

Testing for endocrine disruptors in South African waters: A comparative study employing *in vitro* and *in vivo* screening approaches

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes four original papers published in peer-reviewed journals or books and two unpublished publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Date: December 2016

Abstract

Numerous natural and synthetic chemicals are known to interfere with the endocrine systems of wildlife and humans, i.e., endocrine disruptive chemicals (EDCs). Surface water bodies represent a major sink of EDCs and aquatic vertebrates including fish and amphibians are, therefore, at risk. Although research in the field of endocrine disruption has proliferated globally in the past two decades, only limited work describing the status of surface water has been performed in the South African context.

The aims of this dissertation are: (1) to provide a detailed overview of the present literature on endocrine disruptive potential, i.e., EDC associated biological activity in South African surface waters; (2) address certain literature gaps by screening surface water, collected from different South African water bodies, using *in vitro* reporter gene assays (RGAs) and gene expression based biomarkers in Mozambique tilapia, *Oreochromis mossambicus*, exposed *in vivo*; (3) to compare the results of the *in vitro* hormone receptor RGAs and the *in vivo* gene expression based biomarkers to assess whether the risks predicted correspond; (4) evaluate the potential of *O. mossambicus* as environmental sentinel and source of biomarkers for potential disruption of the reproductive, thyroid and interrenal endocrine systems.

The majority of research on endocrine disruption in South African surface waters has been focused on reproductive targets, whereas potential disruption of the thyroid, adrenal, pancreatic and other metabolism linked endocrine pathways are less well described. An inter-seasonal assessment of the Upper Olifants River (Mpumalanga) indicated alarmingly high concentrations of steroid estrogens and potent estrogenic activity *in vitro* in water collected from the close proximity of a waste water treatment plant (WWTP), indicating local fish may be adversely affected. Future studies evaluating the reproductive systems of wild captured fish inhabiting the Upper Olifants River will be of value. No association could be shown between altered endocrine signalling and the incidence of obesity and pancreatitis in the *O. mossambicus* population inhabiting Loskop Dam (Mpumalanga). Evidence of disrupted thyroid signalling was, however, observed in Loskop Dam fish relative to an alternative population. Gene expression biomarkers representing the reproductive, thyroid and interrenal systems in juvenile *O. mossambicus* suggest limited effects associated with exposure to surface water contaminated with neutralized acid mine drainage, containing high concentrations of Al, Mn, Ni, Co and Cu. In addition, evidence is given indicating the potential risk of crude oil contamination in fresh water to the reproductive and thyroid systems as well as lipid metabolism in wildlife. No significant changes in the expression of a

selection of endocrine linked genes was, however, observed in *Xenopus laevis* tadpoles exposed for a short period to surface water collected from a freshwater pan into which crude oil contaminated water is periodically discharged. Further investigation, and in particular longer term exposures, as well as the evaluation of aquatic fauna collected from (environmental) fresh water bodies contaminated with crude oil are required. Surface water collected from river mouths and harbours in the eThekweni Metropolitan and City of Cape Town was shown to exhibit estrogenic and anti-androgenic activity *in vitro*, suggesting the reproduction of fish populations may be impaired. Future studies evaluating the endocrine systems of wild-captured fish from the aforementioned coastal systems are needed.

The risks associated with exposure to surface water predicted by *in vitro* RGAs did not in all cases correspond to the potential biological activity indicated by *in vivo* gene expression based biomarkers. This study, therefore, provides evidence supporting the use of a combination of *in vitro* and *in vivo* techniques to evaluate surface water for endocrine disruptive activity/risk. Juvenile *O. mossambicus* exposed for a short period was not sensitive to estrogenic substances, but biomarkers relating to thyroid disruption are more promising.

Opsomming

Verskeie natuurlike en sintetiese chemikalieë of metale besit die vermoë om die endokriene sisteme van diere en mense te versteur, i.e., endokriene versteurende verbindings EVV's). EVVs is geneig om te versamel in oppervlakwaterliggame, en hou dus 'n risiko in vir akwatiese werwelidre insluitende visse en amfibieërs. Hoewel die hoeveelheid navorsing gefokus op endokriene versteuring wêreldwyd merkwaardig gegroei het in die afgelope twee dekades, is min bekend oor die toestand van oppervlakwater in Suid-Afrika in terme van potensiële endokriene versteuring.

Die doelstellings van hierdie verhandeling is: (1) om 'n gedetailleerde oorsig van die huidige literatuur oor endokriene versteurende potensiaal, i.e., EVV-geassosieerde biologiese aktiwiteit in Suid-Afrikaanse oppervlakwaterliggame te verskaf; (2) om sekere gapings in die literatuur te vul deur oppervlakwater, wat versamel is uit verskillende Suid-Afrikaanse waterliggame, te toets vir endokriene versteurings potensiaal met die gebruik van *in vitro* verslaggewergeentoetse (VGT's) en geenuitdrukking gebaseerde biomerkers in Mosambiekse kurpers, *Oreochromis mossambicus*, *in vivo* blootgestel; (3) om die resultate van die *in vitro* hormoonreseptor VGT's en die *in vivo* geenuitdrukking gebaseerde biomerkers te vergelyk en sodoende te bepaal of die risiko's wat voorspel word ooreenstem; (4) om die potensiaal van *O. mossambicus* as bron van biomerkers vir moontlike ontwrigting van die voortplanting, tiroïed en adrenale endokriene stelsels te evalueer.

'n Literatuuroorsig het aangetoon dat die meerderheid van navorsing rakende endokriene versteuring in Suid-Afrikaanse oppervlakwaterliggame tot hede gefokus was op die voortplantingsstelsel, terwyl potensiële ontwrigting van die tiroïed, adrenale, pankreatiese en ander metaboliese gekoppelde endokrienebane minder goed beskryf is. 'n Inter-seisoenale studie op die Bo-Olifantsrivier (Mpumalanga) het onrusbarende hoë konsentrasies van steroïde estrogene en kragtige estrogeniese aktiwiteit *in vitro* aangetoon in water versamel in die nabyheid van 'n rioolsuiweringaanleg, wat daarop dui dat die voortplanting van plaaslike vispopulasies nadelig beïnvloed kan wees. Toekomstige studies waarin die hormoonsisteme van visse wat die Bo-Olifantsrivier bewoon bestudeer word sal van waarde wees. Geen assosiasie kon getoon word tussen veranderde endokrienebane en die voorkoms van vetsug en pankreatitis in die Loskopdam (Mpumalanga) *O. mossambicus* bevolking nie. Bewyse van ontwrigte tiroïedeseine is egter waargeneem in Loskopdam kurpers relatief tot 'n ander kurper bevolking. Geenuitdrukking biomerkers wat die voortplanting-, tiroïed- en adrenale sisteme verteenwoordig in jong *O. mossambicus*, het aangetoon dat blootstelling aan oppervlakwater besmet met geneutraliseerde

suurmywater, met hoë konsentrasies van Al, Mn, Ni, Co en Cu, beperkte effekte op die hormoonsisteam het. Daarbenewens toon hierdie verhandeling dat ru-olie besoedeling in varswater die voortplantings- en tiroïed stelsels, asook lipiedmetabolisme in akwatiese werweldiere potensieel kan versteur. Geen beduidende veranderinge in die uitdrukking van 'n seleksie van endokriene-gekoppelde gene is egter waargeneem in platanna *Xenopus laevis* paddavisse blootgestel vir 'n kort tydperk aan water versamel vanuit 'n varswater pan waarin ru-olie besoedelde water van tyd tot tyd gestort word nie. Verdere ondersoek, en in die besonder langtermyn blootstellings, asook die evaluering van werweldiere versamel uit (omgewings) varswaterliggame besmet met ru-olie sal van waarde wees. Oppervlakwater versamel van riviermondings en hawens in die eThekweni Metropool en Stad-Kaapstad het estrogeniese en anti-androgeniese aktiwiteit *in vitro* gehad, wat daarop dui dat die voortplanting van visbevolkings versteur kan wees. Toekomstige studies waarin die endokriene stelsels van visse versamel uit die voorgenoemde kusstelsels ondersoek word is nodig.

