating the hexane in a water bath at 60 ^{o}C and measuring the mass of remaining lipid.

Chapter 5

Effects of environmentally relevant sub-chronic atrazine concentrations on African clawed frog (Xenopus laevis) survival, growth and male gonad development

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Abstract

Sub-chronic toxicity of environmentally relevant atrazine concentrations on exposed tadpoles and adult male African clawed frogs (*Xenopus laevis)* was evaluated in a quality controlled laboratory for 90 days. The aim of this study was to determine the effects of atrazine on the survival, growth and gonad development of African clawed frogs. After exposure of tadpoles to atrazine concentrations of 0 (control), 0.01, 200 and 500 µg L^{-1} in water, mortality rates of 0, 0, 3.3 and 70% respectively were recorded for the 90 day exposure period. Morphometry showed significantly reduced tadpole mass in the 500 µg L^{-1} atrazine exposed tadpoles (p<0.05). Light microscopy on testes of adult frogs exposed to the same atrazine concentrations using hematoxylin and eosin (H&E) and Van Gieson staining techniques revealed gonadal atrophy, disruption of germ cell lines, seminiferous tubule structure damage and formation of extensive connective tissue around seminiferous tubules of frogs exposed to 200 µg L^{-1} and 500 µg L^{-1} atrazine concentrations. Ultrastructural analysis of the cellular organelles using transmission electron microscopy (TEM) revealed significant amounts of damaged mitochondria in testosterone producing Leydig cells as well as Sertoli cells. Biochemical analysis revealed reduced serum testosterone levels in adult frogs at all exposure levels as well as presence of six atrazine metabolites in frog serum and liver. The results indicate that atrazine concentrations greater than the calculated LC50 of 343.7 µg L^{-1} cause significant mortality in tadpoles, while concentrations ≥200 µg L^{-1} adversely affect reproductive health of adult frogs and development of tadpoles sub-chronically exposed to atrazine.

Introduction

Atrazine (CAS# 1912-24-9) is one of the most ubiquitous and extensively used herbicides in the world for the control of broad leaf weeds (Khalil et al., 2017; Schmidt et al., 2017; Zheng et al., 2017). It has unrestricted use in most parts of the world, however its use is banned in the European Union since 2004 (Jablonowski et al., 2011; Yang et al., 2017). The maximum allowable atrazine concentrations in drinking water range from 0.1 to 3 µg L^{-1} in most regions, particularly in Europe, Asia and America (Singh et al., 2018). A recent monitoring study of atrazine across the Ceará state in Brazil revealed mean atrazine concentrations of 7 to 15 µg L^{-1} in reservoir water (Sousa et al., 2016). Atrazine environmental concentrations are sometimes found in much higher concentrations, even over 500 µg L^{-1} (Freeman & Rayburn, 2005; Giddings et al., 2005; Rohr & McCoy, 2010; Storrs & Kiesecker, 2004; Tavera-Mendoza et

al., 2002). Aquatic organisms are likely to be exposed sub-chronically to high atrazine concentrations for periods of up to 3 months in ponds and pools adjacent to fields at the height of the agricultural season as well as chronically to low atrazine concentrations all year round in rivers and lakes downstream (Storrs & Kiesecker, 2004; Wood et al., 2017). Sub-chronic exposures of animals occur over a portion of their life or life cycle (Zhang et al., 2016). Rainfall simulation studies have shown that a high percentage of the atrazine applied on land is lost to ground and surface water sources through surface run-off during the first rain after application, with up to 75% loss occurring over 70 days in loam soils and much less in clay soils where it is prone to transformation into a variety of metabolites (Koskinen & Clay, 1997; Ng & Clegg, 1997; Wallace et al., 2017; Wang et al., 2018).

The widespread presence of atrazine and its metabolites in the environment is reported to be the cause of declining amphibian populations worldwide (Forson & Storfer, 2006; Hayes et al., 2010; Moreira et al., 2017; Shipitalo et al., 2003; Siddiqua et al., 2010). Results of atrazine exposure studies often produce valid but contrasting conclusions (Brodeur et al., 2009; du Preez et al., 2008; Gammon et al., 2005; Kloas et al., 2009; Oka et al., 2008; Rohr & McCoy, 2010; Solomon et al., 2008). It has been proposed that the conflicting results may be due to a combined agonist-antagonist atrazine effect e.g atrazine is proven to both accelerate and delay metamorphosis (Rohr & McCoy, 2010). A variety of frog species exposed to high atrazine concentrations had lower occurrence of gonadal dysgenesis, testicular oocyte development and higher survival rates than frogs exposed to much lower atrazine concentrations (Hayes et al., 2003; Jooste et al., 2005; Storrs & Kiesecker, 2004). This phenomenon described by Storrs & Kiesecker, (2004) as a non-monotonic response has been observed in a wide range of animals. The link between atrazine exposure and development of testicular oocytes in different frog species is one that also remains uncertain (McDaniel et al., 2008; Rohr & McCoy, 2010). It has however been proven that tadpoles exposed to atrazine prior to sexual differentiation, develop aplasia and gonadal dysgenesis (Hayes et al., 2002; Tavera-Mendoza et al., 2002).

Studies on different frog species exposed to atrazine concentrations up to 20 µg L^{-1} (exposure periods from 2 to 75 days) have indicated that there are no significant effects on embryo hatchability and tadpole growth i.e length and mass endpoints (Allran & Karasov, 2001; Diana et al., 2000; Morgan, 1996) and exposures up to 100 µg L^{-1} for 75 days do not significantly affect sexual differentiation and survival (Kloas et al., 2009). Studies by Brodeur et al. (2009) and Rutkoski et al. (2018) have indicated that pre- and prometamorphosis tadpoles are more sensitive to atrazine than embryos. Design flaws and

contamination of controls in some studies have led to questionable results and pronouncement of controversial conclusions (Hayes, 2004; Rohr & McCoy, 2010). Conflicts of interest have also often been singled out as a contributor to questionable results as well as negative results (Hayes, 2004). Trematode and other infections observed in frogs in the environment have been attributed to the presence of atrazine and its metabolites in the aquatic environment (Rohr et al., 2008).

The scope of this work is limited to specific biological and toxicological effects of environmentally relevant atrazine concentrations including a 0.01 µg L^{-1} concentration selected to represent trace concentrations, a documented no observed effect concentration (NOEC) of 200 µg L^{-1} for *X. laevis* tadpoles (Langerveld et al., 2009; Rohr & McCoy, 2010) and a 500 µg L^{-1} concentration which is rarely studied. *X. laevis* was selected for this study as it is a good indicator of environmental pollution due to the frog's semi-aquatic life cycle and properties such as semi-permeable skin. The effect of atrazine on mortality, growth and development of frogs and tadpoles was studied in light of reports that atrazine is responsible for declining global frog populations. The effects of atrazine on qualitative and quantitative sub-cellular testes morphology and serum sex steroid hormone levels of *X. laevis* were assessed to determine the effects of atrazine on gonad development and feminization of male frogs. It is envisaged that this study will provide an unprejudiced assessment of the effects of atrazine on *X. laevis* that would in combination with other similar studies, allow decision makers to gain an informed perspective on risk assessment of environmental atrazine contamination. A 90 day (sub-chronic) exposure was selected as frogs in the wild are usually exposed during short periods between applications and in most cases within the corn growing season spanning three months, though longer exposures are encountered in some lakes, ponds and pools (Solomon et al., 2008).

Materials and methods

Chemicals

30 mg L^{-1} (1.39 x 10^{-4} M) atrazine stock solutions were made up by dissolving 15 mg atrazine (certified reference standard, purity 99.5%) in 500 mL Milli-Q water. Stock solutions for 17-β estradiol, 17-α estradiol, testosterone, atrazine desisopropyl-2-hydroxyl (AD-2OH), desisopropylatrazine (DIA), atrazine-2-hydroxy (A-2OH) and deethylatrazine (DEA) as well as internal standards D7 deethylatrazine, D4 17-β estradiol and D4 estrone were made up to 1 mg mL^{-1} in methanol. Atrazine desethyl-desisopropyl (ADD) and hydroxyatrazine (HA) which have very low solubility in methanol were made up to lower

concentrations of 100 mg L^{-1} in Milli-Q water: methanol (50:50, v/v). Atrazine stock solution for gas chromatography analysis was made up to 100 mg L^{-1} in toluene. All analytical standards had a purity ≥96% and accurate concentrations were made up by gravimetric compensation for standards with <100% purity.

Experimental design and treatment

For this study, two separate atrazine exposures were carried out with adult male frogs (between May and August 2015 and March and June 2016) and two concurrent exposures were carried out with tadpoles between May and August 2015. Sixty laboratory bred adult (263 day old) male African clawed frogs (*Xenopus laevis*) were procured from the African Xenopus Facility (Knysna, South Africa) for each duplicate exposure. The frogs were flown to Johannesburg and on arrival, they were acclimatized for 7 days before being randomly distributed into 4 groups of 15 and exposed to 0 (control), 0.01 µg L^{-1} (4.64 x 10^{-11} M), 200 µg L^{-1} (9.27 x 10^{-7} M) and 500 µg L^{-1} (2.32 x 10^{-6} M) atrazine solutions (prepared in water) in 200 L stainless steel tanks. Adult frogs were fed *ad libitum* with nutritious commercial fish pellets (Koi food) and beef liver pieces once a week.

Sixty laboratory hatched tadpoles were bred at CAS (University of the Witwatersrand Central Animal Services) from a healthy male and a healthy female frog procured from the African Xenopus Facility. The female frog was injected with 250 iu gonadotropin (Sigma Aldrich, USA) hormone into the dorsal lymph sac to stimulate egg production and the male frog was injected with 50 iu gonadotropin 12 hours after injecting the female. The eggs were fertilized by the male frog. The hatched tadpoles were acclimatized up to 10 days old (Nieuwkoop and Faber stage 48), randomly distributed into 8 groups of 15 and exposed in duplicate to 0 (control), 0.01, 200 and 500 µg L^{-1} atrazine solutions (prepared in water) in 80 L glass tanks. Tadpoles were fed *ad libitum* with highly nutritious commercial ornamental fish micro flakes (TetraMini baby®) and received crushed nutritious commercial fish pellets (Koi food) as from 75 days old. The adult *X. laevis* and tadpoles were euthanized at 360 and 100 days old respectively.

Animal housing conditions

Activated carbon filtered and dechlorinated water was used to prepare the control and atrazine exposure water. Adult frogs and tadpoles were exposed in 60 L and 50 L, respectively of the 3 different atrazine concentrations (as well as control). The 30 mg L^{-1} atrazine stock solution was added to the

water in the 0.01, 200 and 500 µg L^{-1} atrazine exposure tanks. The frogs and tadpoles were maintained at the CAS Lab with a 12 h light: 12 h dark cycle (on 06:00, off 18:00). Average water temperatures were maintained at 20 ^{o}C for adult frogs and average tadpole water temperatures were maintained at 22 ^{o}C utilising water heaters. Individual tank thermometers were verified using calibrated thermometers. The adult *X. laevis* were weighed every week and inspected for signs of stress or ill-health. Tadpole water was kept aerated by pumping air bubbles into the water through clean sand and wool filter using a pump. Quality control analysis of water in the 0.01, 200 and 500 µg L^{-1} atrazine exposure tanks showed negligible absorption of atrazine by the sand and wool. Adult frog atrazine exposure solutions were replenished 3 times a week after feeding to maintain a clean environment and to maintain atrazine levels. Water for tadpoles underwent a weekly 40% change for the first 4 weeks to reduce stress to the tadpoles and 100% afterwards. The frogs and tadpoles were euthanized by immersion in 0.2% benzocaine solution (an anesthetic) in Milli-Q water. This work was carried out under permit numbers CPF6 0115 (2015) and CPF6 0120 (2016) from Gauteng Nature Conservation (Appendix 1). All experiments were performed in accordance with the regulations of the Animal Ethics and Control Committee of the University of the Witwatersrand and were approved by the Animal Ethics Screening Committee with ethical clearance number 2014/32/D together with modifications and extensions granted (Appendix 2).

Gravimetric measurements

The length of each tadpole was measured using a ruler placed under a transparent glass beaker containing the tadpole. The tadpole length was measured initially from snout-tail at 41 d old and from snout-vent thereafter, up to 100 d old. The tadpole mass at 41 d old was measured by water volume displacement as the tadpoles were stressed when taken out of the water. From 54 d old, the tadpole mass was measured accurately using a sensitive 4 digit balance as with adult frogs. Testicular volume (both testes) of the adult frogs was measured by water volume displacement and testicular mass (both testes) was measured using a sensitive 4-digit balance.

Histology

Differential staining for light microscopy

Testes were dissected immediately after euthanising, cut into 3 blocks and fixed in 2.5% gluteraldehyde. Blocks were selected randomly, processed and embedded in paraffin. 7 µm thick microtome sections were sectioned and mounted on slides. After deparaffinising and dehydrating, one set of sections was stained with hematoxylin and eosin (H & E). Another set was stained with Van Gieson stain for collagen.

Electron microscopy

Qualitative histological transmission electron microscopy (TEM) of testes from the first set of duplicate exposed adult *X. laevis* was conducted at the University of the Witwatersrand Microscopy and Microanalysis Unit. Quantitative TEM imaging work on testes of the second duplicate set of *X. laevis* was done at the Vrije University, Amsterdam (VU/VUmc) Electron Microscopy Facility. After euthanising the frogs, testes were immediately dissected, sectioned into 3 blocks and fixed in 2.5% gluteraldehyde (in 0.1 M calcium cacodylate ($C_4H_{12}As_2CaO_4$), pH 7) and left at room temperature for 1 h before refrigerating overnight at 4 ^oC. The testes blocks were washed twice in 0.1 M $C_4H_{12}As_2CaO_4$ buffer before transferring to shipping tube containing 30% sucrose in 0.1 M $C_4H_{12}As_2CaO_4$. The tubes were couriered to The Netherlands for processing and imaging.

Tissue blocks were trimmed to 1 x 3 mm blocks and postfixed overnight at 4 ^oC in fresh 2.5% gluteraldehyde (in 0.1 M $C_4H_{12}As_2CaO_4$). The tissue blocks were washed 3 times in 0.1 M $C_4H_{12}As_2CaO_4$ and postfixed with 1% OsO_4/1% $KRu(III)(CN)_6$. Blocks were washed in aqua bidest before dehydration by a series of increasing ethanol concentration (30%, 50%, 70%, 90%, 100%, followed by a second 100%, allowing the tissue block to sink to the bottom before moving to the next higher ethanol concentration). The tissue was washed with propylene oxide before infiltrating with propylene oxide: EPON (epoxy resin) (1:1, v/v) followed by propylene oxide: EPON (2:1 v/v) then embedded in freshly made EPON inside BEEM capsules filled with EPON. The blocks were allowed to sink down to the tip over 2 hours. Polymerisation of the EPON was done at 65 ^oC for 48 hours. The blockface was manually trimmed and 70 nm ultrathin sections were collected on formvar coated copper grids (without carbon) by room temperature ultramicrotomy using a diamond knife. The sections were double contrasted using Reynolds lead citrate and uranyl acetate, before analysing in a JEOL1010 TEM.

Chemical analysis

Serum extraction and cleanup

Immediately after euthanising, the adult frogs were bled out by making an incision on the subclavian artery and blood was collected using a syringe. Blood from 15 frogs was pooled together, allowed to clot for 15 minutes at room temperature, centrifuged to separate the serum and immediately stored at -82 ^{o}C prior to analysis. The serum samples were analysed for atrazine metabolites and steroid hormones. 100 µL of serum sample and 0.5 mL of saturated ammonium sulphate solution were added to an Eppendorf tube, together with 50 µL of 0.1 mg L^{-1} D7 deethylatrazine, 50 µL of 0.4 mg L^{-1} D4 17-β estradiol and 50 µL of 0.4 mg L^{-1} D4 estrone internal standards. The mixture was vortexed for 30 s before centrifuging for 8 min at 3800g (4000 rpm). The clear top layer was removed and transferred to a test tube containing 200 mg PSA/C18 dispersive solid phase extraction (dSPE) sorbents for sample cleanup. 6 mL acetonitrile was added before the mixture was vortexed for 30 s and centrifuged at 3800g for 8 min. The organic extract was transferred to a clean test tube and evaporated to near dryness. Extracts for testosterone analysis were reconstituted in 200 µL of 10% methanol (Milli-Q water: Methanol, 90:10 v/v). Extracts for 17-β estradiol and 17-α estradiol were reconstituted by adding 100 µL of 100 nM $NaHCO_3$ Milli-Q water (pH 10.5) and 100 µL of 1 g L^{-1} dansyl chloride (in acetone: 100 nM $NaHCO_3$ Milli-Q water (pH 10.5), (1:1 v/v)) in an insert inside an LC vial. Due to the low ionization efficiency of 17-β estradiol and 17-α estradiol, with electro-spray ionization (ESI) in Liquid Chromatography-Mass Spectrometry (LC-MS/MS), a chemical derivatisation method was utilized to enhance the sensitivity from a limit of detection (LOD) from 15 ng mL^{-1} to 1 ng mL^{-1}. The derivatisation and LC-MS/MS conditions are described elsewhere (Rimayi et al., 2018a).

Adult frog liver extraction and cleanup

Adult frog liver samples were analysed for atrazine metabolites. 1 g (fresh weight (fw)) finely ground frog liver samples were weighed into 50 mL fluoroethylenepropylene centrifuge tubes. 5 g beef liver (used to feed adult frogs) purchased form a local supermarket was analysed along with the frog liver samples as a quality control (QC) sample. 50 µL of 0.1 mg L^{-1} D7 deethylatrazine internal standard was added to each sample. 4 mL acetonitrile (Sigma-Aldrich, Missouri, USA) and 0.2 g NaCl (Merck, Darmstadt, Germany) were added before manually shaking vigorously for 15 s, vortexing for 15 s at 35 Hz and allowing the mixture to equilibrate for 12 hours. The samples were centrifuged for 15 min at

3800g and the upper organic layer was removed before adding another 4 mL acetonitrile, vortexing for 15 s at 35 Hz and centrifuging for 15 min at 3800g. The second organic layer was removed and combined with the first organic layer in a clean centrifuge tube.

To each centrifuge tube containing a frog liver extract, a QuEChERS kit (containing 50 mg PSA, 50 mg C_{18}, 150 mg $MgSO_4$ (Agela Technologies, Delaware, USA)) was added together with an additional 200 mg $MgSO_4$ (Sigma-Aldrich, Missouri, USA), 200 mg PestiCarb (Agela Technologies, Delaware, USA), 100 mg diatomaceous earth (Sigma-Aldrich, Missouri, USA) and 250 mg basic alumina (Sigma-Aldrich, Missouri, USA). The centrifuge tubes were vortexed for 15 s at 35 Hz, left to settle for 5 minutes and then centrifuged for 8 min at 3800g. Each supernatant was transferred to a clean test tube and evaporated to near dryness using a gentle stream of nitrogen. All extracts were reconstituted in 200 µL of 10% methanol for LC-MS/MS analysis.

LC-MS/MS analysis

Analysis of testosterone, 17-β estradiol and 17-α estradiol and atrazine metabolites in serum and liver extracts was performed on an LC-ESI-MS/MS (1200 series LC system, 6410 triple quadruple MS; Agilent Technologies, Amstelveen, The Netherlands). Analytical instrument conditions for steroid hormone and atrazine metabolite analysis are described by Rimayi et al. (2018a; 2018b), respectively.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Analysis of atrazine exposure water was performed by gas chromatography-mass spectrometry (GC-MS, GC 6890, MS 5975, Agilent Technologies, CA, USA). An Agilent Technologies HP-5MS (5% polydimethylsiloxane) column (30 m x 0.25 mm x 0.25 µm; Chemetrix, Johannesburg, South Africa) was used with an average velocity of 50 cm s^{-1}. GC oven temperature programming was 90 ^{o}C (2 min), 25 ^{o}C min^{-1} to 200 ^{o}C (2 min), 8 ^{o}C min^{-1} to 280 ^{o}C (3 min) with a runtime of 19.4 min. A splitless 1 µL injection was used at 240 ^{o}C. The limit of detection (LOD) of 0.002 µg L^{-1} and limit of quantification (LOQ) of 0.007 µg L^{-1} were estimated at 3x and 10x the signal to noise ratio respectively.