Die risiko's wat verband hou met blootstelling aan oppervlakwater voorspel deur *in vitro* VGT's het nie in alle gevalle ooreengestem met die potensiele biologiese aktiwiteit aangedui deur *in vivo* geenuitdrukking gebaseer biomerkers nie. Hierdie studie bied dus bewyse wat aantoon dat die gebruik van 'n kombinasie van *in vitro* en *in vivo* tegnieke nodig is om oppervlakwater te evalueer vir endokriene versteuring of die risiko daarvan. Jong *O. mossambicus*, blootgestel vir 'n kort tydperk, was nie sensitief vir estrogeniese stowwe nie, maar biomerkers wat verband hou met skildklier ontwrigting in die spesie blyk meer belowend vir toetsingsdoeleindes.

Dedication

To my parents, Nic and Hester Truter

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I thank my supervisor, Hannes van Wyk for his example of integrity in research, gracious attitude towards me, for allowing me freedom to be creative, and for helping me grow as a researcher. I thank my co-supervisor Paul Oberholster and his wife Anna-Maria for their willingness to help, support and for the priceless opportunities and exposure presented to me.

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Finally, but foremost, I thank my Heavenly Father for helping me complete this task. The only condition of grace is a yielded heart. God, thank you for your persistent involvement in my life. Destiny and purpose is revealed in His loving embrace.

“The Lord is gracious and compassionate, slow to anger and abounding in loving kindness and tender mercies.” Psalm 103:8

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Table 4.2: Generalized Linear Model Analysis of Covariance (GLM ANCOVA) of the expression of (a) *thra*, *thrb* and *dio2*, (b) *ppar* and *tshb*, (c) *mr*, *gr1* and *gr2* in the (total) brain tissue of adult *Oreochromis mossambicus* captured from Loskop Dam ($N = 10$) and reference fish maintained in small dams supplied by borehole water ($N = 8$) and irrigation canal water ($N = 8$), respectively. Fulton's condition factor was applied as covariate. Asterisks indicate significant effects.

Table 4.3: Generalized Linear Model Analysis of Covariance (GLM ANCOVA) of the expression of (a) *thra*, *thrb*, *dio2* and *tshb*, (b) *mr*, *gr1*, *gr2* and *pparg* in the (total) brain tissue of *Oreochromis mossambicus* with macroscopically visible pansteatitis ($N = 7$) or without visible pansteatitis ($N = 3$). Fulton's condition factor was applied as covariate. Asterisks indicate significant effects.

Table 4.4: The concentrations ($\mu\text{g.L}^{-1}$) of selected metals and other elements in water collected from three locations within Loskop Dam, as well as dams inhabited by the alternative population evaluated in the current study (i.e., borehole- and irrigation canal supplied dams respectively).

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Table 6.2: Concentrations of polycyclic aromatic hydrocarbons (PAHs) measured in bunker and refinery crude oil water accommodated fractions, as well as the limits of detection (LOD) and recoveries of the individual compounds tested for.

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Table S6.2: Primer sequences for *Oreochromis mossambicus* in the 5' to 3' direction applied for RT-qPCR.

Table S6.3: Expression of a selection of genes in 29 – 31 dpf *Oreochromis mossambicus* (whole body homogenates) and NF48 *Xenopus laevis* exposed for 24 h to the water accommodated fractions of crude oil obtained from and underground bunker as well as a refinery. The concentrations given portray the mass represented relative to the original crude oil mass used to prepare the WAF and 25 g.L^{-1} , therefore, equates 100% WAF. No significant differences were observed among the treatments.

Table S7.1: Sampling locations with descriptions and GPS coordinates. ETH = eThekweni, NM = Nelson Mandela, CPT = Cape Town metropolitan municipalities. The Palmiet Estuary falls outside the City of Cape Town municipality, yet within the metropole's surroundings.

Table S7.2: Concentrations of monoaromatic hydrocarbons and total petroleum hydrocarbons (TPH) detected in water samples collected in Durban Bay, the Amanzimnyama, Umhlatuzana, Umbilo and Umlazi Rivers, and the Isipingo Estuary in the eThekweni metropolitan municipality.

Table S7.3: Concentrations of polycyclic aromatic hydrocarbons (PAH) detected in water samples collected in Durban Bay, the Amanzimnyama, Umhlatuzana, Umbilo and Umlazi rivers, and the Isipingo Estuary in the eThekweni metropolitan municipality.

List of abbreviations

The Xenbase, Zebrafish, and Human Gene Nomenclature Committee (HGNC) gene and protein nomenclature guidelines (amphibians and fish: gene *thrb*, protein Thrb; humans: gene *THRB*, protein THRB) have been applied throughout this dissertation (www.xenbase.org; www.zenfin.org; www.genenames.org).

ABS	Absorbance
ACTB	Beta actin
AMD	Acid mine drainage
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AR	Androgen receptor
BTEX	Isomers benzene, ethylbenzene, toluene, o-xylene, m'p'-xylene
C18	Octadecyl
CAF	Stellenbosch University Central Analytical Facility
cDNA	Complementary DNA
CF	Condition factor
CYP19	Cytochrome P450 aromatase
DDE	1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene
DDT	Dichlorodiphenyltrichloroethane
DEQ	Dihydrotestosterone equivalent concentration
DHT	Dihydrotestosterone
DIO2	Type 2 deiodinase
DMSO	Dimethyl sulfoxide
DNA	Dioxyribonucleic acid
DPF	Days post fertilization
DWAF	Department of Water Affairs and Forestry
DWAF	Department of Water Affairs and Forestry
DWAS	South African Department of Water and Sanitation
E ₂	Estradiol (International nonproprietary name)
EC	Electrical conductivity
EC ₅₀	Half Maximal Effective Concentration
ED	Endocrine Disruption
EDA	Endocrine disruptive action
EDC	Endocrine Disrupting Compound
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EE ₂	Ethinyl-estradiol (International nonproprietary name)
EEQ	Estradiol equivalent concentration
ELISA	Enzyme-linked immunosorbent assay
EPA	US Environmental Protection Agency
ESR	Estrogen receptor
FEQ	Flutamide equivalent concentration
FLU	Flutamide

GLM	Generalized linear model
GPS	Global positioning system
GR	Glucocorticoid receptor
HCG	Human chorionic gonadotropin
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-thyroid
HRE	Hormone response elements
IC ₅₀	Half maximal inhibitory concentration
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
ICP-MS	Inductively coupled plasma mass spectrometry
IEQE ₂	Estradiol induction equivalent
IEQTAM	Tamoxifen induction equivalent
LOD	Limit of detection
LSD	Least significant difference
Milli-Q	Ultrapure water produced using a Millipore system
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
ND	Not detected
NF	Nieuwkoop and Faber developmental stage
NTMP	National Toxicity Monitoring Program
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic aromatic hydrocarbons
PBDE	Polybrominated diphenyl ether
PCA	Principal component analysis
PCB	Polychlorinated biphenyl
PEDC	Programme on Endocrine Disrupting Compounds
PNEC	Predicted no-effect concentration
PPARG	Peroxisome-proliferator activated receptor gamma
PTFE	Polytetrafluoroethylene
RDA	Redundancy analysis
RGA	Reporter gene assay
RO	Reverse osmosis
RPM	Revolutions per minute
RT-qPCR	Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction
SANS	South African National Standards
SPE	Solid phase extraction
SRD5A	Steroid 5 alpha reductase
StAR	Steroidogenic acute regulatory protein
T	Testosterone
T ₃	Triiodothyronine
T ₄	Thyroxine
Ta	Annealing temperature
TBT	Tributyltin
TDS	Total dissolved solids
TEQ	Tamoxifen equivalent concentration

THRA	Thyroid hormone receptor alpha
THRB	Thyroid hormone receptor beta
TPH	Total petroleum hydrocarbons
TSHB'	Thyroid stimulating hormone beta
TTR	Thyroid hormone carrying transthyretin
UK	United Kingdom
US	United States of America
VTG	Vitellogenin
WAF	Water accommodated fraction
WHO	World Health Organisation
WRC	Water Research Commission
WSF	Water soluble fraction
WWTP	Wastewater treatment plant
XEMA	Xenopus metamorphosis assay
YAAS	Yeast anti-androgen screen
YAES	Yeast anti-estrogen screen
YAS	Yeast androgen screen
YES	Yeast estrogen screen
ZA	South Africa

Chapter 1: General introduction

The value of uncontaminated water as resource is increasing rapidly due to factors including population growth, global climate change and industrial expansion, especially in water scarce countries such as South Africa. The mining, manufacturing and agricultural sectors, fossil fuels, i.e., crude oil, natural gas and coal, and the products of fossil fuel combustion, as well as personal care products, pharmaceuticals and other anthropogenic chemicals such as plasticizers, contribute to poor water quality. The pollution of the aquatic environment has implications to man and wildlife because of the associated health risks. A particular group of substances of concern due to biological activity at concentrations below 1 ng.L^{-1} in water, and the potential to modulate the endocrine system and, therefore, various essential physiological functions, are endocrine disrupting compounds (EDCs). These substances have been defined by the US Environmental Protection Agency as:

“Exogenous agents that interfere with the —synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance` of homeostasis, reproduction development and/or behaviour” (Kavlock *et al.*, 1996).

The targets of EDCs are diverse and evidence exist indicating interaction with the reproductive, thyroid, adrenal, pancreatic and other metabolism linked endocrine pathways (Diamanti-Kandarakis *et al.*, 2009; Casals-Casas and Desvergne, 2011). Many studies performed in the past two decades have featured EDCs and the potential effects on vertebrates. There has, however, been, and still is a bias towards reproductive targets in the evaluation of EDCs in research performed globally, as is the case in South Africa (Reviewed in Chapter 2 of this dissertation). The hypothalamus-pituitary-gonadal (HPG) endocrine axis is indeed a vital target of EDCs to study, because reduced fecundity may have effects at population level. However, the assessment of other types of endocrine disruption such as the modulation of thyroid and corticosteroid signalling is important, because an organism may be adversely affected. For example, the vertebrate thyroid system plays an essential role in the regulation of growth and development as well as metabolism and energy homeostasis. Disruption of the hypothalamus-pituitary-thyroid axis during ontogeny may affect critical developmental processes such as neurogenesis (Preau *et al.*, 2015) and the identification of the thyroid disruptive potential of compounds is, therefore, critical to establish.

The aquatic environment represents a major sink of EDCs originating from point and non-point sources. The reality of the risk of EDC exposure to fish was first discovered in the late 1970s when roach, *Rutilus rutilus*, captured from English rivers, at localities impacted by waste water treatment plants (WWTPs), were found to be intersex (Reviewed in Sumpter and Johnson, 2008). These findings lead to a series of investigations locally in the UK and abroad, evaluating potential EDC induced reproductive disorders in wildlife, in field and laboratory situations. In fact, a significant increase in research efforts describing potential targets of EDCs in humans and wildlife occurred during the 1990s, which is ongoing, and the aquatic environment has been the focal point in many investigations. The published reports on endocrine disruptive potential in South African surface waters are, however, limited and studies describing potential disruption of endocrine pathways other than reproduction have been virtually non-existent, prior to the publication of some of the research which forms part of this dissertation (Reviewed in Chapter 2). There is an urgent need for the evaluation of endocrine disruptive potential in South African surface water bodies in order to provide baseline data on EDC loads and assess risks to wildlife.

Studies identifying the links between the land-use of catchments and endocrine disruptive potential in receiving waters will be valuable to the South African and global scientific community. Such data can aid regulatory bodies in identifying culprit industries or organisations responsible for the contamination of surface water with EDCs and, therefore, aid preventative action and conservation.

The first aim of this dissertation was to assess the risk of endocrine disruption in different South African surface water bodies that are subject to a diversity of contamination sources and potential contributors to EDC loads. In addition, associations between different land use areas and specific types of endocrine disruptive potential were identified based on the findings in the different water systems investigated.

Some of the characteristics of EDCs that confound risk assessment and predictive modelling are the non-monotonic dose responses and biological activity at low dosages (Vandenberg *et al.*, 2012). These factors are further complicated when evaluating environmental matrices where hundreds and even thousands of chemicals and elements can occur in mixture. Biological assays and biomarkers in animals exposed *in vivo* or *in situ* present a functional approach for the testing EDC risk in complex samples such as river water. Gene expression is one of the central regulatory mechanisms in physiology, and fundamental to endocrine signalling. The expression of certain candidate genes that constitute key steps in endocrine signalling pathways can be used as biomarkers. Such gene expression based biomarkers are functional as screening tool to identify potential endocrine disruptive effects associated

with EDC exposure, and indicate the need for further investigation (Hutchinson *et al.*, 2006). Some of the advantages of the use of gene expression as biomarkers for endocrine disruptive potential are short exposure periods and the potential evaluation of multiple biological pathways using one technique (Helbing *et al.*, 2003; Ankley *et al.*, 2012).

A further aim of the present dissertation was to compare the results of recombinant yeast *in vitro* reporter gene assays with gene expression based biomarkers in juvenile fish and tadpoles exposed *in vivo*, hence determining whether the predicted risks correspond among the *in vitro* and *in vivo* assays.

Mozambique tilapia, *Oreochromis mossambicus*, is a euryhaline fish species endemic to Southern Africa, with a wide distribution range in rivers, lakes and certain estuaries throughout the sub-continent (Trewavas, 1983). The species is commonly used in aquaculture in South Africa and abroad. Considering the wide distribution range (including fresh and saline waters) and commercial availability due to the aquaculture trade, *O. mossambicus* constitutes a promising species for laboratory and field studies. Previous studies have initiated the identification of gene expression based biomarkers for disruption of the female hormone system using juvenile *O. mossambicus* (Esterhuysen *et al.*, 2008; Esterhuysen *et al.*, 2009; Esterhuysen *et al.*, 2010).

An additional aim of this dissertation was to further explore the potential of *O. mossambicus* as environmental sentinel and source of biomarkers for endocrine disruptive potential, including molecular targets representing the thyroid and interrenal systems.

1.1 Objectives

The objective of this dissertation was firstly to provide an overview of the literature on endocrine disruptive potential, i.e., EDC associated biological activity in South African surface waters, as well as endocrine disruption in wild populations of aquatic organisms (Chapter 2). Surface water collected from a selection of key South African water bodies were subsequently screened for endocrine disruptive activity using a combination of *in vitro* and *in vivo* assays, and in particular, recombinant yeast reporter gene assays and gene expression biomarkers in fish or tadpoles exposed *in vivo*. The systems investigated include: (1) a major river subject to a diversity of point and nonpoint source pollution vectors including mining, agriculture, ill maintained WWTPs and industrial waste water (Chapter 3); (2) a dam in which health disorders associated with metabolism has been observed in wildlife (Chapter 4); (3) a river into which large volumes of neutralized acid mine drainage is discharged (Chapter 5);

(4) surface water contaminated with crude oil from an underground bunker (Chapter 6); (5) selected harbours, river mouths and estuaries on the South African coastline (Chapter 7).

The potential substances responsible for the endocrine disruptive potential observed in the aforementioned systems were identified based on literature reports, taking the land use types in the catchments of the sampling locations into account. The potential sources of EDC contamination (and responsible culprit sectors) could, therefore, be identified, but not confirmed seeing that further investigation is required for the purpose. In addition, the responses observed among the *in vitro* and *in vivo* bioassays were compared within each system evaluated, and discussed in light of the loads of metals or selected organic contaminants present in the samples that were screened, and the present literature.

Eleven candidate genes were selected as biomarkers in juvenile *O. mossambicus* representing the reproductive, interrenal and thyroid systems. The changes in gene expression in response to chemical or surface water exposure were compared to previous literature reports on *O. mossambicus* and other fish in order to assess the sensitivity of the species as sentinel for EDC action. In addition, in one of the case studies of this dissertation (Chapter 6), the expression profiles observed in *O. mossambicus* were compared to that observed in African clawed frog, *Xenopus laevis*, tadpoles exposed to the exact same sample, in order to evaluate relative sensitivity and variation among an amphibian and teleost model organism.

I hope to, by the data communicated through this dissertation, stimulate increased research efforts concerning the endocrine disruption phenomenon in the South African aquatic environment with the end goal of protecting humans and wildlife.