Quality control

The atrazine concentrations in the exposure tanks were measured weekly by GC-MS. 50 mL water samples were sampled before and after water change/recycle. The 50 mL water samples were extracted

by passing through a 200 mg Bond Elut Plexa (Chemetrix, Johannesburg) solid phase extraction cartridge. All atrazine exposure water concentrations were verified to be within ± 6% for 0.01 µg L^{-1}, ± 3.1% for 200 µg L^{-1} and ± 1.6% for 500 µg L^{-1} target concentrations. Atrazine was not detected in the control water. All tadpoles were fed the same quantity and quality of food and adult frogs were also fed the same quantity and quality of food. All tadpoles were kept at the same optimum temperatures and the adult frogs were kept at the same optimum temperatures as well. All chemicals used in this study were certified with a purity ≥97.5% with the exception of D7 deethylatrazine which had a purity of 96%. Analytical standards were supplied by Dr Ehrenstorfer and Toronto Research Chemicals (Industrial Analytical, Johannesburg).

Statistical analysis

Statistical analysis was performed using SPSS ver 16. One-way ANOVA analysis was applied for tadpole mass, length and mortality. Tukey Honestly Significant Difference (HSD) post-hoc analysis was used to determine which atrazine exposure concentrations (from this point, reference to atrazine exposure concentrations includes the control in all instances) differed significantly from others. A *p*-value ≤0.05 was considered statistically significant for all datasets. The Shapiro-Wilk test was applied to test for normality of data and the Levene's test for equality of variances was performed to determine if the differences in the exposure groups were due to random errors or differences between the variances of the frogs/tadpoles. For measurements taken only once, after sacrifice e.g testicular morphology, a one sample t-test was performed with the measurements sampled randomly and meeting the criteria for normal distribution. Data from the second adult frog exposure as well as the duplicate tadpole exposures was used for statistical analysis. Probit analysis software (PriProbit ver. 1.63) was used to compute LC50 values (the lethal dose at which 50% of a population dies within the 90 d exposure period).

Results and discussion

Frog and tadpole morphometry

Adult X. laevis and tadpole growth and motility

The adult *X. laevis* showed good health and weight gain within +3.1g for the second atrazine exposure (Supplementary Information (SI) Table S5.1, n=60) which was used for morphometric and histological analysis. Tadpoles in the control and 0.01 µg L^{-1} showed good health throughout the study with the 0.01 µg L^{-1} exposed tadpoles showing the highest growth rates in terms of weighed mass throughout the entire exposure period (SI Figure S5.1). Only the tadpole mass and the adult frog testicular mass and volume met the assumption of normality (Table 5.1, Shapiro-Wilk test *p*-value >0.05). All the mean adult frog masses from the different atrazine exposures were statistically different (Table 5.1, Tukey HSD *p*-value <0.05). This result is influenced by the fact that the individual frogs within each atrazine exposure group had a different mass.

The mean tadpole masses of the different atrazine exposure concentrations were significantly different (*p*-value <0.05 from one-way ANOVA) as the mean mass of the 500 µg L^{-1} exposed tadpoles was significantly lower than the mean masses of the control and 0.01 µg L^{-1} exposed tadpoles groups (Table 5.1, Tukey HSD *p*-value <0.05). Differences in the tadpole lengths across all atrazine exposure concentrations were statistically insignificant (Table 5.1, *p*-value >0.05 from one-way ANOVA; Tukey HSD *p*-values >0.05 for all exposure concentrations). On observation, the 500 µg L^{-1} exposed tadpoles looked significantly smaller and thinner than the control, 0.01 µg L^{-1} and 200 µg L^{-1} exposed tadpoles throughout the study. Larvae in the 200 and 500 µg L^{-1} atrazine exposures showed signs of stress within 18 h of exposure, with reduced motility. The 0.01 µg L^{-1} exposed tadpoles had the highest average mass of 1.5 g as well as the longest average length of 3 cm throughout the exposure period with the 500 µg L^{-1} exposed tadpoles recording the lowest average mass of 0.8 g and average length of 2.5 cm (SI Figures S5.1 & S5.2). The high atrazine concentrations therefore had a significant adverse effect on tadpole growth.

Frog and tadpole mortality rates

Mortality rates for adult frogs were 0% for all exposure concentrations. Mortality rates of the 500 µg L^{-1} exposed tadpoles were significantly higher than all the other exposure concentrations (Table 5.1, *p*-

value <0.05 from one-way ANOVA). Mortality rates for tadpoles were 0, 0, 3.3 and 70% for the control, 0.01, 200 and 500 µg L^{-1} exposures respectively, for the 90 day exposure period (Figure 5.1, n=120) and did not follow a normal distribution (Table 5.1, Shapiro-Wilk test *p*-value <0.05). Four tadpoles in the 500 µg L^{-1} exposure tanks died within 18 hours of exposure and more gradually continued to die during the 90 day exposure period in both duplicate tanks. Only one death was recorded in one of the duplicate 200 µg L^{-1} atrazine exposure tanks. Similar dose dependant mortalities were discovered in a study by Ji et al. (2016). As with tadpoles exposed to 400 µg L^{-1} atrazine concentrations by Langerveld et al. (2009), the tadpoles exposed to 500 µg L^{-1} experienced high mortality rates.

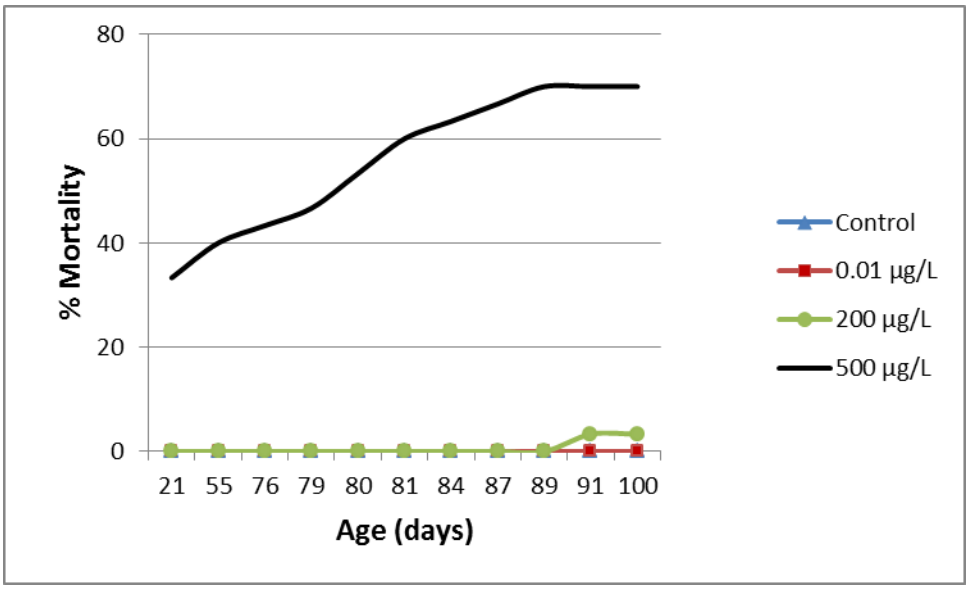

Figure 5.1 Tadpole mortality rate timeline after exposure to different atrazine concentrations (n=120)

A 90 d LC50 of 343.7 µg L^{-1} was computed using Probit analysis software for tadpoles exposed to the different atrazine concentrations (0 to 500 µg L^{-1} exposure range) from 10 days old (Stage 48 Niewkoop and Faber stage). A general LC50 of 410 µg L^{-1} has been suggested for amphibians (Solomon et al., 2008). Atrazine concentrations ≥343.7 µg L^{-1} in breeding sites can therefore be considered catastrophic to pre-metamorphosis tadpoles as they may face significant survival risks at this concentration.

Metamorphosis

The high atrazine concentrations utilized did not significantly influence metamorphosis rates of tadpoles (*p*-value >0.05 from one-way ANOVA). The metamorphosis for the control, 0.01 µg L^{-1} and 500 µg L^{-1} exposed tadpoles was complete within 44 to 50 days, whilst the 200 µg L^{-1} exposed tadpole metamorphosis took longer to complete at 58 days (Figure 5.2). The 500 µg L^{-1} atrazine exposed tadpoles experienced very high mortalities which introduced bias in the metamorphosis rate, as the surviving 20 out of 30 tadpoles completed metamorphosis within 44 days. The data shows a general trend that atrazine increases the time to metamorphosis, though statistically insignificant. It has been previously reported by Solomon et al. (2008) that atrazine exposures of 20 to 200 µg L^{-1} had no effect on development rate, percent metamorphosis and time to metamorphosis. Another study by Freeman & Rayburn (2005) and references therein indicate that atrazine increases the time to complete metamorphosis.

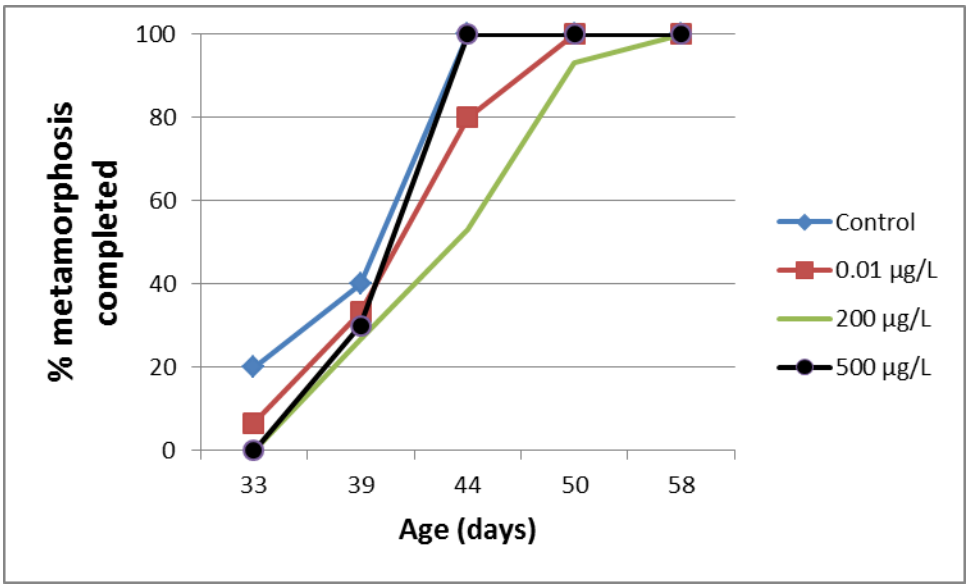

Figure 5.2 *X. laevis* tadpole metamorphosis after exposure to different atrazine concentrations (n=120)

Testicular morphology, mass and volume of adult frogs

There were no statistically significant differences between the adult frog testicular masses (Table 5.1, one sample t-test, T = 0, df = 3, mean = 0.116, *p*-value >0.05) and volumes (one sample t-test, T= -2.54, df = 3, mean = 0.1683, *p*-value >0.05) recorded between the different atrazine exposure concentrations.

The average testicular mass and volume of the control (0.193 g and 0.178 cm^3 respectively) and 0.01 µg L^{-1} exposed frogs (0.167 g and 0.123 cm^3 respectively) were however higher than the 200 µg L^{-1} exposed frogs (0.083 g and 0.11 cm^3 respectively) and 500 µg L^{-1} exposed frogs (0.095 g and 0.1 cm^3, respectively) (Figure 5.3). The average testicular mass of the 500 µg L^{-1} exposed frogs was slightly higher than that of the 200 µg L^{-1} exposed frogs, exhibiting a non-monotonic response. The deleterious effect of atrazine on developing *X. laevis* tadpoles exposed for just 48 hours has been described in great detail by Tavera-Mendoza et al. (2002), with follow-up work carried out by Oka et al. (2008) and therefore do not warrant further investigation for much longer exposures such as 90 days .

The natural phenomenon or otherwise, resulting in occurrence of multiple testes and hermaphrodites as described by Coady et al. (2004); Hayes et al. (2002); Hayes, (2005); Carr et al. (2003); Jooste et al. (2005) and Oka et al. (2008) was not detected in any of the 120 adult male frogs from the two frog batches utilised in this exposure study. This may in part be due to the fact that adult frogs in this study had already developed healthy normal testes in a pristine environment before the atrazine exposure.

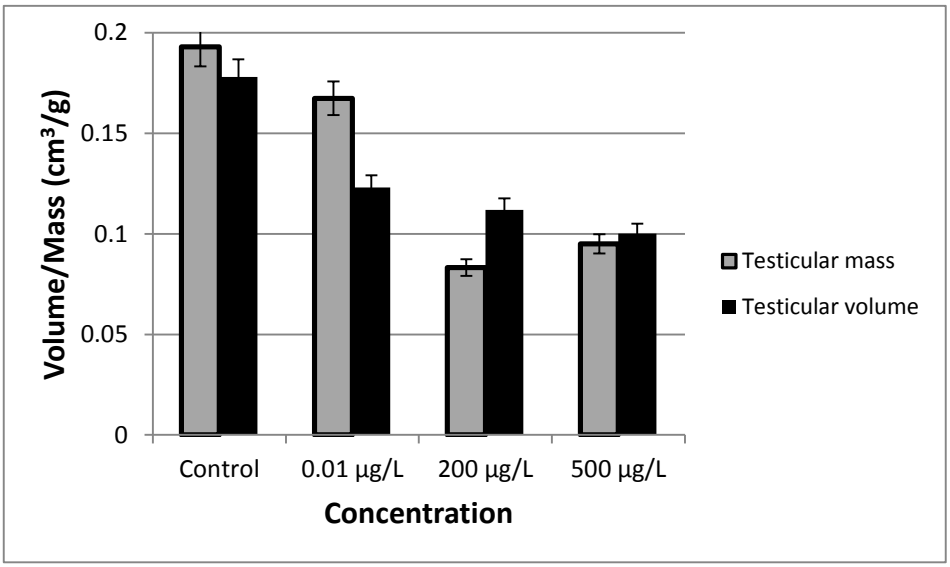

Figure 5.3 Adult *X. laevis* average testicular volume and mass (error bars= standard deviation) measured after atrazine exposure at different atrazine concentrations (n=60)

Table 5.1 frog and tadpole morphometry statistical analysis

Parameter	N	Shapiro-Wilk *p*-value	Levene's *p*-value	One-way ANOVA *p*-value	Post-hoc analysis Tukey HSD *p*-value	T-test *p*-value
Adult frog mass	60	0	0.364**	0***	***p<0.05 all cases	-
Adult frog testicular mass	60	0.51*	-	-	-	1
Adult frog testicular volume	60	0.555*	-	-	-	0.816
Tadpole mass	120	5.35*	0.65**	0.002***	*** p<0.05 for 500 µg L^{-1} exposed tadpoles	-
Tadpole length	120	0	0.994**	0.97	p>0.05 all cases	-
Tadpole mortality	120	0	0.045	0.01***	ϕ	-

*Normal distribution assumed
**Equal variances assumed
***Significant at α= 0.05 probability
ϕTukey HSD invalid because control and 0.01 exposures both have a variance of 0
- Test not applicable
All cases= all atrazine exposure concentrations

Histology

Qualitative histological assessment of duplicate sets of atrazine exposed adult frogs (exposed at different times) showed similar testis histological morphology in the respective atrazine exposure concentrations. Histological morphology of control adult frogs from both groups showed similar histology but showed a range of differences from the atrazine exposed testes.

Adult X. laevis germ cell line

The germ cell line in control frogs was well ordered and well-structured as differentiated cells could be distinguished from others in the sequential order spermatogonium- primary spermatocyte- secondary spermatocyte- spermatid (Figure 5.4 A). The 0.01 µg L^{-1} exposed frogs also showed normal germ cell development. The 200 µg L^{-1} and 500 µg L^{-1} atrazine exposed frogs showed a high incidence of seminiferous tubule degradation with total or occasional significant disruption of the germ cells lines (Figure 5.4 B). The 500 µg L^{-1} atrazine exposed seminiferous tubules showed significant abnormalities such as Sertoli cells (Figure 5.4 B cells 1, 2 & 3) clustered towards the basal lamina (red arrow) not

extending towards the lumen of the seminiferous tubule and with no spermatogonia between them. As a result, spermatids (yellow arrows) were located in many different sections of the seminiferous tubule such as near the basal lamina (Figure 5.4 B, red arrow) instead of in the central lumen of the seminiferous tubule. The seminiferous tubule histological abnormalities and deformities are consistent with findings by Hayes et al. (2011) but contrary to findings by Hecker et al. (2006) who found no evidence of ultrastructural destruction in germ cells. Nucleus-vacuole junctions (black arrows in Figure 5.4 B, sertoli cells 1 & 2) could be observed in the 500 µg L^{-1} atrazine exposed frogs, a sign of cell stress which could result in gonadal dysfunction.

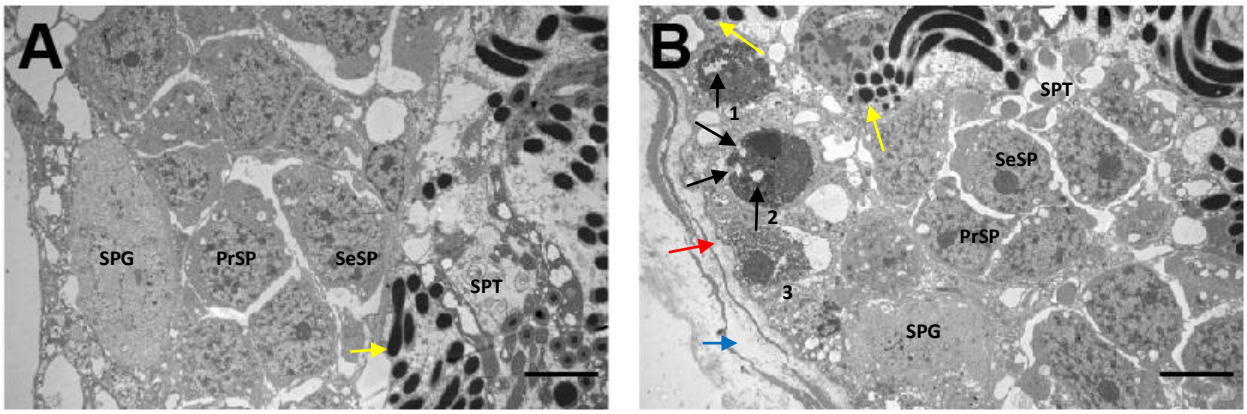

Figure 5.4 Electron micrograph showing germ cells in *X. laevis*.

A- control, B- 500 µg L^{-1} atrazine exposure. Scale bar= 5 µm. SPG= spermatogonium; PrSP= primary spermatocyte; SeSP= secondary spermatocyte; SPT= spermatid; yellow arrow= spermatozoa; black arrow= vacuole in Sertoli cell nucleus, red arrow= basal lamina; blue arrow= smooth muscle (basement membrane).

The absence of testicular ovarian follicules (TOFs) in any of the 120 frogs tested in this study concurs with conclusions in the meta-study by Rohr & McCoy (2010) that development of TOFs in adult frogs is not a natural occurrence. This alludes that the TOFs detected by various researchers cited by Hecker et al. (2006) in both test and control adult *X. laevis* may certainly have been due to other external factors inevitably introduced, particularly during the embryo and larval stages (Hayes et al., 2003). Atrazine contamination is particularly widespread in some laboratory experiments, microcosms, mesocosms and field studies as described by Hayes, (2005).