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Chapter 2: A review of the use of biological assays for the screening of endocrine disruption in the South African context

Abstract

Various natural and anthropogenic compounds have been shown to modulate the endocrine systems of humans and wildlife. Surface water is one of the major sinks of these endocrine disrupting compounds (EDCs), and aquatic organisms are, therefore, at risk. In this review, an overview is firstly given of EDCs including the major chemical classes, biological targets, modes of action, and the biological assays that have been utilized to date as screens for endocrine disruptive potential. The literature describing endocrine disruptive activity (EDA) in South African surface water is then reviewed and discussed. Only a limited number of South African waterbodies have been studied to date, and the research is generally biased towards reproductive endpoints, whereas potential disruption of the thyroid and adrenal endocrine axes and other endocrine targets such as metabolism remains undescribed for the majority of water systems. The present literature indicates the reality of endocrine disruptive potential in various compartments of the South African aquatic environment. Conclusive evidence of adverse health effects in wild populations has, however, not been presented and further research is required. Long-term surveillance of water bodies that are subject to EDC contamination will be valuable in the South African context. Existing governmental initiatives such as the “Green Drop” certification programme and the River Eco-status Monitoring Programme provide promising platforms for government mandated evaluations of EDA in the South African aquatic environment.

2.1 Background

The endocrine system plays a key role in organismal chemical communication being involved in physiological processes such as metabolism, growth and development, reproduction, osmoregulation, behaviour, cardiovascular regulation, immune function and colour change (Molina, 2010). Natural and man-made chemicals and elements have the potential to disrupt the endocrine systems of humans and wildlife. These endocrine-disrupting chemicals (EDCs) are diverse in source and structure, and include pharmaceuticals, personal care products, pesticides, surfactants, hydrocarbons originating from fossil fuels or the combustion thereof, metals, flame retardants, plasticisers and various other chemicals used in the manufacturing industry, and natural hormones excreted by humans and animals (Figure 2.1) (Colborn *et al.*, 1996; Jobling *et al.*, 1998; Casals-Casas and Desvergne, 2011). The presence of EDCs have been shown in virtually every type of aquatic system including rivers, dams, marine environments, groundwater and even tap water (Krimsky, 2000; Kloas *et al.*, 2009; Shi *et al.*, 2012; Kassotis *et al.*, 2016). The identification of potential EDCs and sources of contamination in the environment is,

therefore, of great importance as part of water quality evaluations and from a conservation perspective.

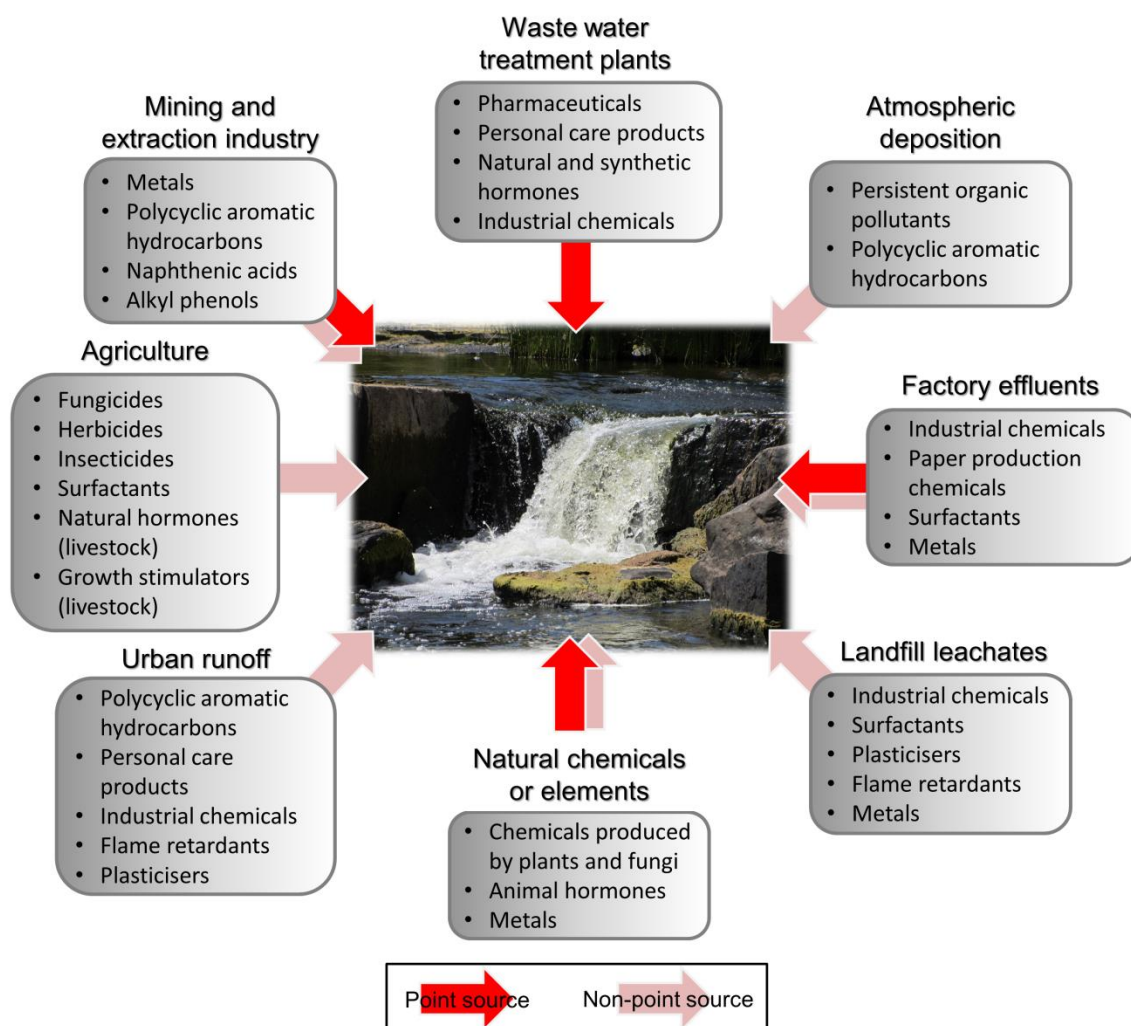


Figure 2.1: The major sources of endocrine disruptors in surface water.

The major targets of endocrine disruptors that have been described to date are the reproductive system (hypothalamus-pituitary-gonadal [HPG] axis), the thyroid system (hypothalamus-pituitary-thyroid [HPT] axis) and adrenal system (or interrenal system in fish) (hypothalamus-pituitary-adrenal [HPA]; hypothalamus-pituitary-interrenal [HPI]) (Figure 2.2) (Diamanti-Kandarakis *et al.*, 2009). There are, however, further hormones and/or pathways, such as those associated with carbohydrate or lipid metabolism which have also been shown to be affected by certain EDCs (Casals-Casas and Desvergne, 2011). A renowned example of EDCs that modulate metabolism is the case of “obesogens”, chemicals that drive a positive energy balance potentially leading to obesity (Grün and Blumberg, 2009).