Adult X. laevis Seminiferous tubule histology

Testes of frogs exposed to 200 and 500 µg L^{-1} atrazine concentrations had a significant degree of gonadal atrophy with poorly developed seminiferous tubules (Figure 5.5 C & D). Seminiferous tubule damage in 200 and 500 µg L^{-1} atrazine exposed frogs is consistent with findings by Chen et al., (2015) and Hayes et al. (2011) as the seminiferous tubules showed signs of disintegration with presence of hollow spaces within seminiferous tubules (Figure 5.5 C & D, red arrows). Van Gieson staining revealed significant fibrosis between seminiferous tubules of the 200 and 500 µg L^{-1} atrazine exposed frogs (Figure 5.5 D, blue arrows). This may cause the reproductive dysfunction and failure to reproduce. The effects of atrazine on adult *X. laevis* seminiferous tubule histology are similar to those reported by Hayes et al. (2010) for *X. laevis* exposed throughout the larval stage.

There are no statistically significant differences between the seminiferous tubule diameters of all atrazine exposure concentrations (Table 5.1, one sample t-test, *p*-value >0.05), however control frogs recorded the longest average seminiferous tubule diameter of 356 µm. The 0.01 µg L^{-1} atrazine exposed frogs recorded a longer average seminiferous tubule diameter of 302 µm than the 200 µg L^{-1} and 500 µg L^{-1} atrazine exposed frogs of 231 and 237 µm respectively (SI Figure S5.3).

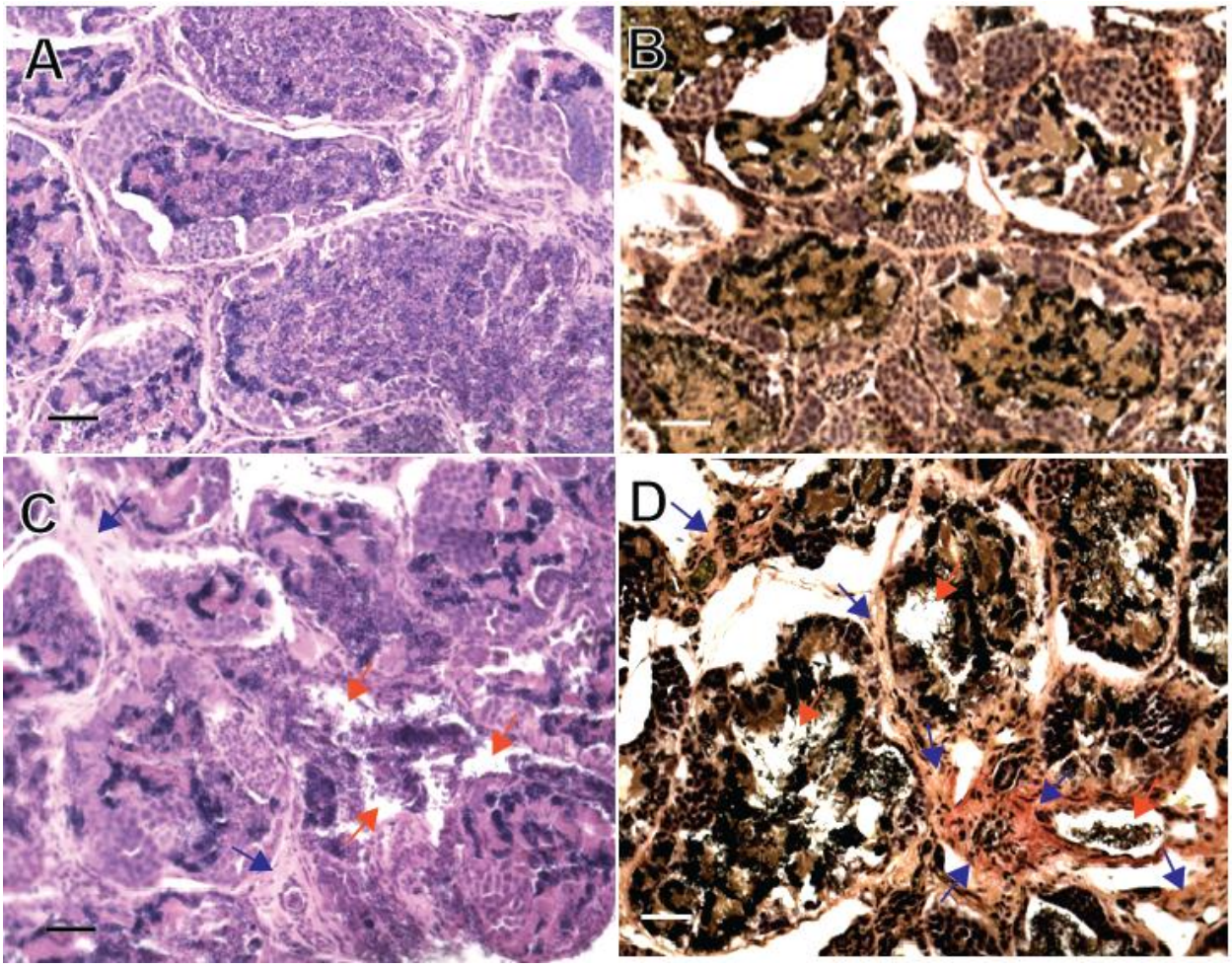

Figure 5.5 Typical seminiferous tubules with H&E staining.

Control (A) and 500 µg L^{-1} atrazine exposures (C), Van Gieson staining control (B) and 500 µg L^{-1} atrazine exposures (D). Scale bar= 70 µm, 20x magnification. Red arrows show empty spaces in seminiferous tubules and blue arrows show abnormal amounts of connective tissue which are more visible with Van Gieson staining.

Adult X. laevis Sertoli cell ultrastructure

The health of the Sertoli cells was assessed by counting the number of mitochondria (n= 82-86 images) and damaged mitochondria present (n=79-86 images). Stressed mitochondria often produce mitochondrial-derived vesicles (MDV) in response to stress inducing conditions (Soubannier et al., 2012; Sugiura et al., 2014). Sertoli cells of frogs exposed to atrazine showed high incidences of mitochondrial

stress and damage as the 0.01, 200 and 500 µg L^{-1} exposed frog Sertoli cell mitochondria appeared vesicular (Figure 5.6 B, C & D respectively, red arrows show vesicles) with incidences of double wall rapture (Figure 5.6 blue arrows), compared to control mitochondria which appeared elongated (Figure 5.6A, yellow arrow).

The Sertoli cells of the 500 µg L^{-1} exposed frogs had significantly lower number of mitochondria, averaging approximately only 3.5 per field of view compared with the control, 0.01 and 200 µg L^{-1} which recorded an average of >5 mitochondria per field of view (Figure 5.7 A). The Sertoli cells of the 500 µg L^{-1} exposed frogs had a very high average percentage of damaged mitochondria of 61% compared to the control, 0.01 and 200 µg L^{-1} recording lower values of 9, 25 and 45% respectively (Figure 5.7 B). As sertoli cell mitochondria have a central role in germ cell development, this function may be impaired due to high incidences of mitochondrial stress and damage.

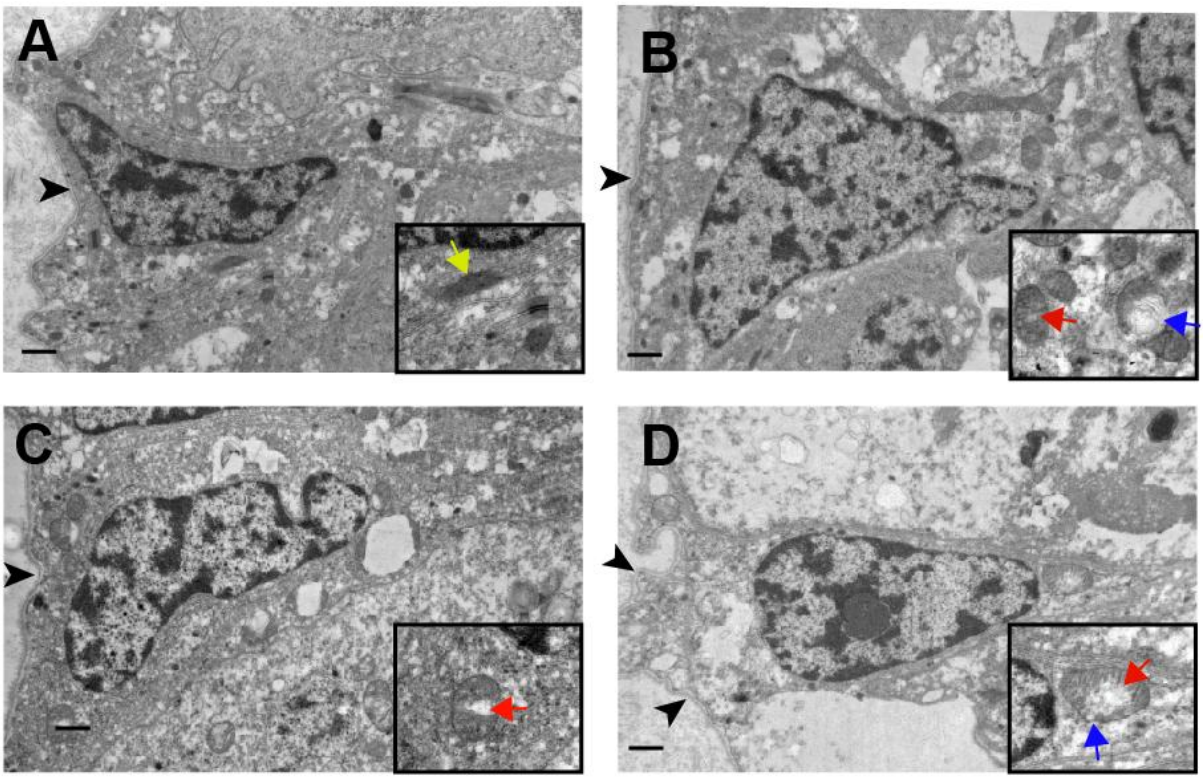

Figure 5.6 Electron micrograph showing typical Sertoli cells of adult *X. laevis*.

A- control, B- 0.01 µg L^{-1}, C-200 µg L^{-1}, and D- 500 µg L^{-1} atrazine exposure. Scale bar= 1 µm. Black arrowheads= the basal membrane, blue arrows= ruptured mitochondrial double membrane; yellow arrow= elongated healthy mitochondria; red arrows= vacuole in mitochondrial matrix.

Adult X. laevis Leydig cell ultrastructure

Leydig cells could be identified by their irregularly shaped cytoplasm and as cells that lie outside the periphery of the seminiferous tubule. The Leydig cells are important in male sexual development as they are the primary testosterone producing cells (Hecker et al., 2005), with synthesis of testosterone in the Leydig cell being mediated in mitochondria and smooth endoplasmic reticulum (Kim et al., 2016). The health of the Leydig cells was assessed by quantifying the number of mitochondria (n=61-79 images) as well as the percentage of damaged mitochondria (n=59-76 images, Figure 5.7 C & D). The Leydig cells of the 200 and 500 µg L^{-1} atrazine exposed frogs showed a significantly lower average number of mitochondria than the control and 0.01 µg L^{-1} exposed frogs (Figure 5.7 C). The 500 µg L^{-1} exposed frogs had significantly higher percentages of damaged mitochondria, averaging 63% compared to 35 and 32% for the 0.01 and 200 µg L^{-1} exposed frogs (Figure 5.7 D). Damaged mitochondria in Leydig cells were characterized by swollen and vesicular appearance (Figure 5.8, red arrows). Cytosolic lipid droplets in the Leydig cells of the 200 and 500 µg L^{-1} atrazine exposed frogs (Figure 5.8 C & D respectively, black arrows) were significantly smaller than cytosolic lipid droplets of the control and 0.01 µg L^{-1} exposed frogs. This can be attributed to suppression of steroidoegenesis in the synthesis of testosterone as healthy Leydig cell cytoplasms are typically rich in large lipid droplets (Figure 5.8 A & B [control and 0.01 µg L^{-1} exposed frogs respectively]).

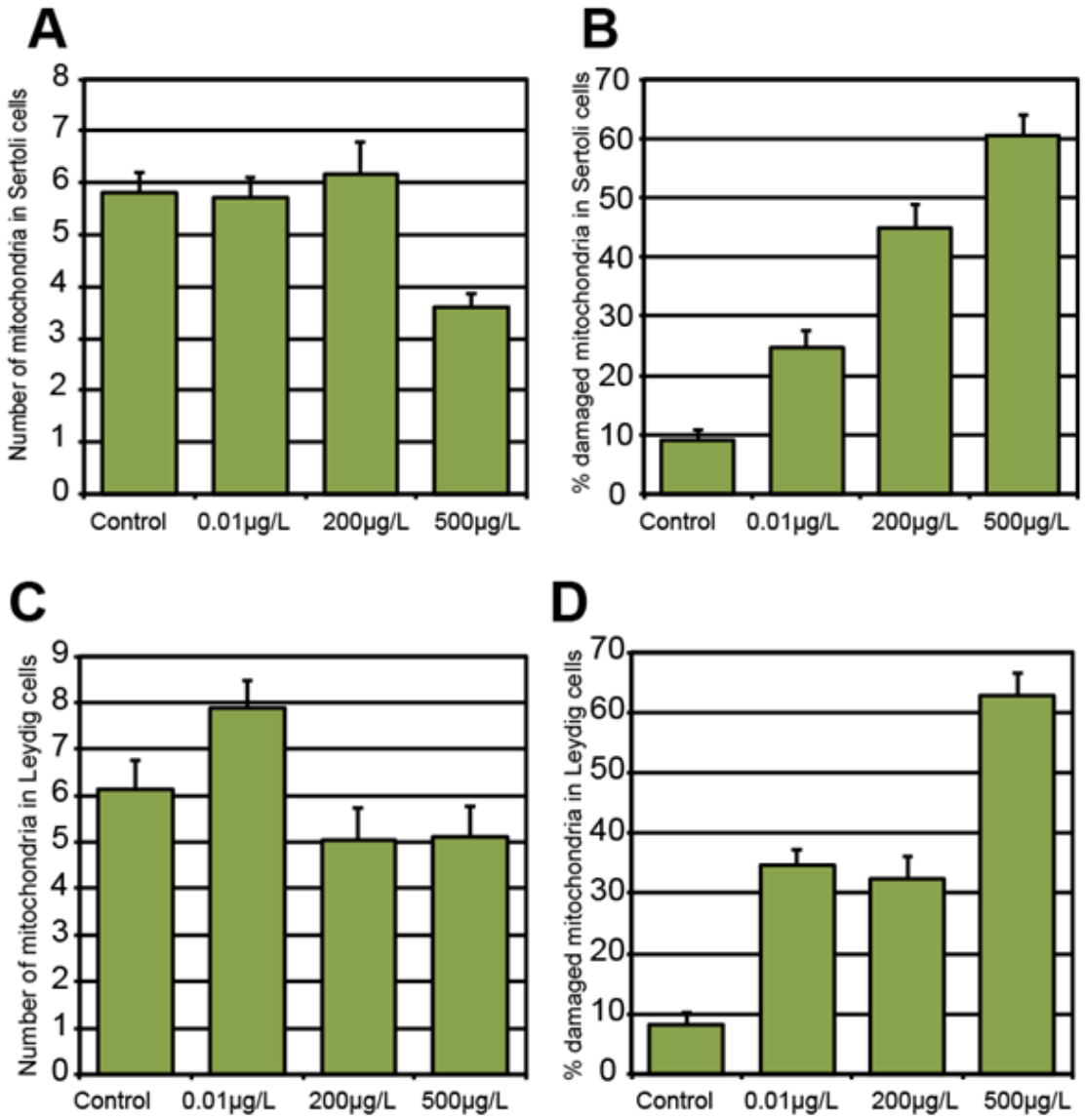

Figure 5.7 Analysis of mitochondria in adult X. laevis Sertoli cells for each exposure concentration

(A- average number of mitochondria and B- average number of damaged mitochondria). Analysis of mitochondria in adult *X. laevis* Leydig cells for each exposure concentration (C- average number of mitochondria and D- average number of damaged mitochondria) after atrazine exposure at different concentrations (error bars= standard deviation).

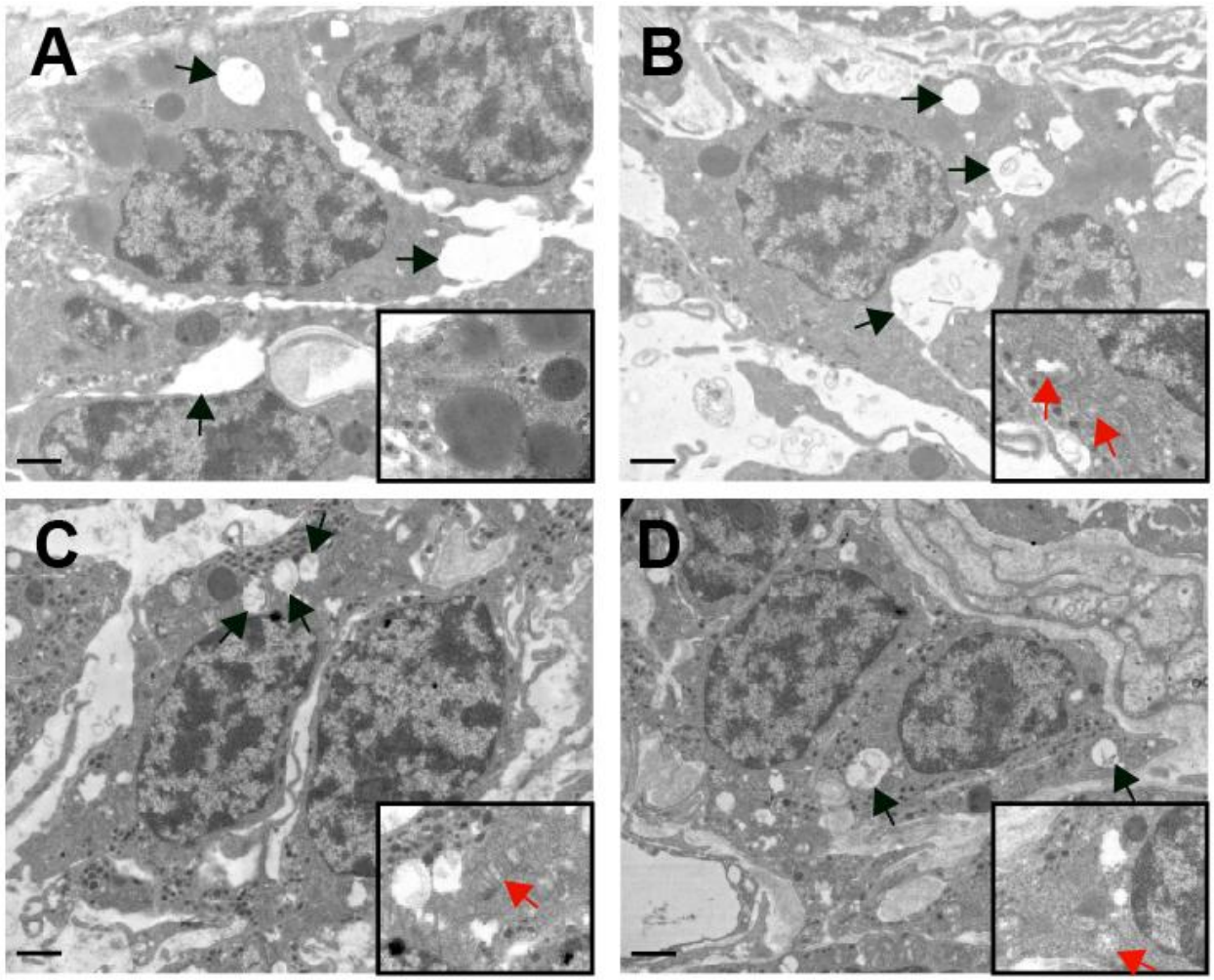

Figure 5.8 Electron micrograph showing typical Leydig cells in adult X. laevis.

A- control, B- 0.01 µg L^{-1}, C-200 µg L^{-1}, D- 500 µg L^{-1} atrazine exposure. Scale bar= 1 µm; Insert zoom shows mitochondria. Black arrows= lipid droplets, red arrows= vesicle/vacuole in mitochondrial matrix

Rough Endoplasmic Reticulum (RER)

The RER in Sertoli cells, Leydig cells and germ cells of all the control and atrazine exposed adult *X. laevis* appeared similar with no signs of swelling. The RER in smooth muscle of 500 µg L^{-1} appeared swollen (SI, Figure S5.4 B) when compared to other treatments, however the incidence of swelling was not consistent within the cells and between different frog testes. RER in both Leydig cells (SI, Figure S5.4 C and D) and Sertoli cells showed no noticeable differences between control and 500 µg L^{-1} exposures.

Biochemical analysis

The serum testosterone concentration of the 0.01 µg L^{-1} atrazine exposed frogs was marginally lower than the control. The serum testosterone concentrations of the 200 µg L^{-1} and 500 µg L^{-1} atrazine exposed frogs were 2 and 3 times lower than control, respectively (Table 5.2). This suggests a graded dose effect as reported by Hayes et al. (2010). 17-β estradiol and 17-α estradiol in serum were below the limit of detection after derivatisation, hence could not be detected. The endocrine disrupting effects of atrazine described by Hayes et al. (2002) were observed in this study.

The presence of atrazine metabolites in frogs is not well documented and data is scanty (Solomon et al., 2008). *In vitro* tests have shown that DEA and DIA induce aromatase activity with the same potency as atrazine (Sanderson et al., 2001). All 6 atrazine metabolites tested were detected in either serum or liver of frogs exposed to 200 and 500 µg L^{-1} atrazine concentrations. For frogs exposed to 500 µg L^{-1} atrazine concentrations, the relative concentrations of ADD, AD-2OH, DIA and DEA in serum (Table 5.2, 16.5 ± 2.3, 0.8 ± 0.2, 72 ± 3.6 and 78.5 ± 2.7 ng mL^{-1} respectively) were all higher than the relative concentrations in the liver (Table 5.3, 1.9 ± 0.6, 0.3 ± 0.1, 26.5 ± 1.2 and 59.7 ± 6.7 ng g^{-1}, respectively). The same trends existed for the 200 µg L^{-1} atrazine exposed frogs with the exception of AD-2OH and DEA which recorded higher concentrations in the liver. Atrazine metabolism in humans and animals has been described as a complex process which can vary from individual to individual (Joo et al., 2010). Only A-2OH and DEA were detected in serum from frogs exposed to 0.01 µg L^{-1} atrazine concentrations. Atrazine metabolites were not detected in both serum and liver of control frogs, liver of frogs exposed to 0.01 µg L^{-1} atrazine concentrations as well as beef liver quality control (QC) sample (Tables 5.2 & 5.3). DIA and DEA recorded the highest concentrations in both frog serum and liver (Tables 5.2 & 5.3), indicating that they are the major metabolites. High concentrations of 78.5 ng mL^{-1} for DEA in serum, as well as 59.7 ng g^{-1} in liver of the 500 µg L^{-1} atrazine exposed frogs may indicate that there is a significant degree of atrazine uptake and metabolism.

Table 5.2 Atrazine metabolites and testosterone levels

(average ng mL^{-1} and standard deviation) measured in *X. laevis* serum (n=15) after exposure to different atrazine concentrations

Exposure concentration	ADD	AD-2OH	DIA	A-2OH	DEA	Testosterone
(µg L^{-1})	(ng mL^{-1})					
500	16.5±2.3	0.8±0.2	72±3.6	2.1±1	78.5±2.7	0.4±0.1
200	9.9±1.5	0.2±0.1	28.8±2	1.9±0.4	27.8±1.4	0.6±0.3
0.01	N.D	N.D	N.D	3.8±0.3	0.6±0.1	1.1±0.5
Control	N.D	N.D	N.D	N.D	N.D	1.3 ±0.6

N.D = Not detected

Table 5.3 Atrazine metabolites measured

(average ng g^{-1} fresh weight and standard deviation) in frog liver (n=5) after exposure to different atrazine concentrations, and unexposed QC sample (beef liver).

Exposure concentration	ADD	AD-2OH	DIA	HA	DEA
(µg L^{-1})	(ng g^{-1} fresh weight)				
500	1.9±0.6	0.3±0.1	26.5±1.2	0.9±0.2	59.7±6.7
200	1.3±0.4	0.4+0.2	15.6±2.6	0.9±0.4	42.1±4.3
0.01	N.D	N.D	N.D	N.D	N.D
0.00	N.D	N.D	N.D	N.D	N.D
QC sample	N.D	N.D	N.D	N.D	N.D

N.D = Not detected

Conclusions

Exposure of *X. laevis* tadpoles to high sub-chronic atrazine concentrations of 500 µg L^{-1} lead to a significant reduction in tadpole mass and a significant increase in tadpole mortality, whilst exposure to 0.01 and 200 µg L^{-1} atrazine concentrations did not lead to significant mortality or reduction of tadpole growth and development. Exposure of adult *X. laevis* to 200 and 500 µg L^{-1}atrazine concentrations lead to seminiferous tubule structure damage and gonadal atrophy, with significant disruption of normal germ cell lines. Atrazine also caused significant stress and damage to Sertoli and Leydig cell mitochondria and as a result led to diminished serum testosterone levels in the atrazine treated groups. The data generated indicates that environmentally relevant atrazine concentrations adversely affect

frog gonadal development and tadpole survival at concentrations above the calculated LC50 value of 343.7 µg L^{-1}.

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Chapter 5 – Supporting Information

Tables:

Table S5.1 Mean adult frog mass

(g ± standard deviation) for second exposure (± SD)

Frog age (d)	Control (n= 15)	0.01 µg L^{-1} (n= 15)	200µg L^{-1} (n= 15)	500 µg L^{-1} (n= 15)
277	43.7 ±2.6	47.2 ±3.8	41.1 ±3.7	39.9 ±2.8
284	44.4 ±2.3	48.5 ±3.4	41.3 ±4.3	41.2 ±3.3
293	44.9 ± 3.1	48.3 ±3.7	41.4 ±4.5	41.0 ±2.6
300	44.6 ± 2.7	48.7 ±3.8	42.6 ±4	41.4 ±2.7
307	44.1 ± 3.3	49.2 ±4.1	42.5 ±4.6	41.7 ±2.7
320	45.22 ± 3.6	49.6 ±3.6	42.3 ±4.2	41.5 ±2.4
328	45.6 ± 3.3	49.4 ±3.7	42.2 ±4.4	41.3 ±2.8
334	45.2 ±2.9	49.8 ±3.6	42.7 ±4.1	41.2 ±3.3
341	45.5 ±3.2	50.2 ±4.1	42.4 ±3.8	41.5 ±3.5
348	45.9 ± 3.4	49.9 ±3.9	43.5 ±4	41.8 ±2.6
355	46.2 ±3.6	50.3 ±3.7	43.5 ±3.9	42.0 ±3.2
360	46.8 ±3.2	50.1 ±3.4	43.8 ±4.2	42.3 ±3.2
*Weight gain	3.1	2.9	2.7	2.4

* Weight gain= Net average weight gain during exposure period

SD= Standard deviation

Figures:

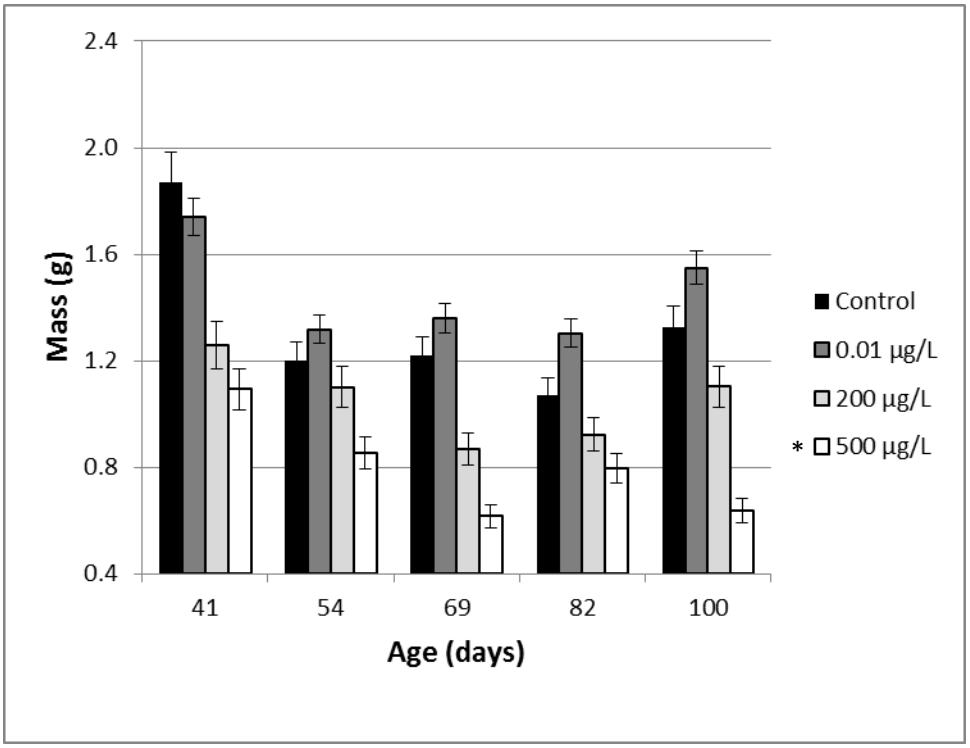

Figure S5.1 Tadpole/juvenile frog mean mass (error bars show standard deviation) after exposure to atrazine exposure at different concentrations (n=120).

The measurements at 41 d old were performed by water volume displacement, whereas the other day's measurements were performed by weighing on an accurate balance.

*Statistically significant

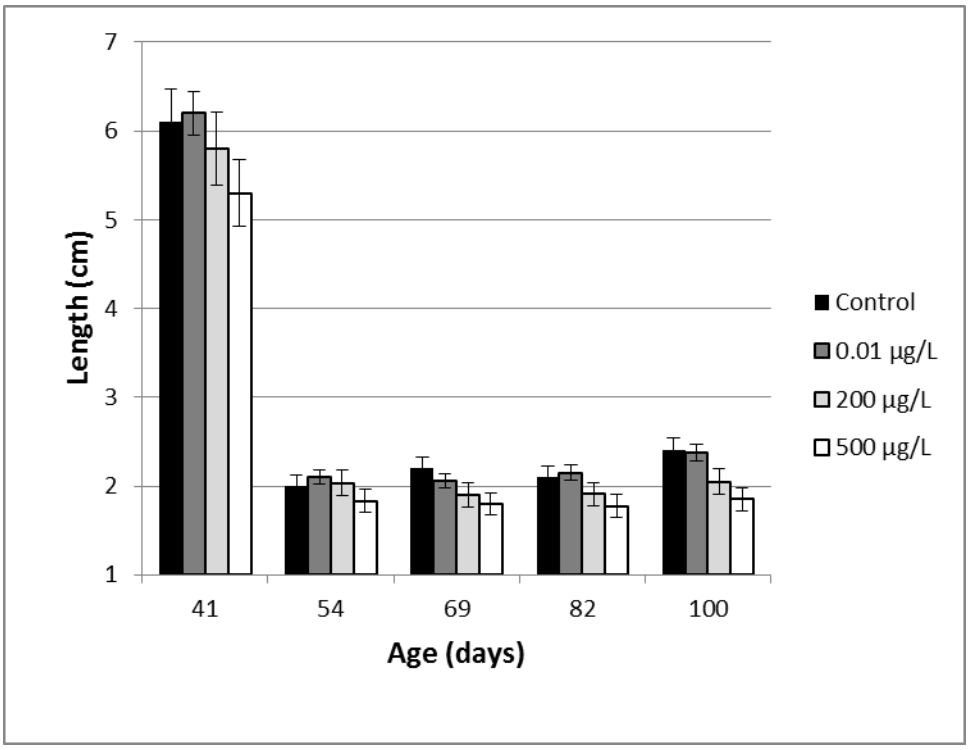

Figure S5.2 Tadpole/juvenile frog mean lengths

(error bars show standard deviation) measured from snout to tip after exposure to atrazineat different concentrations (n=120). At 41 d the tadpole's length was measured from snout to tail and from snout to vent thereafter.

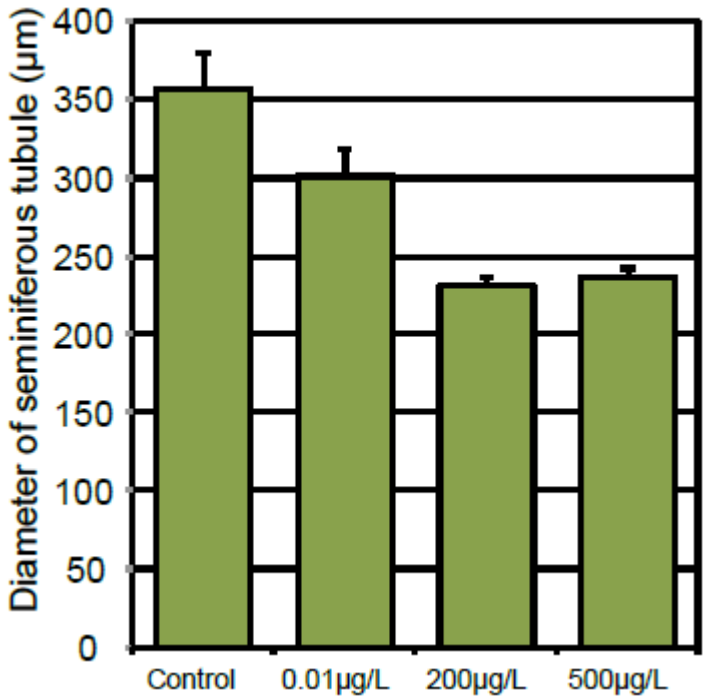

Figure S5.3 Adult *X. laevis* Seminiferous tubule average diameters after exposure to different atrazine concentrations

(error bars show standard deviation).

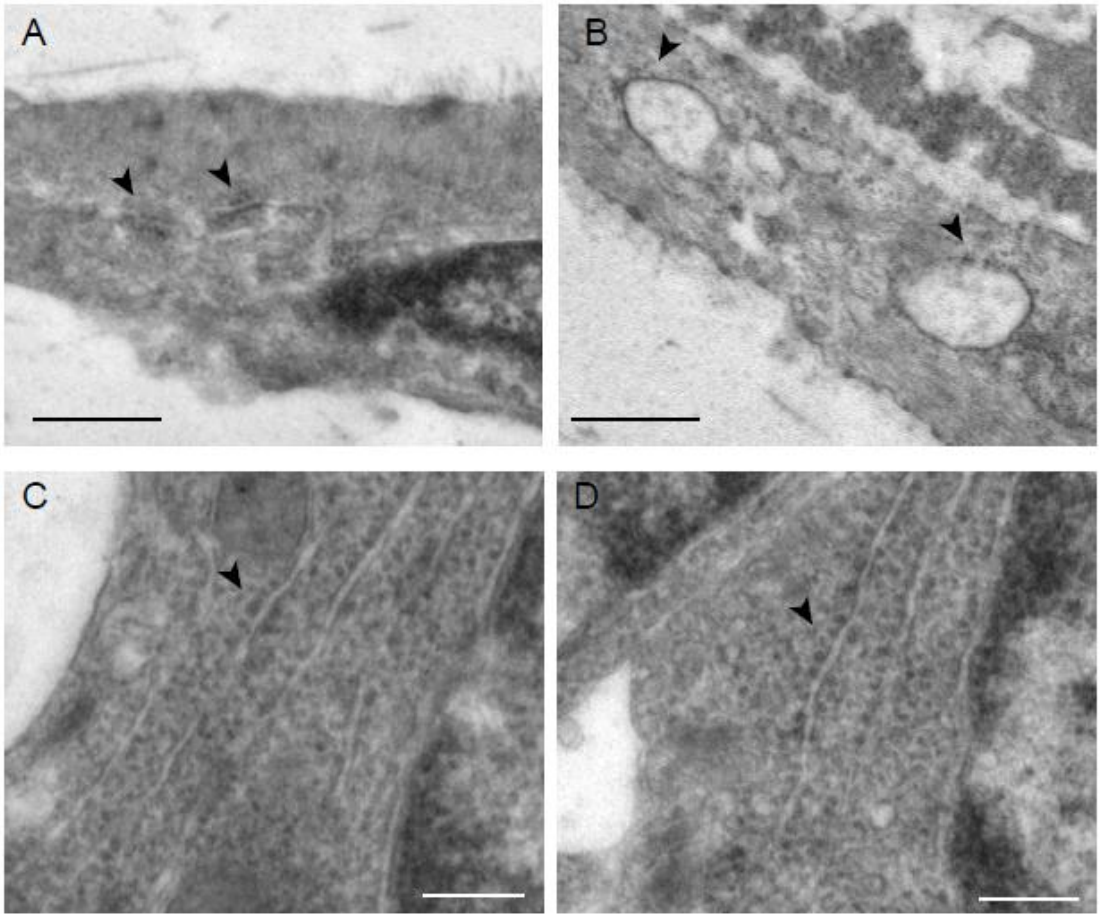

Figure S5.4 Electron micrographs of adult X. laevis RER structures in smooth muscle and Leydig cells.

A-smooth muscle control, B- smooth muscle 500 µg L^{-1} atrazine exposure, C- Leydig cell control and D-Leydig cell 500 µg L^{-1} atrazine exposure. Arrowheads indicate the RER structures. Scale bar represents 500 µm for A and B and 200 µm for C and D.

Chapter 6

Contaminants of emerging concern in the Hartbeespoort Dam catchment and the uMngeni River estuary 2016 pollution incident, South Africa

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Abstract

A quantitative assessment of pollutants of emerging concern in the Hartbeespoort Dam catchment area was conducted using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to establish the occurrence, source and distribution of 15 environmental pollutants, including 10 pharmaceuticals, 1 pesticide and 4 steroid hormones. Seasonal sampling was conducted in the Hartbeespoort Lake using sub-surface grab sampling to determine the lake's ecological status and obtain data for establishment of progressive operational monitoring. The Jukskei River, which lies upstream of the Hartbeespoort Dam, was sampled in the winter season. Five year old carp (*Cyprinus carpio)* and catfish (*Clarias gariepinus*) were also sampled from the Hartbeespoort Dam to study bioaccumulation in biota as well as to estimate risk associated with fish consumption. In the Jukskei River, the main source of 11 emerging pollutants (EPs) was identified as raw sewage overflow, with the highest ∑11 EP concentration of 593 ng L^{-1} being recorded at the Midrand point and the lowest ∑11 EP concentration of 164 ng L^{-1} at the N14 site located 1 km downstream of a large wastewater treatment plant. The Jukskei River was found to be the largest contributor of the emerging contaminants detected in the Hartbeespoort Dam. In the Hartbeespoort Dam EP concentrations were generally in the order efavirenz> nevirapine> carbamazepine> methocarbamol> bromacil> venlafaxine. Water and sediment were sampled from the uMngeni River estuary within 24 hours after large volumes of an assortment of pharmaceutical waste had been discovered to be washed into the river estuary after flash rainfall on 18 May 2016. Analytical results revealed high levels of some emerging pollutants in sediment samples, up to 81 ng g^{-1} for nevirapine and 4 ng g^{-1} for etilefrine HCL. This study shows that efavirenz, nevirapine, carbamazepine, methocarbamol, bromacil and venlafaxine are contaminants that require operational monitoring in South African urban waters. The high cost of analysis and high capital cost of purchasing LC-MS/MS instrumentation is a major constraint in maintaining operational monitoring programs in South Africa.