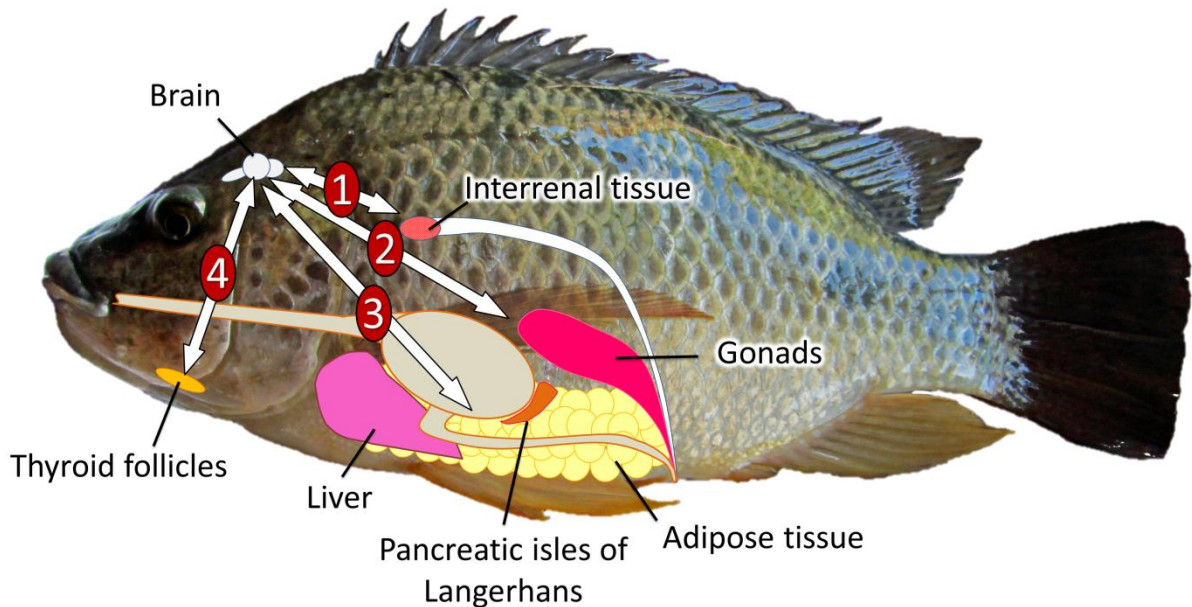


Figure 2.2: Selected components of the fish endocrine system with the major targets of endocrine disruptors known to date indicated namely: (1) Hypothalamus-pituitary-interrenal axis; (2) hypothalamus-pituitary-gonadal axis; (3) metabolism (with linkage to brain indicated due to central control of certain potentially disrupted aspects of metabolic homeostasis such as appetite); (4) hypothalamus-pituitary-thyroid axis.

Aquatic fauna are some of the organisms most impacted by EDCs in the environment, because chemicals of diverse sources end up in waterbodies (surface waters) via point-source and non-point source contamination vectors (Figure 2.1). Waste water treatment plants (WWTPs) that discharge treated wastewater directly into rivers, lakes or marine water are significant sources of EDC pollution, due to incomplete removal, especially in the effluents of less advanced plants lacking final purification steps such as activated carbon or reverse osmosis membranes (Janex-Habibi *et al.*, 2009; Grover *et al.*, 2011). The reality of the potential impacts of WWTP effluents, as source of EDCs, on fish was realised in the United Kingdom when a nationwide survey revealed widespread intersex condition (testicular oocytes) in fish (roach, *Rutilus rutilus*) captured from the close proximity of WWTPs (Jobling *et al.*, 1998). Intersex have since been observed in fish inhabiting rivers downstream of WWTPs in France (Minier *et al.*, 2000), the United States (Woodling *et al.*, 2006) and Canada (Tetreault *et al.*, 2011) among other countries.

A further well known case of endocrine disruption in a wild population is the American alligators, *Alligator mississippiensis*, inhabiting the polluted Lake Apopka, Florida, US. In particular, both males and female alligators were shown to exhibit developmental abnormalities in their reproductive systems, leading to decreased fecundity and population

viability, which persists to this day (Guillette *et al.*, 1994; Guillette *et al.*, 1996). Moreover, the thyroid systems of juvenile Lake Apopka alligators were shown to be significantly impacted in regards to altered plasma thyroid hormone levels, and follicular hypoplasia and colloid depletion in thyroid glands (Crain *et al.*, 1998; Boggs *et al.*, 2013).

Certain EDCs exhibit biological activity at low doses (ng.L^{-1} range in water) (Vandenberg *et al.*, 2012). For instance, the predicted no effect concentration (PNEC), for fish reproductive impairment, of the synthetic estrogen 17 α -ethynylestradiol (EE₂) (commonly used in contraceptives and, therefore, a product of WWTP effluents) is 0.1 ng.L^{-1} (Caldwell *et al.*, 2012). The reality of the risk of EDCs at low dosages was shown in a classic experiment performed in the Canadian Experimental Lakes Region, where the fathead minnow, *Pimephales promelas* population inhabiting a lake, dosed with an environmentally relevant concentration of EE₂ (i.e., 5-6 ng.L^{-1}), was monitored. Male fish were feminized, and the population collapsed after three years, illustrating the potential population level effects of low dosages of EDCs (Kidd *et al.*, 2007).

Like vertebrates, the endocrine systems of aquatic invertebrates can be affected by exogenous chemicals, although the targets and responses differ markedly among these groups, due to the phylogenetic divergence among chordates and other phyla (Rodriguez *et al.*, 2007; Sooin and Smagghe, 2007; Oehlmann *et al.*, 2007). Likely the most well-known case of endocrine disruption in aquatic invertebrates is in imposex in molluscs (i.e., male sexual organs developing in females) resulting from tributyltin exposure (TBT, a chemical commonly used in antifouling paints) (Matthiessen and Gibbs, 1998). The unequivocal evidence showing the TBT induced imposex have, however, been fruitful and the use of TBT containing antifouling paints is now banned globally by the International Maritime Organization.

South Africa has been classified as “water scarce”, and the conservation of water resources is of importance (Conley, 1996). As signatory of the Stockholm Convention on Persistent Organic Pollutants (<http://www.pops.int>), South Africa is obliged to protect its aquatic environment from anthropogenic pollution and initiate programs, which facilitate the assessment of waterbodies. Moreover, the National Water Act (Act 36 of 1998) warrants the protection of the country’s aquatic environment, through law enforcement, and in fact offenders are liable to be fined or receive up to five years imprisonment during their first conviction, and up to 10 years during subsequent conviction (section 151, National Water Act). Although the Department of Water Affairs and Forestry (DWAF) initiated the development of the National Toxicity Monitoring Program (NTMP) in 2003, since the sampling design was finalized and tested in 2008, no systematic toxicity screening has taken

place in South Africa. The NTMP includes the measurement of a number of commonly known endocrine disruptors including aldrin, endosulfan and atrazine, yet, the screening of EDC activity using bioassays were never part of the NTMP objectives. The reality, however, is that South African waterbodies are subject to a myriad of potential endocrine disruptors, with the main sources being WWTPs, agriculture and industries (including mining) (see Figure 2.1). Waste water treatment plants as source of EDCs in the South African aquatic is of special concern. The South African Department of Water and Sanitation (DWS) Green Drop initiative awards WWTPs with “risk ratings” based on (1) the amount of water processed relative to the design capacity of the plant, (2) effluent quality and (3) existing technical skills. (<http://www.dwaf.gov.za/greendrop/>). The 2014 Green Drop report indicated that 25.73% of the 824 WWTPs evaluated were classified as being in “a critical state”, and a further 31.43% as “high risk” based on the Green Drop (evaluation) framework. Because WWTP effluents in general are known to contain a diversity of endocrine active contaminants, waterbodies receiving effluents from poorly functioning South African WWTPs are at risk of contamination by EDCs, due to incomplete chemical removal (Janex-Habibi *et al.*, 2009).

Agriculture is known to be a major source of EDCs in the aquatic environment due to pesticides (reaching water through surface run-off and spray drift) and natural and synthetic hormones originating from intensive livestock farming practices (e.g., e.g., feedlots and piggeries) (Diamanti-Kandarakis *et al.*, 2009; Casals-Casas and Desvergne, 2011). The agricultural sector is a further important potential source of EDC contamination in South African surface waters, as has been confirmed by a nationwide assessment facilitated by the South African Water Research Commission (WRC) (Burger and Nel, 2008). A degree of regulation for pesticide residues (including selected EDCs) in tissues of agricultural produce is facilitated by end-users in export markets such as through the Global GAP Certification. However, the potential contamination of rivers and dams by agricultural practices is not regulated in South Africa. The aim of this review is to provide an overview of the screening technologies that have been applied to date in the South African context to evaluate the endocrine disruptive potential of environmental surface water. Background on EDC mechanisms of action and the testing methods currently applied globally to screen for endocrine disruptive compounds in surface water is first given.

2.2 The mechanisms of EDCs action

There are four major hormone classes namely: steroids, glycoproteins, polypeptides and amino acid-derived hormones. The steroid hormones, estrogens, androgens, progestins, glucocorticoids and mineralocorticoids, as well as thyroid hormones are ligands which bind

with high affinity to intracellular (nuclear) receptors. These hormone-receptor complexes subsequently initiate the expression of genes containing the appropriate hormone response elements (HRE) in their promotor regions (Molina, 2010). Certain EDCs can interfere with hormone receptor transactivation through agonism, antagonism (le Maire *et al.*, 2010), by affecting the recruitment of cofactors or transcription factors (Shanle and Xu, 2011), post transcriptionally through altered miRNA expression (Klinge, 2015), or by epigenetic modification of genes associated with endocrine signalling (i.e., DNA methylation or histone modification) (Skinner, 2016).

Enzymes are important counterparts in the endocrine system, and fulfil an essential role in the biosynthesis and breakdown of hormones. Certain EDCs are known to target enzymes involved with hormone biosynthesis through direct inhibition or by altering the expression of enzyme coding genes (Hampl *et al.*, 2016). In addition, EDCs can modulate hormone biosynthesis by altering the abundance of essential molecular building blocks. For example, in the case of steroid hormones, cholesterol availability can be affected by EDCs targeting the steroidogenic acute regulatory protein (StAR) (Clark and Cochrum, 2007); whereas, iodine levels can be altered due to disruption of sodium/iodide symporter (NIS) function, hence affecting thyroid hormone biosynthesis (Agretti *et al.*, 2011). Another important EDC mode of action is interference with hormone transport by affecting transport proteins such as hormone-binding globulins or other transporters such as thyroid hormone carrying transthyretin (TTR) (Schussler, 2000; Hampl *et al.*, 2016). Endocrine disruptors can also affect organisms at central level, which may lead to phenotypic or behavioural changes. Such anomalies may be a legacy of EDC exposure during ontogeny, affecting neural development, or it may be associated with short term exposure targeting neurons, dopaminergic and serotonergic circuits, and, therefore, responsive and not due to developmental defects (Le Page *et al.*, 2011; Frye *et al.*, 2012).

2.3 The detection of EDC activity

Two characteristics of many EDCs that complicate environmental risk assessments concerning these substances are low-dose effects and nonmonotonic dose response curves (Vandenberg *et al.*, 2012). In addition, the activity of certain EDCs are known to be affected when in mixture, due to the cocktail effect (Kortenkamp, 2007), which further complicates risk assessment.

The use of bioassays provides a holistic view of the endocrine disruptive potential in complex samples such as surface water or whole effluents, describing (biological) endocrine disruptive activity (EDA) rather than presence. Factors such as nonmonotonic responses

and mixture interactions are, therefore, accounted for. The disadvantage, however, of the sole use of bioassays is that the exact chemicals that are responsible for the particular EDA (e.g., estrogenicity) remains unknown, unless chemical analyses have been performed in tandem, allowing the identification of potential EDCs responsible for the observed activity. Chemical analyses are, however, expensive due to equipment costs, the need of specific reference and internal standards, and because methods need to be optimized for each compound tested for. These factors are of special relevance when the testing of whole effluent samples or surface waters are considered, because 100s and even 1000s of potential EDCs may be present in for example a location where WWTP effluents are released.

2.3.1 *In vitro* assays relevant to screen EDA in surface water

Because the principal mechanism of hormone action is receptor mediated gene expression, *in vitro* assays describing interaction with hormone receptors are likely the most widely used, and include receptor binding assays (Baker *et al.*, 1999), and yeast-, bacterial- or mammalian cell reporter gene assays (RGAs) (Routledge and Sumpter, 1996; Chakraborty *et al.*, 2011; Liang *et al.*, 2011). Further *in vitro* bioassays presently applied to screen for EDA (relevant to the testing of surface water samples) include enzyme activity assays (using microsomes or recombinant enzymes) (Hinfrey *et al.*, 2006; Ekuase *et al.*, 2011), co-factor or co-repressor binding assays (Johnson *et al.*, 2011), cell proliferation assays (Soto *et al.*, 1995) and hormone biosynthesis screens (e.g., steroidogenesis in adrenocarcinoma cells) (Hecker *et al.*, 2006).

In addition, primary cultures from fish or amphibians as source of biomarkers are commonly applied with endpoints such as vitellogenin (Vtg) production by hepatocyte cells (Dobbins *et al.*, 2008) or liver slices (Hurter *et al.*, 2002), spiggen production by stickleback fish kidney cells (Jolly *et al.*, 2009), steroidogenesis in ovary explants (Villeneuve *et al.*, 2007), hormone production by testis tissues (Loomis and Thomas, 2000) or thyroid gland explants in culture (Hornung *et al.*, 2010), and excised tadpole tails in culture as source of biomarkers for thyroid signalling (Helbing *et al.*, 2003). Other biomarkers from primary cultures include leptin production by adipocyte cells, and phosphorylation of protein kinase B in primary adipocyte cells as biomarker for insulin signalling (Sargis *et al.*, 2012).

The advantages of *in vitro* bioassays are short exposure periods, high throughput, the absence of animal sacrifices (in cell based assays) and small volumes of environmental samples required. Moreover, *in vitro* bioassays are in many cases more sensitive to EDA

than *in vivo* assays using fish and amphibians as models, although this is not always the case (Scholz *et al.*, 2013).

2.3.2 *In vivo* assays relevant to screen EDA in surface water

There are a number of biomarkers in fish and amphibians exposed *in vivo* that are relevant to reproductive, thyroid, adrenal/interrenal and metabolic disruptors, and these include plasma hormone levels, protein levels (plasma and whole body homogenate; e.g., vitellogenin in male fish) (Ankley *et al.*, 2001), enzyme activity (e.g., aromatase) (Geraudie *et al.*, 2011) and gene expression (Hutchinson *et al.*, 2006). Gene expression can be quantified by quantitative or semi-quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR or RT-PCR), DNA micro-arrays, RNA-seq (next-generation sequencing) (Baker and Hardiman, 2014) or *in situ* hybridisation (Dong *et al.*, 2013). A further application of gene expression in *in vivo* screening is transgenic fish or tadpoles, expressing a (recombinant) reporter gene which causes fluorescence when transactivation of a specific pathway occurs (e.g., thyroid hormone receptor beta agonism) (Terrien *et al.*, 2011). These aforementioned endpoints (i.e., hormones, proteins, enzymes and gene expression) are measured in embryos, juveniles or adults, and can be quantified after short or longer term exposures, because responses at molecular level are rapid and prolonged exposures are not essential (Ankley *et al.*, 2012). Further *in vivo* biomarkers relevant to the four major classes of endocrine disruptors, centered on morphological changes, typically require longer term exposure. It is well known that ontogeny is one of the critical windows of susceptibility for adverse effects associated with EDC exposure (Sumpter and Johnson, 2008). Many of the *in vivo* assays to assess endocrine disruptive potential based on long-term exposure are, therefore, performed using embryos and developing fish or tadpoles.

By far the most biomarkers (from *in vivo* exposure) for endocrine disruptive potential described to date are related to the reproductive systems of animals. Such endpoints/biomarkers in fish and amphibians include: secondary sexual characteristics, e.g., nuptial pads and facial tubercle development in fathead minnows (Ankley *et al.*, 2001), sex ratios after developmental exposure (Goleman *et al.*, 2002), time to puberty (Meier *et al.*, 2011), gonad histology, reproductive output (Ankley *et al.*, 2001), sperm count (Raut and Angus, 2010) and behaviour (Bertram *et al.*, 2015).

Biomarkers, in fish and amphibians exposed *in vivo*, for the screening of thyroid, adrenal/interrenal or metabolic disruption, apart from the biomarkers previously described in this section (i.e., plasma hormone concentration, protein levels, enzyme activity and gene expression), are limited. Histology of thyroid glands in amphibians or thyroid follicles in fish,

and frog metamorphosis assays (Opitz *et al.*, 2005) are commonly applied to evaluate thyroid disruptive potential. As an endpoint for disruption of the adrenal/interrenal system, cortisol production can be measured in artificially stressed animals, such as fish removed from water for short periods. Reduced glucocorticoid release (relative to controls) indicates disruption of the hypothalamus-pituitary-interrenal (HPI) axis (Harvey, 2016). Biomarkers for metabolic disruption in fish exposed *in vivo*, which have been used to date include body mass index, fat accumulation, appetite and feeding behaviour (Reviewed by Nguyen *et al.*, 2013).

The effects of EDC exposure on F1, F2 and further offspring are also tested frequently and most of the endpoints in animals exposed *in vivo* already described in the sub-section can be evaluated in such studies. The testing of such endpoints is important considering the known potential transgenerational effects of EDC exposure (Skinner, 2016).