Introduction

An emerging contaminant is an unregulated compound that is not included in regular environmental monitoring programs and for which there is only limited knowledge about its toxicity and behavior in the environment, but has the potential to cause adverse effects to the ecosystem (López-Doval et al., 2017; Pal et al., 2010; Sauvé & Desrosiers, 2014). Emerging contaminants are seldom monitored in South African waters, as the significance of monitoring them is poorly understood. Therefore, the focus is

largely on legacy contaminants and toxic persistent organic pollutants. Emerging pollutants include active pharmaceutical ingredients (APIs) or biologically active compounds, some of which have been recently formulated, but widely used due to their efficacy (Metcalfe et al., 2014; Maruya et al., 2014; Pal et al., 2010). The analytical power of instrumentation and techniques to screen and quantify emerging contaminants has significantly improved over the past two decades, enabling the discovery of more emerging pollutants (Raldúa et al., 2011; Sauvé & Desrosiers, 2014; Valavanidis et al., 2014). Due to the large number of APIs present in water bodies impacted by wastewater treatment plant effluents as well as hospital and manufacturing waste, prioritising which API's to monitor may prove to be a challenge (Cadwell et al., 2014; Besse & Garic, 2008). API's are more likely to be found in the water fraction than in the sediment and air fractions due to their high polarity and low volatilities (Crane et al., 2006).

Ideally, predicted effect concentrations provide an acceptable way of identifying pollutants of high priority for monitoring in the environment, along with exposure, persistence, bioaccumulation and toxicity (EPBT) data (Ebele et al., 2017; Fick et al., 2010; Mansour et al., 2016). However much of this information is scanty for emerging pollutants (Besse & Garic, 2008). A constant exposure to API contaminants in aquatic reservoirs may have effects on non-target organisms such as altering homeostasis of exposed aquatic organisms, acute toxicity and development of pharmaceutical drug resistance in microorganisms due to chronic exposure at low concentrations (Agunbiade and Moodley, 2014; Ebele et al., 2017; Farré et al., 2008; Lei et al., 2015; Maruya et al., 2014; Pereira et al., 2015). As traces of antibiotics in water have been found to induce antibacterial resistance, the threat of antiretroviral resistance due to ubiquitous antiretroviral (ARV) drugs and metabolites in South African waters may be worth investigating (Sauvé & Desrosiers, 2014). A study by Swanepoel et al. (2015) has detected ARV drugs in groundwater as well as tap water sampled from the Gauteng Province of South Africa and ARV drugs in groundwater have been previously reported by K'Oreje et al. (2016) in Kenya. ARV drugs have also been discovered in surface water in the Gauteng, North West and Free state Provinces of South Africa in a study by Wood et al., (2015).

The effects of pharmaceuticals on the ecosystem may not be noticeable without considerable scrutiny, however, there is growing evidence of adverse effects of some pharmaceuticals on exposed aquatic animals (Klatte et al., 2017). With the exception of 17-β-estradiol and 17-α-estradiol, the majority of pharmaceuticals monitored in the environment do not appear to cause significant endocrine disruption or toxic effects in aquatic animals as they are usually below the therapeutic concentrations (Brumovský

et al., 2016; Caldwell et al., 2014; Crane et al., 2006; Jálová et al., 2013; Schoenfuss et al., 2016). However, the effect of a cocktail of pharmaceutical contaminants in water may produce a significant adverse effect compared to a single pharmaceutical contaminant (Schoenfuss et al., 2016). Antidepressants and central nervous system drugs including venlafaxine and methocarbamol may be linked to neurobehavioural disorders in aquatic animals with reports of diminished predator evasive behavior and less aggressive nest defence in exposed fish (K'Oreje et al., 2012; Schoenfuss et al., 2016). Morphological examinations of fish exposed to pharmaceuticals show enlarged livers, an observation that may be linked to the fact that most ARV drugs cause variable degrees of liver damage and hepatotoxicity in humans (Schoenfuss et al., 2016).

The Hartbeespoort Dam is situated downstream of the largest wastewater treatment plant in Johannesburg, Gauteng Province and is one of the major agricultural dams in South Africa. The Madibeng municipality draws water from the Hartbeespoort Lake for treatment and supply to the nearby Hartbeespoort Dam community, however it has also been implicated for contaminating the Hartbeespoort Dam with sewage overflow. Biologically active pharmaceuticals originating from the Northern wastewater treatment works (WWTW) and raw sewage spills contaminating the Jukskei River may potentially contaminate aquatic life and irrigated crops downstream as most wastewater treatment plants are not designed to eliminate active pharmaceutical ingredients, unmetabolised pharmaceuticals and other organic compounds (Barbosa et al., 2016). Operational and investigative monitoring of emerging contaminants in water bodies may provide useful information to water resource managers, particularly in times of episodic contamination. An episodic pollution event recorded on 18 May 2016 caused large quantities of improperly disposed medical waste to be deposited on the uMngeni River estuary, located north of Durban city centre in KwaZulu-Natal province, as well as the adjacent beach after sudden flash rainfall. As a result, the beach was closed to the public for massive cleanup campaigns which took more than 3 days to complete. In spite of the pollution incident, the beach was re-opened to the public within 24 hours after the incident was discovered, before any scientific tests could be conducted on the water quality. During the estuary cleanup numerous pharmaceutical bottles containing mainly expired medicines were found on the uMngeni estuary and beach front, including multivitamins as well as ARV and hypertension drugs. The source of the pharmaceutical waste was never identified, hence accountability as stipulated in the South African National Environmental Management Act 107 0f 1998 (which states that the waste holder should take steps to ensure that waste does not

endanger health or the environment) was not implemented because investigations were inconclusive. Despite South Africa having formidable environmental laws, environmental compliance monitoring and enforcement has often been viewed as being ineffective (DEA, 2012).

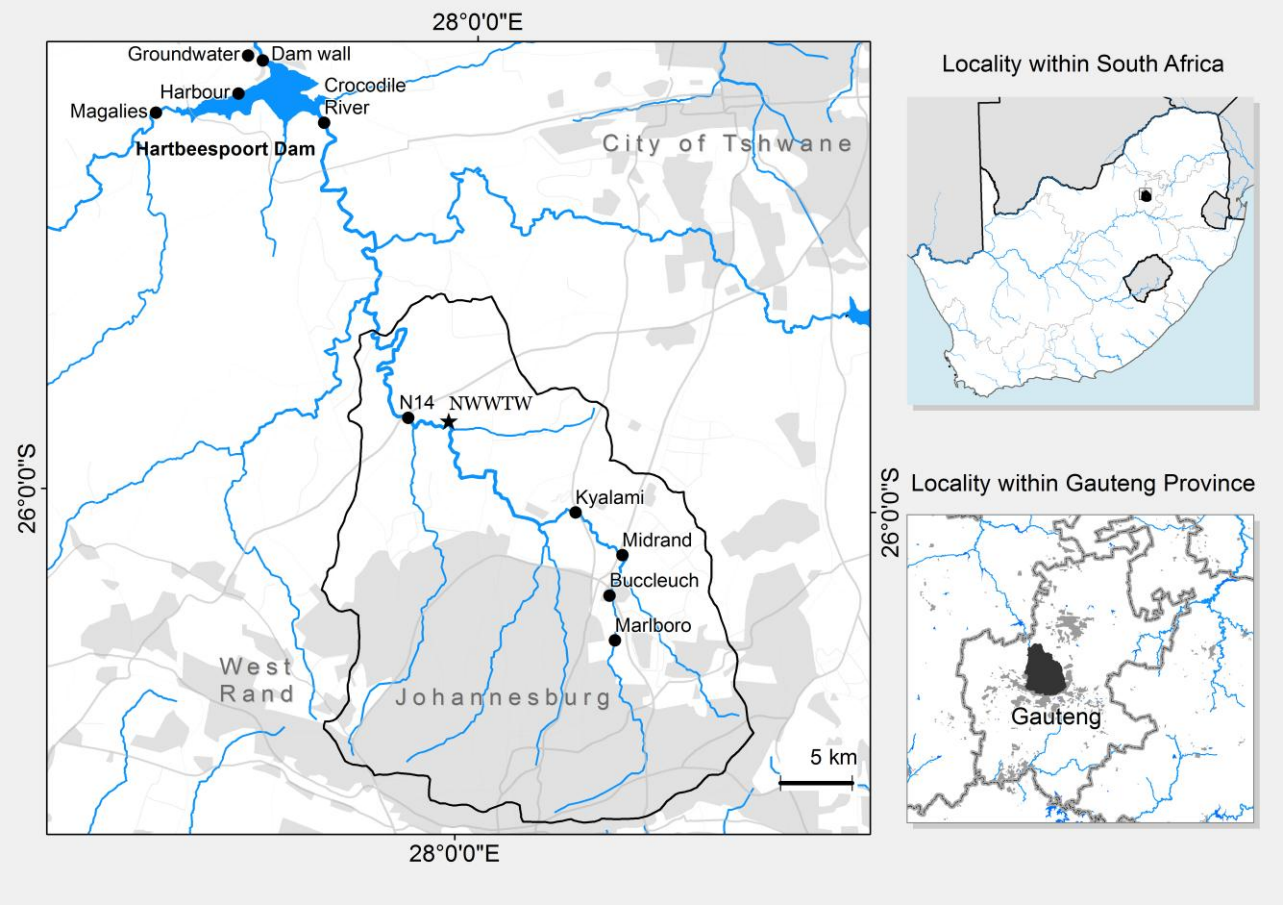
The aim of this research was to study the seasonal variation of selected emerging pollutants (EPs) and to assess their occurrence and transport in the Hartbeespoort Dam catchment, which is impacted by a large wastewater treatment plant and numerous informal settlements which contribute raw sewage into the catchment. It was also aimed to assess the impact of an episodic event of improperly disposed pharmaceutical waste washing up on the uMngeni River estuary by analysis of the 15 EPs in water and sediments sampled within 24 hours after the pollution incident was discovered.

Materials and methods

Study area and sampling

Five sites located primarily around the major inlets and outlet of the Hartbeespoort Dam (Figure 6.1) were sampled to determine the point with the greatest influence on the Hartbeespoort Dam pollution. Another 5 points located at pollution hotspots upstream of the Hartbeespoort Dam, in the Jukskei River (Figure 6.1) were selected for the study. The GPS coordinates are listed in Supporting Information (SI) Table S6.1. The Jukskei River and the Hartbeespoort Dam are effluent dominated waters (Wimberly & Coleman, 1993). The Jukskei River passes through the largest and most densely populated and industrialised region in South Africa. The Jukskei River sampling points of Marlboro, Buccleuch, Midrand and Kyalami (Figure 6.1) are located in areas heavily impacted by raw sewage where numerous informal settlements with no municipal sewage facilities are sources of organic pollutants, particularly upstream of the Marlboro site as well as downstream of the Midrand site. Other point sources include municipal and industrial waste waters as well as the Northern WWTW which is the largest wastewater treatment plant in Johannesburg, serving 1.6 million people with a sewage capacity of 360 million L day^{-1}. The Northern WWTW is located adjacent to the Jukskei River, upstream of the N14 site where the effluent is discharged (Amdany et al., 2014). The sites were selected to determine the effect of the wastewater treatment plant as well as untreated sewage continuously contaminating the Jukskei River between the Marlboro and Midrand sites. At the Hartbeespoort Dam, the groundwater site is situated in close proximity to the Dam wall point (1.1 km away) and the water is used for drinking purposes by the surrounding community and government offices.

The uMngeni River estuary lies downstream of an expanse of municipal, rural, industrial and agricultural lands (Matongo et al., 2015). A composite sub-surface water sample was sampled across the uMngeni River estuary from a boat, along site U2 (Figure 6.2) using sub-surface (2-3 cm below the water surface) grab sampling, filling a clean 4 L amber glass bottle. Sediment samples were taken at sites U1 to U4 (Figure 6.2) to assess the risk and concentrations of medical waste and expired pharmaceuticals (suspected to have been dumped along the uMngeni River) washed downstream by flash rainfall. All samples were taken in a random manner within a 2 m radius and placed in a cooler box with ice packs before transporting by air to the laboratory cold room maintained at 5 ^{o}C. Site U4 is located on the mouth of the uMngeni River estuary (Figure 6.2). Sites U1, U2, U3 are located 700 m, 250 m and 120 m from the mouth of the estuary (U4) respectively. The GPS coordinates are listed in SI Table S6.2.



NWWTW = Northern Wastewater Treatment Works (Municipal sewage treatment plant). The Jukskei River catchment is shown in black border

Figure 6.1 Sampling area- Hartbeespoort Dam catchment, Gauteng province, South Africa

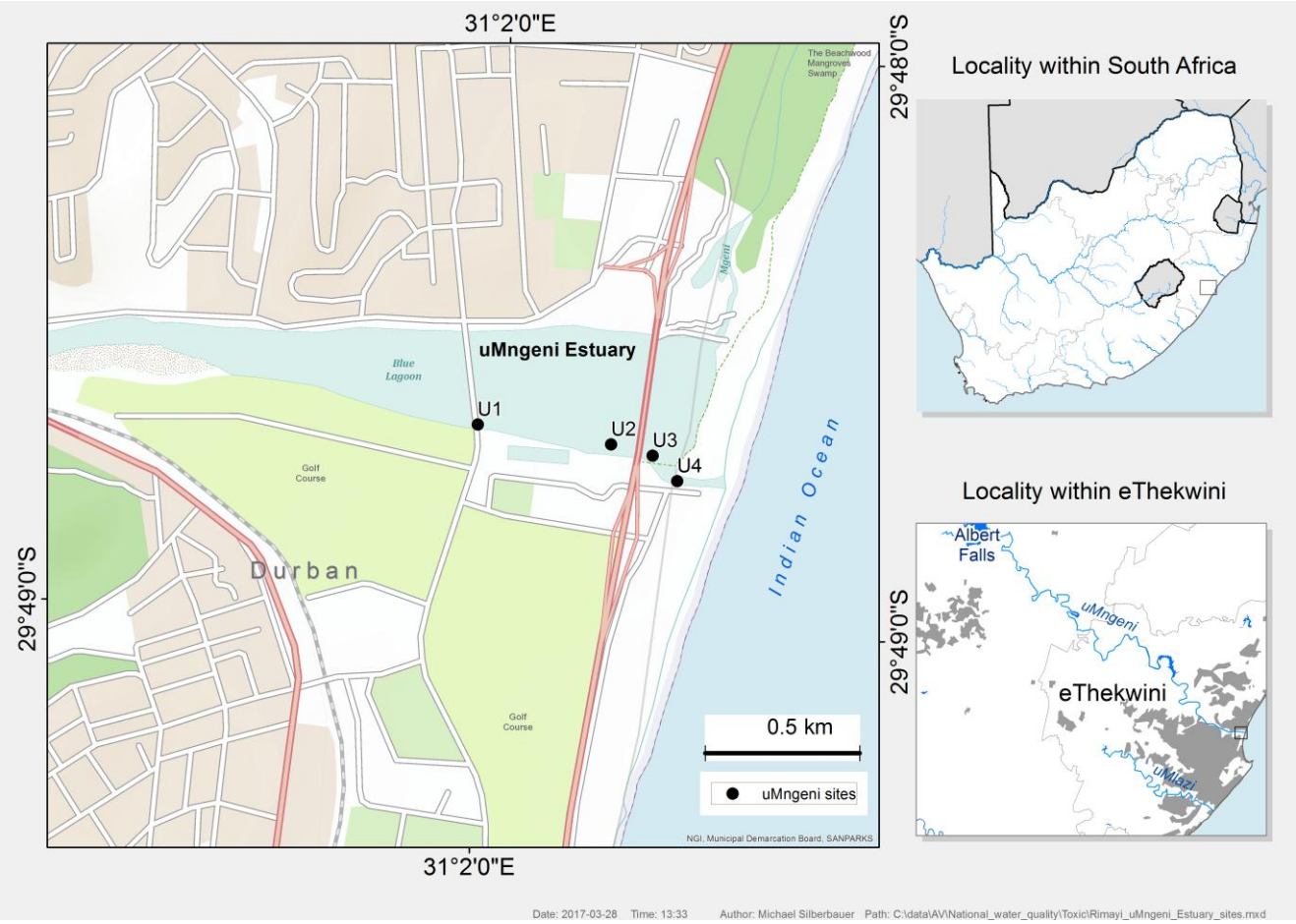


Figure 6.2 Sampling area- uMngeni River estuary

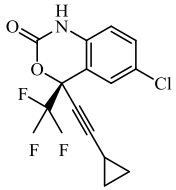
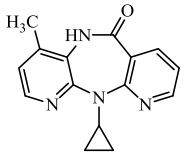
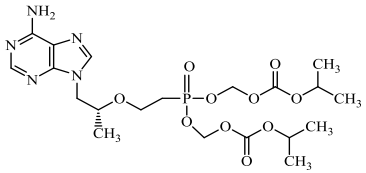
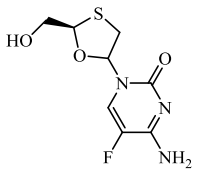
Sampling in the Hartbeespoort Dam was conducted between November 2014 and September 2015 (SI Table S6.3), over 4 seasons and the Jukskei River was sampled in May 2015 (winter season). The uMngeni River estuary lies in the eThekwini municipal area (Figure 6.2) and was sampled on 19 May 2016. Sediment samples (top 0-5cm) were sampled using a Van Veen grab sampler and collected in a clean 500 mL glass jar. Water samples were sampled using sub-surface (2-3 cm below the water surface) grab sampling and a bailer for the Hartbeespoort Dam wall point at 5 m and 30 m depths. The water samples were collected in a clean 4 L amber glass bottle. All samples were taken in a random manner within a 2 m radius from within a boat and placed in a cooler box with ice packs before being transported to the laboratory where they were stored at 5 ^{o}C prior to analysis. Three 5 year old catfish (*Clarias gariepinus*) and three 5 year old carp (*Cyprinus carpio*) were caught on 14 August 2015 from the Hartbeespoort Dam using mobile nets and immediately frozen at the Hartbeespoort Dam site before

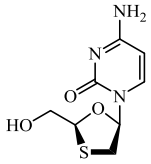
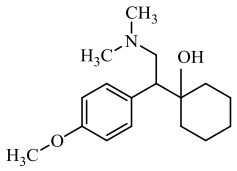
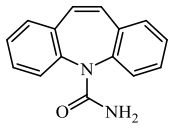
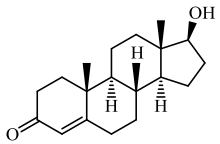
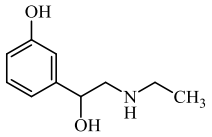
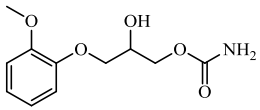
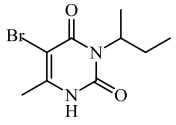
transporting to the laboratory freezer in a cooler box packed with ice. The age of the catfish was estimated by using length-weight ratios and the age of the carp was estimated using a combination of length-weight ratios and scale annuls, utilising scales taken from between the lateral line and pectoral fin. The seasons (SI Table S6.3) and climate of the sampling area has been described in detail elsewhere (Rimayi et al., 2018).

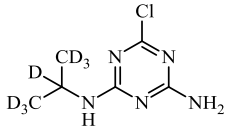
Chemicals

All internal and native standards had a purity ≥96% (Tables 6.1 & 6.2) and were supplied by Dr. Ehrenstorfer and Toronto Research Chemicals (Industrial Analytical Johannesburg, South Africa). All solvents had a purity >99.9%. Ultrapure Milli-Q water used in all preparation work was produced by a Millipore Advantage system (Merck, Johannesburg, South Africa) with a TOC <3 mg L^{-1}.

Table 6.1 Target database and internal standards

Compound	Structure	Purity (%)	Compound type	Quantification ion	Confirmation ion 1 (Collision Energy)	Confirmation ion 2 (Collision Energy)	Therapeutic dose side effects on humans*
Efavirenz CAS# 154598-52-4 $C_{14}H_9ClF_3NO_2$ logP: 4.46		100	NNRTI	53.2 (60)	167.5 (20)		Moderate to severe liver problems
Nevirapine CAS# 129618-40-2 $C_{15}H_{14}N_4O$ logP: 2.49		100	NNRTI	78.2 (68)	51.1 (112)		Hepatotoxicity causing fatal liver problems, life threatening skin reaction
Tenofovir disoproxil CAS# 202138-50-9 $C_{19}H_{30}N_5O_{10}P$ logP: 2.65		98	NRTI	176.5 (56)	159.4 (64)	136.5 (72)	Severe liver problems, severe lactic acidosis
Emtricitabine (mikromol) CAS# 143491-57-0 $C_8H_{10}FN_3O_3S$ logP: -0.90		100	NRTI	130.3 (8)	85.3 (56)	58.3 (72)	Lactic acidosis, liver damage, skin reaction

Lamivudine CAS# 134678-17-4 $C_8H_{11}N_3O_3S$ logP: -1.10		99.7	NRTI	112.3 (8)	52.2 (76)	95.3 (44)	Severe and fatal liver problems, high blood acid levels (lactic acidosis), skin reaction
Venlafaxine CAS# 93413-69-5 $C_{17}H_{27}NO_2$ logP: 2.74		100	Selective serotonin reuptake inhibitor (SSNRI) antidepressant	58.2 (16)	77.3 (72)	51.2 (132)	Skin reaction, difficulty breathing
Carbamazepine CAS# 298-46-4 $C_{15}H_{12}N_20$ logP: 2.77		99.5	Anticonvulsant and mood stabilizer	165.4 (52)	191.5 (56)	62.2 (152)	Ataxia (loss of control of body movement), dizziness, drowsiness
Testosterone CAS# 58-22-0 $C_{19}H_{28}O_2$ logP: 3.37		99	Natural androgen	79.2 (20)	77.2 (52)	51.2 (192)	Endocrine disruptor
Etilefrine (β-blocker) CAS# 709-55-7 $C_{10}H_{15}NO_2$ logP: 0.23		100	Cardiac stimulant	164.5 (8)	77.2 (56)	65.2 (56)	Anxiety, tremors, headaches
Methocarbamol CAS# 532-03-6 $C_{11}H_{15}NO_5$ logP: 0.45			Muscle relaxant	118.3 (8)	199.6 (4)	77.3 (52)	Syncope (temporary loss of consciousness), anaphylaxis, hives, Abdominal pain
Bromacil CAS# 314-40-9 $C_9H_{13}BrN_2O_2$		99	Herbicide	54.2 (36)	205.4 (4)	52.2 (40)	Poisoning and toxicity

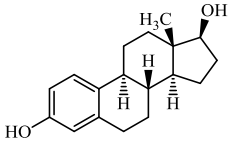
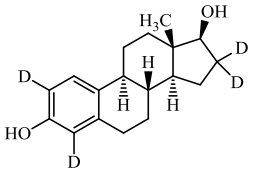
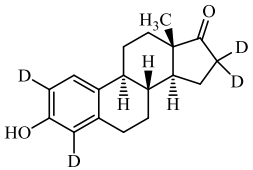
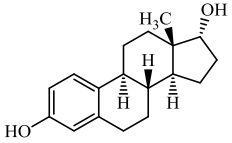
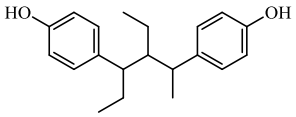
logP: 2.51 D7 deethylatrazine CAS # 1216649-31-8		96	Internal standard	195.6 (4)	147.5 (16)	68.2 (48)	n/a

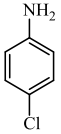
NNRTI = Non-Nucleoside Reverse Transcription Inhibitor (Antiretroviral drug)

NRTI= Nucleoside Reverse Transcription Inhibitor (Antiretroviral drug)

*information available on https://www.drugs.com

Table 6.2 Derivatised (dansylated) target database and internal standards

Compound	**Structure**	Purity (%)	Compound type	Dansylated Derivative Mw	Dansylated derivative Mf	Dansylated derivative transition (Collision Energy)	Confirmation ion 1 (Collision Energy)	Confirmation ion 2 (Collision Energy)
17-β-Estradiol CAS# 50-28-2 $C_{18}H_{24}O_2$ logP: 3.75		100	Natural steroid hormone	505.6	$C_{30}H_{35}NO_4S$	506.2→171.6 (56)	156.3 (64)	
D4 17-β-Estradiol $C_{18}HD_4H_{20}O_2$ logP: 3.75		99	Internal standard	509.1	$C_{30}H_{39}NO_4S$	510.3→171.5 (36)	156.3 (60)	
D4 Estrone CAS#53-16-7 $C_{18}D_4H_{18}O_2$ logP:		99	Internal standard	507.2	$C_{30}D_4H_{29}NO_4S$	508.2→171.5 (36)	156.4 (68)	
17-α estradiol 58-22-0 logP 3.75		99	Natural steroid hormone	505.6	$C_{30}H_{35}NO_4S$	506.2→171.6 (36)	154.4 (68)	115.3 (100)
Benzestrol CAS# 85-95-0 $C_{20}H_{26}O_2$ logP: 6.10		98	Non steroidal synthetic estrogen	764.9	$C_{32}H_{37}NO_4S$	765.3→281.5 (44)	156.3, (62)	171.6 (58)

p-Chloroaniline CAS# 106-47-8 $ClC_6H_4NH_2$ logP: 1.75 		98	Antimicrobial and pesticide and dye precursor	362.1	$ClC_{18}H_{17}N_2O_2S$	363.1→168.4 (48)	115.3 (72)	156.5 (44)

Mw= molecular weight
Mf= molecular formula

Sample analysis

Water sample analysis

1 L water samples for LC-MS/MS analysis were extracted using 200 mg Agilent Bond Elut Plexa (Stryrene divinyl benzyl) solid phase extraction cartridges (Chemetrix, Johannesburg, South Africa) according to the method described by Rimayi et al. (2018). The sample extracts were reconstituted with 200 µL of 10% methanol (Milli-Q water: Methanol, 90:10 v/v).

Fish analysis

The modified Quick Easy Cheap Efficient Rugged Safe (QuEChERS) analytical procedure used for the analysis of the 5 year old free range carp and catfish muscle has been described elsewhere (Rimayi et al. 2018).

Sediment analysis

Sediments were freeze dried using a Christ Alpha 1-4 LD plus freeze dryer (Lasec, Johannesburg, South Africa), granulated with a mortar and pestle and sieved through a 2 mm sieve before transporting to The Netherlands under frozen conditions where extraction was performed according to the EPA method 3545A using dichloromethane and acetone 1:1 (v/v) under conditions described by Rimayi et al. (2016).

Particle size distribution

The particle size distribution was measured using an automated Retsch AS200 filterflow instrument set at aptitude 60 for 2 min, using ISO 3310-1 certified sieves with mesh sizes of 2000, 424, 100, 50 and 25 µm. The sieved fractions were weighed using a calibrated Precisa 180A, 5 decimal place balance. Stones and debris >2 mm were removed using a 2 mm mesh size sieve.

Total organic carbon

A modified Walkley-Black method was used to calculate the total organic carbon (TOC) in sediments according to the method described by Rimayi et al. (2016). The sediment samples were weighed into a digestion tube before adding 8 mL of 1 N $K_2Cr_2O_7$ (Minema, Johannesburg, South Africa) and digesting the mixture for 30 min at 150 ˚C using a digestion block. After cooling, the digestant was transferred to a conical flask and rinsed with 25 mL deionised water before adding 2 mL H_3PO_4 to eliminate interfering

Fe^{3+} ions. Eight drops of 0.2% barium diphenylamine-4-sulfonate (Sigma-Aldrich, Missouri, USA) colour indicator solution were added before back titrating the residual $K_2Cr_2O_7$ to a green endpoint with 1 M ferrous ammonium sulphate ($Fe(NH_4)_2(SO_4)_{2*}6H_2O$) solution (BDH Merck Limited, Poole, England).

LC-MS/MS method

An LC-ESI-MS/MS (1200 series LC system, 6410 triple quadruple MS; Agilent Technologies, Amstelveen, The Netherlands) instrument utilising a 100 x 2.1 mm Kinetex 2.6 µm Biphenyl liquid chromatography (LC) column (100Å) maintained at 25°C was used. The mobile phase for compounds not derivatised (Table 6.1) consisted of Solvent A: 5 mM ammonium formate in Milli-Q water (pH 4, formic acid) and Solvent B: 1.5% formic acid in methanol. Solvent A gradient was held at 100% for 2 min before increasing solvent B linearly to 20% (hold 8 min), increasing solvent B linearly to 95% (hold 5 min) and returning to 100% solvent A at 15.5 min (hold 14.9 min). A 10 µL injection volume was used with a constant flow of 0.3 mL min^{-1}. Tandem mass spectrometry was (MS/MS) was coupled to an Electron Spray Ionization (ESI) source in positive mode with source spray voltage 4 kV, transfer capillary temperature 350 ^{o}C, capillary voltage +4000 V, nitrogen drying gas flow 9 mL min^{-1} and nebulizer pressure 40 psi. For dansylated compounds (Table 6.2), solvent A consisted of 1% formic acid in Milli-Q water and solvent B consisted of 1.5% formic acid in methanol. A dansyl chloride derivatisation procedure was utilized to achieve a calibration range of 8 to 0.1 ng L^{-1} for 17-β estradiol, 17-α estradiol, D4 estrone, *p*-chloroaniline and benzestrol. The derivatisation procedure employed is described elsewhere (Nelson et al., 2004) and yielded monodansylated 17-β estradiol, 17-α estradiol and *p*-chloroaniline as well as bidansylated benzestrol. A calibration range of 200 to 0.2 ng L^{-1} was used for the underivatised compounds. Data was acquired in dynamic MRM mode and analysed on a computer with MassHunter quantitative analysis software (Palo Alto, USA).

Evaluation of method performance

All native and internal standards were made up to a 1 mg mL^{-1} stock solution in methanol with gravimetric correction for standards with <100% purity. The LC-MS/MS analytical method robustness was successfully tested for precision and repeatability (n=6 samples, SI Table S6.4). A combination of standard addition and labelled internal standard calibration was used to calculate recoveries and compensate for matrix effects. Water sample recoveries ranged from 73 to 112% for underivatised compounds and 58 to 87% for derivatised compounds (SI Table S6.4). Sediment sample recoveries

ranged from 84 to 123% for underivatised compounds and 69 to 86% for derivatised compounds (SI Table S6.4). Limit of detection (LOD) and limit of quantification (LOQ) were calculated at 3x and 10x the signal to noise ratio, respectively. 17-β estradiol, 17-α estradiol, benzestrol and *p*-chloroaniline had poor ionization in both positive and negative ionization which resulted in high detection limits. A derivatisation method with dansyl chloride was utilized to lower their limit of detection from ranges of 10-0.125 ng L^{-1} to 0.1-0.04 ng L^{-1} in positive ionization mode. The use of D4 17- β estradiol internal standard was validated against a D4 estrone internal standard to prove that there are no interferences. 17-α estradiol, 17-β estradiol and D4 17- β estradiol were validated and proved to be free from interference. 17-β estradiol and 17-α estradiol peaks were completely resolved by retention time, eluting at 15.488 min and 15.048 min respectively. 17-β estradiol and D4 17-β estradiol coeluted at 15.488 min but were resolved by optimising the dansylated transitions 506.2→171.6 and 510.3→171.5 respectively.

Results and discussion

Hartbeespoort Dam water pollutants

Efavirenz, nevirapine and carbamazepine were the only EPs found in all water samples in the Hartbeespoort Dam samples, with concentrations generally in the order efavirenz > nevirapine > carbamazepine (Table 6.3). Methocarbamol, bromacil and venlafaxine could be quantified in 81, 76 and 62% of the water samples, respectively. Steroid hormones analysed could not be detected in any of the water samples tested. On analysis of the surface water ∑11 EP concentrations, the Crocodile River and Magalies River points had the highest and lowest ∑11 EP concentrations respectively in all four seasons (Figure 6.3). The other two surface water points of Dam Wall 30 m and Harbour showed trends of varying and fluctuating levels between the four seasons. Unlike herbicides, which are abundant in the summer rainy season, EPs were present in the lowest concentrations in summer which may be attributed to dilution by the high seasonal summer rainfall. Seasonal pollutant concentrations which were in the order spring > winter > autumn > summer were influenced by the rainy seasons as the spring and winter seasons receive the least rainfall.

The most abundant pollutants found in the groundwater were nevirapine and carbamazepine, followed by efavirenz (Table 6.3). As humans depend on the groundwater as a source of drinking water this

exposure pathway may lead to adverse health effects. Nevirapine concentrations in groundwater showed fairly constant levels of 8, 10, 13 and 13 ng L^{-1} for summer, autumn, winter and spring respectively with a relative standard deviation of only 2% for the 4 seasons sampled. Groundwater ∑11EP concentrations were lower than surface water concentrations in three of the four seasons, except spring where the groundwater and Magalies River points recorded ∑11EP concentrations of 29 and 27 ng L^{-1} respectively. Generally, the Magalies River is the least EP impacted surface water point in the Hartbeespoort Dam as it is not located downstream of any major point source, with its water source being the pristine Magalies mountains. The Magalies River passes through rich agricultural lands before pouring into the Hartbeespoort Dam.

There were no significant differences between the Hartbeespoort Dam 5m and 30m in EP concentrations in spring. The Crocodile River point which recorded the highest ∑11EP concentrations has the greatest influence on the Hartbeespoort Dam pollution particularly as it has significantly higher flows and contributes 90% of the water in the Hartbeespoort Dam (Amdany et al., 2014).

Jukskei River water pollutants

In the Jukskei River, the Midrand point, which is affected by multiple point source contamination from raw sewage, recorded the highest ∑EP concentration of 593 ng L^{-1} (Table 6.3). This is more than twice as much as the Crocodile River winter concentration of 213 ng L^{-1}. The rampant raw sewage contamination may be the primary source of high emerging pollutant levels detected at the Midrand site. The furthermost upstream point of Marlboro recorded the second highest ∑11EP concentration of 368 ng L^{-1}. The Marlboro site is downstream of the Alexandra Township whose municipal sewage facilities are overloaded with up to 5 times the volume it was originally designed for. This situation therefore results in frequent unabated raw sewage overflows into the Jukskei River. The Marlboro site is also located downstream of riverside informal settlements such as the Stjwela community in Alexandra which is located right on the bank of the Jukskei River with no municipal sewage facilities.

The Kyalami point located immediately downstream of the Midrand point recorded a significantly lower ∑11EP concentration of 226 ng L^{-1} compared to the upstream Midrand point. Despite being located downstream of the Northern WWTW, the furthermost upstream Jukskei River point of N14 recorded the lowest ∑11EP concentration of 164 ng L^{-1}. This may be due to the dilution effect as the Jukskei River is joined by three tributaries (Figure 6.1) before the Northern WWTW. The Northern WWTW does not

contribute polar organic pollutants as significantly as the raw sewage contaminating the Jukskei River at Alexandra, upstream of the Marlboro point as well as the informal settlements upstream of the Midrand point. The stench of raw sewage was quite strong in most of the Jukskei River sampling points. Fluctuations in the ∑11EP concentrations along the Jukskei River may be attributed to temporal pollution fluxes due to raw sewage overflows that are rampant along the Jukskei River, particularly upstream of the Northern WWTW.

Jukskei River ∑11EP concentrations were in the order Midrand>Marlboro>Buccleuch>Kyalami>N14. Further downstream of the N14 point, the Crocodile River point had the highest contribution of emerging pollutants into the Hartbeespoort Dam, particularly highest for the spring and winter with ∑11EP concentrations of 577 and 213 ng L^{-1} respectively, indicating that the Jukskei River contributes the greatest emerging pollutant concentrations towards the Hartbeespoort Dam. The Jukskei River between the Midrand, Kyalami and Marlboro has historically been described as similar to sewage in the dry season sampled (Wimberly & Coleman, 1993) and to date, still has the same characteristics. Efavirenz was found in the highest concentrations in all Jukskei River points with significantly higher concentrations in all points compared to the Hartbeespoort Dam points averaging, 6-fold higher in the winter season. Emtricitabine was only detected in the N14, Marlboro and Buccleuch sites throughout the Hartbeespoort Dam catchment with low concentrations of 0.5, 13 and 2 ng L^{-1} respectively (Table 6.3). *P*-chloroaniline was detected at very low concentrations, below the LOQ in all the Jukskei River points with the exception of the Kyalami site which had no detectable *p*-chloroaniline. Steroid hormones could not be detected in all Jukskei River water samples and fish muscle tested. In the fish muscle tested, only bromacil could be detected above the LOQ with a concentration of 1 ng g^{-1} in catfish (Table 6.3), indicating that bioaccumulation of polar emerging compounds in fish muscle is negligible.

uMngeni River water pollutants

In the uMngeni River composite water sample, efavirenz was found in the highest concentration of 138 ng L^{-1} (Table 6.3). Carbamazepine and nevirapine were also detected at high concentrations of 94 and 68 ng L^{-1}. Other pollutants were identified at lower concentrations (Table 6.3) and the steroid hormones analysed could not be detected in the uMngeni water samples. Comparing the ∑11EP water concentrations of the uMngeni River estuary and Jukskei River points, the uMngeni River estuary ∑11EP concentration across the U2 point which recorded 323 ng L^{-1} was only lower than the Midrand and

Marlboro points which recorded high values of 593 and 368 ng L^{-1}. The downstream Buccleuch point in the Jukskei River with a ∑11EP concentration of 303 ng L^{-1} is considered a polluted water body where swimming and bathing is prohibited. Based on the concentrations of pollutants detected, re-opening the Umgeni beach to the public after 24 hours may have been too hasty, particularly as the cleanup operation was still underway and bottles containing a variety of pharmaceuticals, soaked with water inside were still in the water as well as on the estuary islands and the bank of the uMngeni River estuary.

uMngeni River sediment pollutants

Nevirapine was found at the highest concentration of 81 ng g^{-1} at U1 (700 m upstream of the uMngeni River mouth) sediments and 11 ng g^{-1} at the U2 site sediments (250 m upstream of the uMngeni River mouth, Figure 6.2). Only efavirenz and *p*-chloroaniline could be detected near the sandy estuary mouth (U3 and U4 sites) whilst all other compounds tested were detected at the upstream sites (Figure 6.2). Organic pollutants experience dynamic sorption and desorption between the water phase and organic material in sediment. Sediments with high organic carbon content have a greater pollutant sorption capacity. Finer sediment particles consist of higher clay content which have higher organic pollutant sorption characteristics compared to larger sand particles. The sediment composition therefore plays an important role in retention and sorption of organic pollutants. The trends observed may be attributed to the significantly lower TOC and the more sandy samples at U3 and U4 as indicated by the high proportion of sandy particles (2000-425 µm, Figure 6.4) particularly at U4 site with no particles at all <100 µm. U1 had the highest proportion of clay particles (<100) µm and U4 had the and highest proportion of sandy particles (2000-425 µm) respectively. This sediment composition may explain the higher detection rates of the pollutants at U1. Carbamazepine has previously been detected in the uMngeni River catchment sediments with concentrations ranging from 1 to 2.3 ng g^{-1} (Matongo et al., 2015). U1 recorded a similar concentration of 2 ng g^{-1} and U2 recorded a higher concentration of 3 ng g^{-1}. Steroid hormones could not be detected in all the uMngeni estuary sediment samples tested.