Gene expression is one of the central mechanisms of endocrine regulation. The evaluation of the expression of genes associated with endocrine signalling is a powerful tool to assess the potential of a chemical or mixture of chemicals to disrupt endocrine signalling and furthermore to identify the mechanism of action through which disruption occurs. A significant advantage of the use of gene expression in exposed animals as screening tool for endocrine disruptive potential is the ability to evaluate multiple biological pathways using a single technique. For example, potential interference with reproductive, thyroid and adrenal endocrine axes and at multiple levels within the hormone signalling cascade can be evaluated using a single cDNA sample (reverse transcribed from RNA). In contrast, other techniques such as enzyme activity assays, protein concentrations, plasma hormone concentrations or other biomarkers applied to evaluate potential targets within the reproductive, thyroid and adrenal endocrine axes, will require multiple biological test kits or chemical standards. The entire transcriptome of an individual animal (present in a particular tissue type) can be characterised, and the expression profiles of each gene described by RNA-seq using next-generation sequencing platforms (i.e., transcriptomics) (Mehinto *et al.*, 2012). This approach is currently the avant-garde of toxicogenomics, but is expensive to use and requires advanced bioinformatics expertise due to the large quantities of data produced. On the other hand, the expression of a limited number of genes can be quantified with relative ease through RT-PCR or RT-qPCR. Such evaluations based on a limited number of candidate genes as biomarkers for endocrine disruption enables the testing of key pathways, is functional as a screening tool, and can advocate the need for further more detailed investigation (Hutchinson *et al.*, 2006).

2.3.3 Organisms collected from the aquatic environment

Virtually all the biomarkers for EDA measured in fish and amphibians exposed *in vivo* listed in the previous section can also be evaluated in individuals collected from environmental waterbodies. Endpoints that are commonly measured in wild-captured fish and amphibians are plasma hormone levels, enzyme activity (Hecker *et al.*, 2007), protein levels and histopathology (Jobling *et al.*, 1998). The advantage of using wild-captured organisms is that chronic exposure is accounted for, as well as effects such as dietary uptake of chemicals. The assessment of wild-captured (chronically exposed) animals furthermore allows researchers to confirm chemistry and/or bioassay based predicted effects regarding endocrine disruption, for a particular water body.

2.4 Evaluations of endocrine disruptive potential in South African surface waters

The purpose of this section of the review is to provide an overview of the research that has been performed describing EDA in South African surface waters, and, therefore, excludes reports of WWTP effluents, drinking water and other water sources potentially contaminated with EDCs.

The South African WRC has been the principal sponsor of research on EDA in South African surface water, and a number of official reports have been published on the subject (Van Wyk *et al.*, 2005; Bornman *et al.*, 2007; Slabbert *et al.*, 2008; Burger, 2008; Bornman *et al.*, 2009; Bornman *et al.*, 2010; Van Wyk, 2013; Van Wyk *et al.*, 2014). In an attempt to strategically approach EDC research in South Africa, the WRC initiated the Programme on Endocrine Disrupting Compounds (PEDC) in 2001 (Burger, 2009). The outcomes of the PEDC were to assess the status of EDCs and the screening thereof in South Africa. A coordinated research programme to determine the occurrence and sources of EDCs in South African waters was subsequently launched. Firstly, the PEDC described a research plan (Burger, 2005), followed by an initial scoping study, in which a number of laboratories from both the private and academic sectors participated (Burger, 2008). Although all WRC funded research on EDA has been overseen and audited by the commission, Burger (2008) is the only study to date focussed on EDA in surface water managed by the commission itself. The study included the screening of four waterbodies (i.e., Hartbeespoort Dam, North West; Makhatini flats, KwaZulu-Natal; Vaal River Barrage, Gauteng and Rietvlei Dam, Gauteng) for EDA using a combination of *in vitro* and *in vivo* biological assays. In particular, the Yeast Estrogen Screen (YES) and Yeast Androgen Screen (YAS) RGAs were applied to measure estrogenicity and androgenicity *in vitro*. Estrogenicity was furthermore evaluated using Vtg

production by African clawed frog, *Xenopus laevis* liver explants in culture as biomarker (i.e., *Xenopus* liver slice bioassay), alkali-labile phosphate (ALP) levels in whole body homogenates as indicator of Vtg induction in *D. rerio* exposed *in vivo* (i.e., Vtg/ALP screen), and plasma Vtg (quantified using immunoassays) levels in *D. rerio* exposed *in vivo*. Surface water was collected during the summers of 2003 and 2004, as well as winter, autumn and spring 2004 from 10 sampling locations per study area. Two composite samples (consisting of a mixture of five samples each) were analysed per area. The YES assay indicated estrogenicity at each of the locations sampled throughout the sampling regime, with estradiol equivalents (EEQs) ranging between 0.18 and 2.04 ng.L⁻¹. The PNEC for fish reproduction (Caldwell *et al.*, 2012) was, however, exceeded in only a single instance, i.e., Rietvlei Dam, Summer 2004. Androgenicity was observed in only two samples, namely Makhatini flats and Hartbeespoort Dam, in both cases during autumn 2004, and with testosterone equivalents (TEQs) of 15.05 and 22.02 ng.L⁻¹ respectively. In addition, the *Xenopus* liver slice bioassay indicated estrogenicity in two of the samples tested, i.e., Vaal Barrage and Rietvlei Dam, winter 2004, whereas the zebrafish Vtg/ALP screen suggested a significant Vtg induction in water from the Vaal Barrage and Hartbeespoort Dam, during the winter and summer of 2004. Unlike expected, the results of the zebrafish plasma Vtg screen differed from the Vtg/ALP screen, and indicated significant Vtg induction after exposure to one of the Rietvlei Dam and one of the Makhatini flats samples representing autumn 2004, and in both the Hartbeespoort Dam samples collected during summer 2004 (Burger, 2008).

In general, the *in vitro* and *in vivo* results did not correspond, and the *in vitro* data did not predict the responses observed with the *in vivo* bioassays when samples were considered individually. Considerable seasonal variation in the risk to wildlife in the waterbodies studied was furthermore observed. Burger (2008) concluded that “spot sampling” may be misleading and that more detailed sampling campaigns are required. Burger (2008) furthermore stated: “There is an urgent need for a comprehensive surveillance programme in the country. Without the data produced in such a study, the risk to the human population and to wildlife cannot be determined.” However, such a “surveillance programme” describing endocrine disruption in South African waterbodies is yet to be mandated.

2.4.1 Agriculture

2.4.1.1 Agricultural regions in the Western Cape Province

Various agrochemicals are known EDCs (Diamanti-Kandarakis *et al.*, 2009; Casals-Casas and Desvergne, 2011). The first work performed in South Africa relating to the screening of environmental surface waters for EDA was centred on agriculture impacted waterbodies in

the Western Cape (Van Wyk *et al.*, 2005). In particular, the estrogenic potential of surface waters from three agricultural regions, i.e., Hex River catchment (predominantly vineyards); Grabouw (predominantly orchards: pome and stone fruit); Bredasdorp (predominantly wheat), were evaluated during 1998 and 1999 using plasma Vtg as biomarker in adult male frogs exposed *in situ* in cages for 21 days. The exposures were performed using 10 frogs per cage at six to 10 locations per region and were repeated between seven and eight times. Significant increases in plasma Vtg in exposed relative to unexposed frogs were observed at three instances in the pome and stone fruit growing region (three localities), four instances in the wheat growing region (three localities), and none in the grape growing region. The data, therefore, suggest a reduced risk to frogs in grape growing regions. There were no definite temporal trends in the incidence of estrogenicity apart from the wheat growing region during August 1999, when a significant increase in plasma Vtg was observed associated with all three locations from which frogs could be recovered. In general, the incidence of estrogenicity was sporadic, being observed during only seven of the 146 instances when frogs in cages were recovered (Van Wyk *et al.*, 2005).

In a further investigation, five river samples collected from the Hex River valley, as well as five dams located in the proximity of pome and stone fruit orchards in the Grabouw region were screened for estrogenic potential using the *Xenopus* liver slice bioassay. The dates of sample collection were not specified. Only one sample from the Hex River valley as well as a single sample from Grabouw was estrogenic. The potency of estrogenicity and, therefore, relative risk, was not reported (Van Wyk *et al.*, 2005).