Table 6.3 Emerging pollutants determined in the Hartbeespoort Dam catchment and uMngeni River estuary water samples (ng L^{-1}), the uMngeni River estuary sediment (ng g^{-1} dry weight) and Hartbeespoort Dam fish muscle samples (ng g^{-1} fresh weight)

Sample	Nevirapine	Efavirenz	Lamivudine	Emtricitabine	Tenofovir disoproxil	Carbamazepine	Methocarbamol	Etilefrine HCL	Venlafaxine HCL	Bromacil	*p*-Chloroaniline	∑EP site
LOD X3	0.2	0.09	0.05	0.04	0.06	0.04	0.05	0.05	0.06	0.2	0.04	
LOQ X10	0.67	0.3	0.15	0.13	0.2	0.13	0.15	0.15	0.2	0.67	0.13	
Summer												
Croc. R.	35	20	N.D	N.D	N.D	37	7	N.D	1	2	<0.13	102
Dam wall 30m	25	10	N.D	N.D	N.D	19	6	N.D	N.D	4	N.D	64
Harbour	17	35	N.D	N.D	N.D	27	5	N.D	0.3	3	N.D	87
Magalies R.	6	8	N.D	N.D	N.D	4	1	N.D	<0.2	<0.67	N.D	19
Groundwater	8	3	N.D	N.D	N.D	6	N.D	N.D	N.D	N.D	N.D	17
Autumn												
Croc. R.	42	27	N.D	N.D	N.D	46	20	N.D	3	13	<0.13	151
Dam wall 30m	40	17	N.D	N.D	N.D	38	15	N.D	2	7	N.D	119
Harbour	39	17	N.D	N.D	N.D	41	12	N.D	1	7	N.D	117
Magalies R.	30	6	N.D	N.D	N.D	28	8	N.D	0.3	5	N.D	77
Groundwater	10	5	N.D	N.D	N.D	8	N.D	N.D	N.D	N.D	N.D	23
Winter												
Croc. R.	44	80	N.D	N.D	N.D	45	34	N.D	3	7	<0.13	213
Dam wall 30m	39	55	N.D	N.D	N.D	39	33	N.D	1	6	N.D	173
Harbour	43	54	N.D	N.D	N.D	44	28	N.D	2	7	N.D	177
Magalies R.	35	3	N.D	N.D	N.D	23	13	N.D	0.4	5	N.D	79
Groundwater	13	2	N.D	N.D	N.D	11	<0.15	N.D	N.D	N.D	N.D	26
Spring												
Croc. R.	71	303	N.D	N.D	N.D	94	96	N.D	10	3	<0.13	577
Dam Wall 5m	31	32	N.D	N.D	N.D	33	24	N.D	1	5	N.D	126
Dam Wall 30m	37	62	N.D	N.D	N.D	31	31	N.D	N.D	4	N.D	165
Harbour	35	82	N.D	N.D	N.D	35	20	N.D	1	5	N.D	178
Magalies R.	6	12	N.D	N.D	N.D	5	3	N.D	<0.2	1	N.D	27

Groundwater	13	3	N.D	N.D	N.D	13	<0.15	N.D	N.D	N.D	N.D	29
Jukskei River Winter												
N14	23	134	N.D	0.5	N.D	4	0.4	N.D	<0.2	2	<0.13	164
Kyalami	18	167	N.D	N.D	N.D	17	6	N.D	2	16	N.D	226
Midrand	45	354	N.D	N.D	N.D	75	86	N.D	26	7	<0.13	593
Buccleuch	57	168	N.D	2	N.D	16	48	N.D	3	9	<0.13	303
Marlboro	N.D	302	N.D	13	N.D	18	22	N.D	4	9	<0.13	368
uMngeni River water winter												
Composite	68	138	<0.15	8	N.D	94	3	N.D	1	10	0.76	323
uMngeni River sediment winter			**(ng g $^{-1}$ dw)**									
U1	81.0	N.D	0.4	1.0	0.2	2.0	0.6	4.0	0.3	8.0	5	103
U2	11.0	N.D	0.6	0.6	0.3	3.0	0.4	4.0	0.8	9.0	0.4	30
U3	N.D	3.0	<0.15	<0.13	<0.2	<0.13	<0.15	<0.15	<0.2	<0.67	0.2	3
U4	N.D	2.0	<0.15	<0.13	<0.2	<0.13	<0.15	<0.15	<0.2	<0.67	0.2	2
Hartbeespoort Dam fish muscle			**(ng g $^{-1}$ fw)**									
Catfish	N.D	N.D	N.D	N.D	N.D	N.D	N.D	<0.15	<0.2	1	N.D	1
Carp	N.D	N.D	<0.15	N.D	N.D	N.D	N.D	N.D	N.D	<0.67	N.D	0

N.D =Not Detected

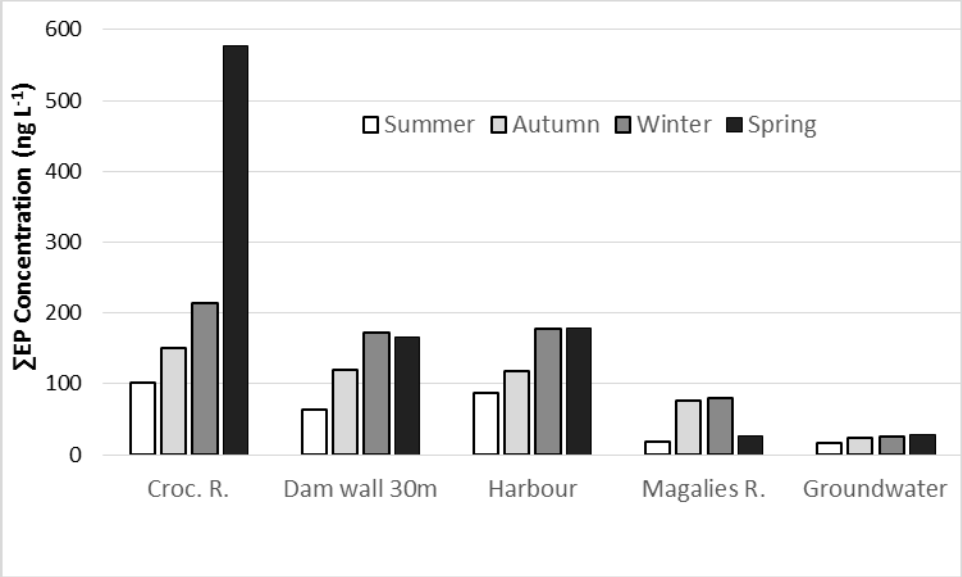


Figure 6.3 Seasonal ∑EP concentrations in the Hartbeespoort Dam

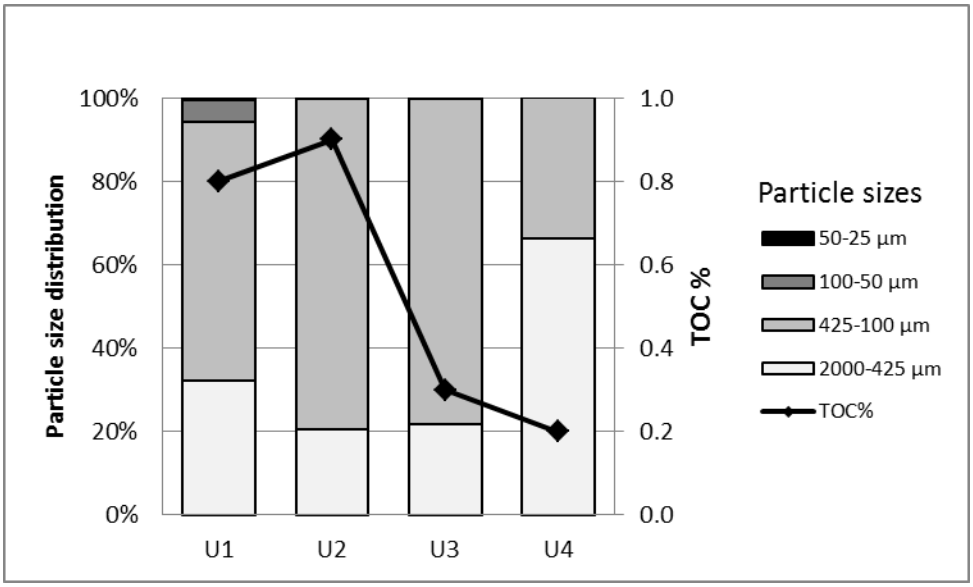


Figure 6.4 uMngeni River sediment TOC and particle size distribution

Limitations of the study

This study is limited to a single sampling regime, meaning that it can be affected by temporal fluxes in API concentrations within the river channels and dam. Though the continuous and steady contamination of the Jukskei River with raw sewage and wastewater treatment plant effluent is not expected to cause

major fluxes in API's, this can be addressed through use of a high sampling frequencies or the use of passive samplers to calculate time weighted averages. The analysis of sediments in the uMngeni River estuary may reflect historic pollution which was not evaluated. Useful EP degradation and stability studies throughout the sample collection and extraction stages were not carried out to determine the stability of each compound during sample handling. Development of methodology tailored for diverse emerging pollutants in water and sediments could assist to analyse a wider range of emerging pollutants such as acidic polar organic compounds. Development of enantioselective methods for profiling chiral APIs which can interact differently with biological organisms, exhibiting different pharmacokinetics is gaining interest in the scientific community and is recommended in future studies.

Conclusions

It is proposed that, along with the antibiotics of common usage, the emerging contaminant candidate list in South African urban waters should include nevirapine, efavirenz, carbamazepine, methocarbamol, venlafaxine (hydrochloride) and bromacil. The authors suggest for effluent dominated waters, a contaminant candidate list be published by regulatory authorities and either operational or investigative monitoring be conducted using LC-MS/MS. The presence of emerging contaminants in the Hartbeespoort Lake is a cause of major concern for the ecosystem and particularly for fish health and survival as some of the EPs have been proven to cause diminished predator evasive behavior. The Hartbeespoort Dam is a valuable source of fish for the surrounding community and the water is primarily used for irrigating crops, hence the presence of EPs may have an effect on the quality and grade of food. The Crocodile River exerts the most significant impact on the Hartbeespoort Dam pollution, particularly in the dry winter and spring seasons. Further investigation may be conducted for polar emerging pollutants not detected in water by grab sampling by using passive samplers such as polar organic chemical integrative sampler (POCIS) or Chemcatcher.

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and sampling point selection in the Hartbeespoort Dam and Bart Fokkens for assistance with the sampling boat in the uMngeni River estuary. This work was made possible by the South African National Research Foundation (NRF) travelling grant numbers KICI5091018149662 and 98818 that allowed the first author to spend time in The Netherlands as well as the South African Department of Water and Sanitation, Human Resource Development Directorate for provision of the bursary award.

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Chapter 6 – Supporting Information

Tables:

Table S6.1 Sampling sites and GPS coordinates

Site	Latitude (South)	Longitude (East)
Crocodile River	-25.76596	27.89394
Dam wall	-25.72808	27.84971
Harbour	-25.74929	2783328
Magalies River	-25.76267	27.77582
Groundwater	-25.72528	27.83922
N14	-25.94933	27.95878
Kyalami	-26.0056	28.07836
Midrand	-26.03139	28.11222
Buccleuch	-26.05700	28.10400
Marlboro	-26.08494	28.10881

Table S6.2 Sampling sites and GPS coordinates

Site	Latitude (South)	Longitude (East)
U1	-29.810971	31.032892
U2	-29.811350	31.037415
U3	-29.811612	31.038824
U4	-29.812316	31.039699

Table S6.3 Seasons in South Africa

Calendar dates	Season	Sampling dates
1 September to 30 November	Spring	14 November 2014
1 December to 28/29 February	Summer	13 February 2015
1 March to 31 May	Autumn	15 May 2015
1 June to 31 August	Winter	14 August 2015

Table S6.4 Method validation data

Compound	RT (min)	Precision (%RSD) (n=6)	Repeatability (%SD, n=6)	Water Recovery (%)	Sediment recovery (%)	Regression (r^2)	Fit
Efavirenz	11.821	6.7	3.6	86	92	0.999	Linear
Nevirapine	10.036	5.9	4.2	92	84	0.999	Linear
Tenofovir disoproxil (fumarate)	11.192	6.2	4.9	73	87	1	Linear
Emtricitabine (mikromol)	6.297	7.1	6.7	107	116	0.999	Linear
Lamivudine	5.729	5.4	5.3	94	104	0.998	Linear
Venlafaxine (hydrochloride)	9.593	4.9	6.2	79	94	1	Linear
Carbamazepine	10.910	5.6	4.8	112	123	1	Linear
Testosterone	12.674	7.3	6.5	86	93	0.998	Linear
Etilefrine	4.752	5.8	5.9	92	88	1	Linear
Methocarbamol	9.303	6.8	5.6	84	73	1	Linear
Bromacil	9.895	6.4	5.6	93	98		Linear
17-β-Estradiol (dansylated)	15.488	8.3	7.3	71	84	0.998	Linear
17-α estradiol (dansylated)	15.048	7.6	6.5	58	76	0.998	Linear
Benzestrol (bi ansylated)	14.840	4.4	3.1	79	69	1	Linear
p-Chloroaniline (dansylated)	12.756	5.2	4.3	87	86	1	Linear
D7 deethylatrazine (dansylated)	8.275	7.6	5.3	IS	IS	IS	IS
D4 17-β-Estradiol (dansylated)	15.488	6.4	4.8	IS	IS	IS	IS
D4 Estrone (dansylated)	18.110	5.3	5.6	IS	IS	IS	IS

IS= Internal standard

RT= Retention time
% RSD= percentage relative standard deviation
%SD = percentage standard deviation

Chapter 7

Discussion, concluding remarks and recommendations

General discussion

The main chemical pollutants of concern in South African urban rivers are the triazine herbicides atrazine and terbuthylazine, pharmaceutical residues, PCBs, PAHs, PCDDs and PCDFs. This study was targeted on analysis of these pollutants in South African urbanised ecosystems. It is well known that South Africa faces serious water quantity and quality challenges, both in inland urban provinces such as Gauteng and coastal provinces such as KwaZulu-Natal which were sampled. South Africa's economy is heavily dependent on mechanised and energy-intensive industries such as mining, construction, manufacturing and agriculture. This has caused a recent boom in the demand for energy for which the South African power utility, Eskom had not anticipated. This led to a crippling energy shortage crisis from 2008 to 2015 and necessitated the construction of two new mega power stations with a combined energy generation of 9600 MW (Kusile Power station situated in Mpumalanga Province, on the outskirts of the Gauteng Province in close proximity to the main study area and Medupi Power station in Limpopo Province, further north of and adjacent to Gauteng Province). The two power stations are both reported to become the largest coal-fired dry cooled power stations in the world. Construction of mega power stations comes with environmental pollution challenges as the new mega power stations, along with the majority of the power stations currently in operation run on coal as fuel to generate a total of 93% of the country's energy demands. The percentage of power produced from coal is set to significantly increase by 2019 when all the coal fired power plants under construction become fully operational. It is expected that there will be a rise in PCDD, PCDF and particularly PAH concentrations in the environment as a result. The relationship between energy demand and environmental pollution has historical roots as Eskom has been the primary importer of equipment containing polychlorinated biphenyls (PCBs), which are now ubiquitous pollutants in the South African urban environment. The quantities of PCBs and PCB containing equipment in South Africa are unknown as there is currently no comprehensive PCB inventory for South Africa.

Seasonal sampling in this study has shown that EPs are present in some South African urban rivers and lakes at detectable levels of pseudo persistent pollutants, which are characterised by consistent ubiquitous concentration levels that arise from facilities such as wastewater treatment works which operate continuously, releasing effluent. The organic pollutant pseudo persistent concentrations are due in part to the presence of many poor performing and sometimes dysfunctional WWTPs which sometimes discharge raw sewage into rivers. The highest concentrations of emerging pollutants in the

Jukskei River have been found at sites adjacent to informal settlements along the Jukskei River, with concentrations higher than in sites located in close proximity (downstream) of WWTP discharge points. The high EP concentrations at sites adjacent to informal settlements are due to poor and sometimes unavailable sanitation in river bank dwelling populations in Johannesburg. Overloaded sewer systems often leak into the Jukskei River, creating and maintaining unacceptable pseudo-persistent pollutant levels in water resources. Antiretroviral, antidepressant and anticonvulsant drugs have been discovered to be ubiquitous in urban waters contaminated by raw sewage and WWTP effluent. The ramifications of poor management and enforcement of environmental regulations on land and water use activities in upstream catchments often have serious implications in downstream catchments. Extremely high pollution levels at sites in the headwaters of the Jukskei River adversely affect the Hartbeespoort Dam located 80 km downstream as the Hartbeespoort Dam acts as a sink for pollutants contaminating the Jukskei River. Water resource managers often have to deal with pollution problems that originate in areas or districts beyond their jurisdiction or area of authority.

The Department of Water and Sanitation initiated an innovative system of assessing the level of performance of WWTPs called the Green Drop certification program for wastewater quality management regulation. The Green Drop programme measures and compares the performance WWTPs and awards them with Green Drop status if they comply with wastewater regulations or penalises them for non-compliance. The vast majority of WWTPs in South Africa do not have adequate instrumentation for assessing the quality of wastewater discharged or measuring the removal rates of organic pollutants before discharging the wastewater. It is understood that South Africa faces challenges in analysis of emerging pollutants and persistent organic pollutants in terms of lack of instrumentation and human capital with knowledge on analytical techniques and expertise. It is expected that sensitive LC-MS/MS instrumentation required for the analysis of non-volatile compounds such as triazine herbicide metabolites and emerging pollutants as well as GCxGC for the analysis of dioxin-like compounds will become common in the next decade. It is also expected that skilled a workforce with competence in complex analytical techniques for sample extraction, cleanup and analyte/congener identification will become more abundant.

Illegal dumping of medical waste is a common occurrence throughout South Africa where truckloads of medical waste and expired medicines are often found dumped illegally by unscrupulous business people in various locations where they are sometimes washed away by rain into rivers. Such activities put

pressure on urban water quality. Over 90% of medical waste in South Africa is incinerated, which creates another environmental problem. Small clinics and hospitals performing their own incineration in small furnaces with poor combustion efficiencies have been identified as a significant risk in the production and release of PCDD/Fs particularly as chlorine based disinfectant products and a lot of plastics containing PVC and are used, which creates conditions conducive for PCDD/F production. The situation is exacerbated as the vast majority of the incineration units are not fitted with air pollution control devices or scrubbers.

Methodology

Analysis of PAHs, PCBs and PCDD/Fs in water yielded low detection levels and therefore were not reported in this study. This is due to their high K_{ow} coefficients and lipophilic nature, which leads them to partition more strongly onto organic matter and sediments. Sediment analysis enabled trends and PAH, PCB and PCDD/F contamination patterns to be deduced. Source characterisation of PCB mixtures and source identification of petrogenic and pyrogenic PAHs was possible in Jukskei and Kilp River sediments. Extraction of PCDD/Fs, PCBs, PAHs and emerging pollutants from sediment samples was conducted by ASE according to the EPA method 3545A. For extraction of non-polar analytes, PCDD/Fs, PCBs and PAHs a mixture of hexane and acetone was used. This was followed by sulphuric acid silica and GPC cleanup for DR-luc analysis. For PCDD/F analysis, the ASE extracts were digested with concentrated H_2SO_4, followed by a GPC cleanup procedure to remove sulphur and other interferences. This was followed by fractionation on activated carbon cartridges prior to GCxGC-µECD analysis.

Fractionation of ASE sample extracts for PCB and PAH analysis over silica could separate them into their respective two fractions before GC-MS analysis. For polar emerging pollutants, a more polar solvent combination of DCM and acetone was used for ASE extraction. A dSPE method was utilised for further cleanup of emerging pollutant ASE extracts. A modified Walkley-Black method was utilised to selectively measure TOC, eliminating interferences from inorganic carbon. A modified QuEChERS method was used to extract chloro-s-triazines from frog liver and fish muscle, which included the addition of PestiCarb and diatomaceous earth which facilitates supported liquid extraction (SLE). For frog serum analysis, a saturated ammonium sulphate solution protein precipitation method was used before conducting a dSPE cleanup method using a PSA/C18 dSPE sorbents. Water samples for emerging pollutant analysis

were successfully extracted using solid phase extraction (SPE) with a styrene divinylbenzene sorbent phase.

Selectivity and sensitivity challenges are often encountered during analysis of PCDD/Fs when methods other than the GC-HRMS utilising a magnetic sector analyser are used. Selectivity of PCDD/F analysis was enhanced by (i) digesting the sample matrix with H_2SO_4 (ii) GPC elimination of matrix and (iii) fractionation of PCDD/Fs over activated carbon cartridges. The use of a sensitive micro-ECD detector coupled with a GCxGC allowed good selectivity and sensitivity to be achieved. Identification of *ortho* substituted indicator PCBs could be achieved by identifying their specific retention times by GC-MS analysis. Identification of emerging contaminants could be achieved by selecting suitable precursor and confirmation ions in positive ionisation mode, utilising LC-ESI-MS/MS. It is intended to utilise the developed methods routinely for operational monitoring in South Africa, given the limited resources available for purchase, maintenance and routine operation/analytical costs of complex GC-HRMS instrumentation.

Importance and relevance of findings

All sediment samples tested showed the presence of dioxin-like toxicity. This shows that there may be a risk of dioxin-like compound toxicity in the Gauteng province. The DR-Luc bioassay is important to rapidly screen samples and identify areas which are at risk of dioxin-like compound toxicity. Selective chemical analysis of specific PCDD/Fs is important to identify and quantify the PCDD/F congeners and this is important when monitoring emissions and enforcing legislation to control the release of dioxin-like compounds. The analysis of indicator PCBs is important for compliance with the Stockholm Convention as well as national legislation on management of PCB contaminated sites and control of PCB containing equipment. The discovery of the main contaminating PCB technical mixtures, Aroclor 1254 and 1260 will aid South Africa manage PCB remediation like countries in Europe, which have experienced the same Aroclor contamination.

Source characterisation of PAHs is important in determining PAH apportionment and to establish appropriate legislation for the protection of the environment. Determination of the distribution of PAH pollutants in the environment also enables resource managers to establish the main contributors to PAH

pollution and enables policymakers to advocate and legislate for practices and technologies that provide a cleaner environment such use of wind power generators and hydroelectric power stations instead of coal fired power stations. In large areas of South Africa where the use of coal is indispensible, the use of coal of a higher quality may be advocated as well as the use of higher combustion efficiencies in both small scale and industrial coal furnaces.

The discovery of pseudo-persistent triazine herbicide conditions in the Jukskei River as well as the Magalies River and in the Hartbeespoort Dam throughout the four seasons will enable policymakers to advocate for use of more biodegradable and less toxic alternative herbicides. Toxicological studies on frogs and tadpoles exposed to atrazine have practically shown the nature of atrazine toxicity through the use of different toxicological endpoints. This study provides decision makers with sufficient data to make informed decisions on matters related to atrazine use, required to prevent environmental catastrophes in the future. The high concentrations of pharmaceutical compound residues detected in the Jukskei River catchment shows that poor sanitation has significant adverse impacts on water resources and provides motivation for provision and enforcement of good sanitation and environmental practices. The need for use of technologies with high pharmaceutical residue removal efficiencies in WWTPs has also been highlighted.

Limitations of study and future work

The study of PCDD/Fs, PCBs and PAH was limited to water and sediment samples only. As expected, the water samples had very low PCDD/F, PCB and PAHconcentrations;hence those were not reported. The inclusion of high volume air sampling techniques is encouraged in future studies for better identification of sources and transport of organic pollutants and the inclusion of biological matrices such as fish muscle can help to determine bioaccumulation and evaluate environmental risk assessment. The majority of sampling sites in the Witwatersrand catchment, however, had no viable fish populations. The DR-Luc *in vitro* dioxin-like studies were performed on sulphuric acid digested sample extracts. The DR-Luc*in vitro* analytical technique can be improved by fractionating the sulphuric acid digested sample extracts into specific compound classes such as PCDDs, PCDFs, mixed chlorinated/brominated dibenzo-*p*-dioxins and dibenzofurans, polychloriated biphenyls (PBBs) and mixed chlorinated/brominated biphenyls, thereby eliminating interferences. This study did not analyse for the presence of PCDD/F and

PCB metabolites, which have dioxin-like activity. Therefore the GC congener specific chemical analysis may underestimate the dioxin-like toxicity. Sampling for PCDD/F, PCB and PAH analysis in the Witwatersrand catchment was limited to two months; hence seasonal and long term contamination trends could not be deduced. The sampling for analysis of triazine compounds and emerging pollutants in the Hartbeespoort Dam catchment was limited to only one year, hence long term trends could not be determined. The laboratory exposure studies of atrazine were limited to only one frog species, the African clawed frog. Exposure to different frog species and other aquatic organisms such as fish and crabs could provide a broader atrazine ecotoxicological risk assessment study.

Concluding remarks and recommendations for policymakers

As an advanced emerging economy, South Africa is susceptible to high pollution levels if industrial, domestic and agricultural emissions are not adequately monitored and regulated. As a water-scarce country, pollution may easily lead to a water quantity and quality crisis if pollution incidences are left unabated. Monitoring and evaluation of pollutant levels in the environment is an important tool in managing available resources sustainably, protecting the environment from pollution and prevention of environmental catastrophes. The South African economy has been struggling to meet domestic energy demands over the past decade, necessitating the construction of bigger coal-fired power stations which operate at the expense of the environment. Water scarcity in South Africa has necessitated the implementation of more environmentally friendly technologies such as dry cooling technology in the Medupi and Kusile coal fired mega power stations. Data on environmental pollution in South Africa is scanty when compared to China, India, Europe and the USA, making fair comparison impractical. However, air quality monitoring is expected to significantly increase in the next decade, particularly with the introduction of the new legislation enforcing compulsory monitoring of air quality by all entities holding atmospheric emission licenses as from 1 April 2015.

Ecotoxicological studies on emerging pollutants are required to ascertain the level of risk posed by emerging and legacy pollutants. The use of bioassays for estimating toxicity provides a useful tool as chemical analysis is unable to determine biological responses of exposure to chemicals. As toxicity is a biological phenomenon, bioassays can bridge the gap between chemical contamination and ecological status. Bioassays can conclusively give an estimation of the total toxicity of a sample as well as the

combined effect of a cocktail of pollutants as they can cover a broad range of toxicity mechanisms, which may not be possible with chemical analysis as it is not always possible or practical to characterise all potential pollutants in a contaminated sample.

In vivo studies are as important as *in vitro* studies because they can additionally provide important information on effects of pollutants on different organs as well as cell types. *In vivo* studies also provide useful information on complex inter-dependant homeostatic and endocrine mechanisms such as such as the suppression/inhibition of aromatase activity, which can result in increased levels of testosterone, coupled with simultaneous deficiency of estrogens. In situations where elimination of contaminating pollutants in the environment can prove to be impractical in the interim or long-term, bioassays can also be used to deduce EC50 and LD50 concentrations as well as predicted no-effect concentrations, which are vital parameters in the management of pollutants and remediation programs. New and existing wastewater treatment plants must be designed to incorporate technologies with high emerging pollutant removal efficiencies and better town planning strategies need to be implemented to ensure that sewer systems and other amenities are not overloaded beyond their carrying capacities. Given the unavailability of minimum residue level (MRL) standards or environmental quality standards (EQS) for contaminants in water, sediments or biota in South Africa, it is recommended to review and implement regulatory frameworks and monitoring programs, such as, for example, the European Union Water Framework Directive (Directive 2000/60/EC).

Main conclusions

- All sediment samples from the Witwatersrand catchment (Jukskei, Klip and Vaal Rivers) tested positive for dioxin-like toxicity with DR-Luc *in vitro* analysis.
- The Witwatersrand catchment is generally at a low risk of dioxin-like compound contamination.
- PCBs and PAHs are ubiquitous organic pollutants in the Witwatersrand catchment area.
- PCB congener pattern contamination in the Witwatersrand catchment area is characteristic of contamination by Arochlor 1245 and 1260 technical mixtures.
- PCB congeners 138, 153 and 180 are the most abundant indicator PCBs in the Witwatersrand catchment area.
- PAH contamination patterns in the Witwatersrand catchment area is characteristic of both pyrogenic and petrogenic PAH contamination.
- Fluoranthene and phenanthrene are the most abundant contamination PAHs in the Witwatersrand catchment sediments.
- Atrazine, simazine and terbuthylazine are found in the Hartbeespoort Dam surface water in all four seasons.
- Triazine herbicide contaminationin the Hartbeespoort Dam was in the order atrazine>simazine>propazine>ametryn>prometryn.
- Hartbeespoort Dam groundwater is contaminated by atrazine with concentrations >130 ng L^{-1} for all seasons except spring.
- Hartbeespoort Dam surface water triazine herbicide contamination was highest in summer and gradually decreased in successive seasons of autumn, winter and spring.
- African clawed frog (*Xenopuslaevis)* tadpole atrazine (in water) exposure LC50 is 343.7 µg L^{-1} (for a 90 day sub-chronic exposure).
- Atrazine exposure causes reduced testosterone serum levels in adult African clawed frogs.
- Atrazine exposure causes gonadal atrophy, disruption of germ cell lines, significantly reduced seminiferous tubule diameter, seminiferous tubule structure damage and damage to mitochondria in testosterone producing Leydig cells as well as Sertoli cells of adult African clawed frogs.
- The most abundant emerging pollutants found in theHartbeespoort Dam groundwater over four seasons are nevirapine, carbamazepine andefavirenz.

- For effluent dominated waters in South Africa, a contaminant candidate list consisting of antibiotics of common usage, nevirapine, efavirenz, carbamazepine, methocarbamol, venlafaxine (hydrochloride) and bromacil is recommended.

Permits and Ethical Clearance

PREMIER OF THE PROVINCE OF GAUTENG
NATURE CONSERVATION

CPF6 № 0115

PERMIT TO HUNT AND/OR COLLECT AND CONVEY A WILD ANIMALS FOR SCIENTIFIC PURPOSES

Issued in terms of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)

Name of permit holder: Chongetanji Cornelius Rimayo

DATE STAMP

Residential address: Dept of water Affairs
Roodeplaatdam
Moloto Road
Pretoria

2014 -10- 24

Name and address of institution or department on whose behalf shall be hunted and conveyed:

PARTICULARS OF WILD ANIMALS WHICH MAY BE HUNTED AND CONVEYED

Number	Species	Sex
360 (three hundred & sixty)	African Clawed frogs (Xenopus laevis)	

In terms of and subject of the provisions of the **Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)** and the regulations framed thereunder, the abovementioned person is hereby authorised, subject to the conditions appearing on this permit to hunt and convey the wild animal/s referred to above during the period of validity of this permit on behalf of the institution or department referred to above.

PARTICULARS IN CONNECTION WITH THE HUNT

Number	Species	Sex	Date hunted and conveyed

Valid until 23/10/2015

Ridongeni

SIGNED ON BEHALF OF THE PREMIER: MEC

SIGNATURE OF PERMIT HOLDER

(See conditions on reverse side)

PREMIER OF THE PROVINCE OF GAUTENG
NATURE CONSERVATION

CPF6 **№ 0120**

PERMIT TO HUNT AND/OR COLLECT AND CONVEY A WILD ANIMALS FOR SCIENTIFIC PURPOSES

Issued in terms of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)

Name of permit holder: Chergetayi Cornelius Rimayi

Residential address: Dept of Water Affairs (DWA)
Roodeplaat dam
Moloto Road
Pretoria

DATE STAMP
GAUTENG PROVINCIAL GOVERNMENT
DEPARTMENT OF AGRICULTURE AND RURAL DEVELOPMENT
23 DEC 2015
DIRECTORATE OF CONSERVATION
PERMITS OFFICE
PRETORIA 2000

Name and address of institution or department on whose behalf shall be hunted and conveyed:

PARTICULARS OF WILD ANIMALS WHICH MAY BE HUNTED AND CONVEYED

Number	Species	Sex
180 (One Hundred & Eighty)	African Clawed Frogs (Xenopus laevis)	
See attached permit Conditions		

In terms of and subject of the provisions of the **Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)** and the regulations framed thereunder, the abovementioned person is hereby authorised, subject to the conditions appearing on this permit to hunt and convey the wild animal/s referred to above during the period of validity of this permit on behalf of the institution or department referred to above.

PARTICULARS IN CONNECTION WITH THE HUNT

Number	Species	Sex	Date hunted and conveyed

Valid until 22/12/2016

SIGNED ON BEHALF OF THE PREMIER MEC

SIGNATURE OF PERMIT HOLDER

(See conditions on reverse side)

Conditions to Permit: CPF6-0120

- The applicant is required to provide a copy of his animal ethics clearance certificate from the University of the Witwatersrand.
- An electronic copy of any publications that arise from this work that are relevant to Gauteng Province must be provided to the GDARD Ornithologist within one month of publication.

For MEC: Pdlangani

GAUTENG PROVINCIAL GOVERNMENT
DEPARTMENT OF AGRICULTURE AND RURAL DEVELOPMENT
23 DEC 2015
DIRECTORATE OF CONSERVATION
PERMITS OFFICE
P.O. BOX 8769, JOHANNESBURG 2000

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2014/32/D

APPLICANT: Mr CC Rimayi

SCHOOL: Chemistry

LOCATION: Faculty of Sciences

PROJECT TITLE: *Frogs (Xenopus laevis) as an indicator for environmental organic pollution*

Number and Species

360 Frogs (180 adult and 180 tadpoles)

Approval was given for to the use of animals for the project described above at an AESC meeting held on 27 May 2014. This approval remains valid until 26 May 2016.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None.

Signed: _____ (Chairperson, AESC) Date: 3/11/2014

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 3rd Nov 2014

cc: Supervisor: Prof L Chimuka
Director: CAS

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Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Chengetayi Cornelius Rimayi.....

b. Department: Chemistry.....

c. Experiment to be modified / extended **AESC NO:2014/32/D**

Original AESC number	2014	32	D
Other M&E's : n/a			

d. Project Title: Frogs (Xenopus laevis) as an indicator for environmental organic pollution

e. Number and species of animals **originally approved**:	360	
f. Number of additional animals **previously allocated on M&Es**:	n/a	
g. Total number of animals **allocated** to the experiment to date:	360	
h. Number of animals **used** to date:	360	

i. Specific modification / extension requested:
   a) To include the use of the scheduled hormone, Gonadotropin for inducing spawning in female frogs for production of the required tadpoles. N.B an authorised registered worker will purchase and use the gonadotropin on Mr Rimayi's behalf.
   b) Inclusion of co-workers Jaclyn Asouzu and Lynette Rufaro Sena who will also care for the frogs and also conduct histological analyses on the frog organs I will not be conducting histological analysis on and which I would otherwise be discarding.

j. Motivation for modification / extension:

   a) It would be less expensive to produce the tadpoles using the Gonadotropin hormone at the Wits Central Animal Service than to buy them from an external organisation due to limited funds

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Curriculum vitae

Chengetayi Cornelius Rimayi was born in the 24th of June 1985 in Harare, Zimbabwe. After completing a BSc Honours degree in Food Science and Nutrition at Midlands State University was employed as an Analytical Chemist at the Government Analyst Laboratory in Zimbabwe. After resigning to concentrate on research, he enrolled with Vaal University of Technology- Vanderbijlpark for a part-time master's degree in Chemistry and joined the Department of Water Affairs- South Africa in 2009. He later on graduated with a Masters degree in Biotechnology in 2012. In 2013 he commenced part-time joint PhD studies with the University of the Witwatersrand, Johannesburg and Vrije Universiteit, Amsterdam.

List of publications

C. Rimayi, D. Odusanya, J. M. Weiss, J. de Boer, L. Chimuka, F. Mbajiorgu. 2018. Effects of environmentally relevant sub-chronic atrazine concentrations on African clawed frog (Xenopus laevis) survival, growth and male gonad development. ***Aquatic Toxicology* 199, 1–11**.

DOI 10.1016/j.aquatox.2018.03.028.

C. Rimayi, D. Odusanya, J. Weiss, J. de Boer and L. Chimuka. 2018. Contaminants of emerging concern in the Hartbeespoort Dam catchment and the Umngeni River estuary 2016 pollution incident, South Africa. ***Science of The Total Environment* 627, 1008-1017.** DOI: 10.1016/j.scitotenv.2018.01.263.

C. Rimayi, D. Odusanya, J. Weiss, J. de Boer and L. Chimuka. 2018. Seasonal variation of chloro-s-triazines in the Hartbeespoort Dam catchment, South Africa. ***Science of The Total Environment* 613-614, 472-482**. DOI: 10.1016/j.scitotenv.2017.09.119.

C. Rimayi, L. Chimuka, D. Odusanya, J. De Boer and J. Weiss. 2017. Source characterisation and distribution of selected PCBs, PAHs and alkyl PAHs in the Klip and Jukskei River Sediments, South Africa. ***Environmental Monitoring and Assessment* 189, 1-16.** DOI: 10.1007/s10661-017-6043-y.

C. Rimayi, L. Chimuka, D. Odusanya, J. De Boer and J. Weiss. 2016. Distribution of 2,3,7,8-substituted polychlorinated Dibenzo-p-dioxins and polychlorinated dibenzofurans in the Jukskei and Klip/Vaal catchment areas in South Africa. ***Chemosphere* 145, 314-321**. DOI: 10.1016/j.chemosphere.2015.11.088.

C. Rimayi, F. Mtunzi, D. Odusanya and S. Tsoka. 2015. Alternative calibration techniques for counteracting the matrix effects in GC-MS-SPE pesticide residue analysis - A statistical approach. ***Chemosphere* 118, 35-43.** DOI: 10.1016/j.chemosphere.2014.05.075.

C. Rimayi, F. Mtunzi, C. Van Wyk and D. Odusanya. 2012. Influence of the matrix on selected organochlorine pesticide residue analysis by Solid Phase Extraction coupled with GC-MS. ***International Journal of Computational Methods and Experimental Measurements* 2, 71-91.**

C. Rimayi, F. Mtunzi, C. Van Wyk and D. Odusanya. 2012. Method development for the influence of matrix on selected organochlorine pesticides residue analysis in surface water by GC-MS. ***WIT Transaction on Ecology and Environment***. ISSN: 1746-448X, Digital ISSN 1743-3541.

List of oral presentations

Department of Science and technology and Department of Environmental Affairs seminar, Pretoria, South Africa, June 2016

C. Rimayi, D. Odusanya, J. Weiss, J. De Boer, L. Chimuka. POPs research at the University of the Witwatersrand

Water Institute of Southern Africa (WISA) 2016 Conference, Durban, South Africa, May 2016

C. Rimayi, D. Odusanya, J. Weiss, J. De Boer, L. Chimuka. Evaluation of the ecological significance of PCB contamination in the hydrologic connectivity of the Hartbeespoort dam and the upstream Jukskei River

Emerging Frontiers For Sustainable Water – A Trilateral Partnership Africa-India-UK 2015, Johannesburg, South Africa, August 2015

C. Rimayi, D. Odusanya, J. Weiss, J. De Boer, L. Chimuka. Applications of passive samplers to screen organic pollutants in South African water bodies

South African Chemical Institute (SACI) Convention, Durban, South Africa, November 2015

C. Rimayi, D. Odusanya, J. Weiss, J. De Boer, L. Chimuka. Spatial distribution and trends of selected PCBs, PAHs and alkyl PAHs in the Klip and Jukskei River sediments, South Africa

ANALITIKA 2014 Conference, Parys, South Africa, September 2014

C. Rimayi, D. Odusanya, J. Weiss, J. De Boer, L. Chimuka. Determination of 2,3,7,8-chlorine substituted dioxins and furans using DR-Luc bioassays and two dimensional gas chromatography with micro electron-capture detection in sediments from the Klip/Vaal and Jukskei River catchment areas in Gauteng, South Africa

Water Institute of Southern Africa (WISA) 2014 Conference, Nelspruit, South Africa, May 2014

C. Rimayi, D. Odusanya, J. Weiss, J. De Boer, L. Chimuka. Environmental sediment-water partitioning of organic pollutants in the Kilp/Vaal River channel, Gauteng, South Africa

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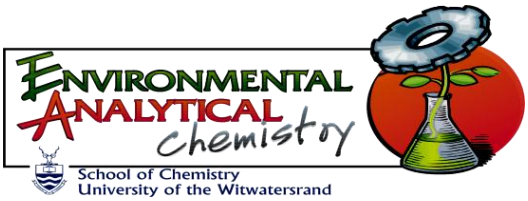
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