Moreover, the *Xenopus* liver slice bioassay was applied to screen the estrogenic potential of surface water collected from a single lake and single farm dam within an unspecified agricultural area in the Western Cape Province (Hurter *et al.*, 2002; Van Wyk *et al.*, 2005). The exact locations and date of sample collection was not reported. Both the samples tested were shown to be estrogenic.

Tilapia sparmanii were collected from three rivers in the Western Cape (Palmiet, Lourens and Hout Bay River). Water samples were furthermore collected from the same rivers and screened for estrogenicity using the frog liver slice bioassay. The presence of Vtg was shown in 60% of the male *T. sparmanii* collected from the Lourens River; whereas, none of the surface water samples that were screened exhibited estrogenicity (Van Wyk *et al.*, 2005).

In a recent investigation, selected dams located in the Stellenbosch Winelands were studied for potential EDA (Archer, 2014). In particular, surface water samples collected during the

summer and winter of 2013 from nine dams located in the proximity of vineyards and/or orchards as well as a control dam surrounded by natural vegetation were screened for estrogenicity, androgenicity and anti-androgenicity using the YES and YAS recombinant yeast RGAs. Moreover, the potential of interference with steroidogenesis of compounds in the aforementioned samples were screened using a rat minced testis assay (Archer, 2014).

Androgenicity was observed in surface water from three of the dams tested during summer; whereas, anti-androgenicity was observed in five samples at the time, which included the reference dam. In addition, two of the samples collected during summer were estrogenic. Interference with steroidogenesis was also observed during summer in rat testis cells exposed to surface water from two of the locations. Interestingly, none of the bioassays applied indicated EDA in samples representing the winter season. It is of note that the most intense pesticide application occurs during spring and early summer in the areas investigated by Archer (2014), and the EDA observed in samples collected during summer, and absence of activity during winter suggest an association with pesticide application.

Archer (2014) furthermore collected adult male frogs from a reference dam as well as three of the nine waterbodies tested using the *in vitro* screens ($N = 10$ frogs per dam). A number of biomarkers for endocrine disruption were evaluated including plasma estradiol, dihydrotestosterone, testosterone and VTG levels, as well as histology of the testis tissues and nuptial pads (a secondary sexual characteristic, known to be sensitive to anti-androgenic action). In addition, the expression of the thyroid linked genes *thyroid hormone receptor beta (thrb)* and *type 2 deiodinase (dio2)* were evaluated in liver tissue collected from the wild captured frogs as well as a laboratory control group, maintained in buffered reverse osmosis water (Archer, 2014).

Significantly higher levels of plasma estradiol were observed in frogs collected from two dams compared to the reference group, as well as increased Vtg (which was observed in the group shown to have the highest estradiol levels). Moreover, a significant increase in plasma testosterone was observed in the frogs captured from one of the dams, and increased dihydrotestosterone in frogs from a further dam. Some evidence of thyroid disruption was furthermore observed, and in particular the expression of *thrb* was significantly downregulated in the livers of male frogs from one of the dams relative to control frogs (Archer, 2014).

The percentage of spermatogonia, spermatocytes and spermatozoa present per seminiferous tubule varied significantly in the testis tissue of frogs collected from one of the agriculture impacted dams, relative to frogs collected from the reference dam. No significant

differences in the morphology of nuptial glands were observed among the different frog groups. Notably, the dam in which male frog testes varied significantly from reference frog did not test positive for androgenicity or anti-androgenicity with the *in vitro* screens, but was shown to be estrogenic (Archer, 2014).

Surface water from eight out of the 10 dams tested by Archer (2014) were shown to exhibit at least one form of EDA *in vitro*; but, this number would have been only two, had estrogenicity been the sole endpoint screened for, three if only androgenicity was evaluated, five in the case of anti-androgenicity and one based on steroidogenic disruption. Moreover, considering the endpoints assessed in the wild captured frogs, changes were observed in reproductive hormones in the Dam 2 and Dam 3 groups, and increased Vtg in Dam 3 frogs, but the expression of the marker gene for thyroid signalling (i.e., *thrb*) was significantly different in Dam 4 frogs relative to the control. The results of Archer (2014), therefore, illustrate the need for the evaluation of multiple pathways to describe EDC risk in water impacted by agricultural activity.

2.4.1.2 Atrazine

Evidence exists suggesting that the herbicide atrazine adversely affects reproduction in *X. laevis* (Hayes *et al.*, 2002). Impaired reproduction or effects such as sex reversal is expected to affect population structure on the long term. Du Preez *et al.* (2005) compared the population structure of *X. laevis* among areas where atrazine is periodically sprayed and areas (theoretically) unimpacted by atrazine. Frogs were collected during 2001 from five ponds in a maize growing area (Viljoenskroon) as well as three ponds from an area where maize is not cultivated (control, the ponds did not have maize fields in their catchments) (Potchefstroom) in the North West Province. No significant differences were observed in sex ratios or age structure among the populations representing maize growing and non-maize growing regions, providing evidence that atrazine and other agrochemicals applied in the maize growing region studied do not significantly affect population structure in *X. laevis*.

Plasma estradiol and testosterone levels as well as aromatase activity were measured in *X. laevis* collected during the autumn of 2002 from the same waterbodies representing a maize-growing region and non-maize-growing region sampled by Du Preez *et al.* (2005) (Hecker *et al.*, 2004). Estradiol levels were significantly lower in females captured from the maize-growing region. Similarly, testosterone was significantly lower in both males and females collected from the maize-growing region relative to the non-maize growing region. In addition, a significant negative correlation was observed between estradiol levels in female frogs and the concentrations of atrazine and its metabolite, deethylatrazine measured in the

waterbodies from where the frogs were captured (Hecker *et al.*, 2004). The findings of Hecker *et al.* (2004) indicate the reality of endocrine disruption in frogs inhabiting waterbodies subject to atrazine and/or other pesticides utilized by maize farmers, but the physiological changes observed does not necessarily imply that reproduction will be adversely affected.

Smith *et al.* (2005) assessed the laryngeal muscles and testicular cell types of *X. laevis* collected during the autumn of 2002 from the exact same waterbodies as the aforementioned two investigations (i.e., Hecker *et al.*, 2004; Du Preez *et al.*, 2005) in order to evaluate the potential influence of atrazine exposure on the male phenotype of these frogs. There were no significant differences in laryngeal mass, testicular cell types or the incidence of testicular oocytes among the populations representing maize growing and non-maize growing regions (Smith *et al.*, 2005).

The incidence of intersex was evaluated in *X. laevis* captured from nine localities in South Africa, representing a major maize growing region, a minor maize growing region, and areas where maize is not cultivated (and theoretically not contaminated with atrazine). Testicular oocytes were observed in frogs collected from two locations in the maize-growing regions in the northern parts of South Africa as well as in frogs captured from two further waterbodies in the Western Cape Province. Conversely, none of the frogs collected in the Cape Town and Stellenbosch Winelands areas had testicular oocytes. Du Preez *et al.* (2009) argued that the varied incidence of intersex is an artefact of genetic differences and not chemical exposure, because the frogs captured from the Cape Town and Stellenbosch Winelands regions were shown to be part of a phylogenetic clade distinct from the other populations sampled (Du Preez *et al.*, 2009).

In summary, the research performed in South Africa on *X. laevis* populations inhabiting waterbodies contaminated with atrazine, failed to provide evidence indicating that reproduction is adversely affected in this species.

2.4.2 Areas where DDT is applied in Limpopo Province

Dichlorodiphenyltrichloroethane (DDT) application is currently banned throughout the developed world due to the persistence, bioaccumulation potential and health risks (which include multiple targets within the endocrine system) associated with DDT and metabolites of DDT (Turusov *et al.*, 2002). However, DDT is still applied presently in parts of South Africa for malaria control.

A series of investigations have been performed in the Limpopo Province in order to evaluate the endocrine disruptive potential in surface water, as well as the endocrine systems of aquatic fauna collected from regions where DDT is presently applied for malaria control. The first such study was performed by Bornman *et al.* (2009) who collected surface water samples and fish from the Thohoyandou region. In particular, three impoundments were investigated, namely: the Nandoni Dam (an impoundment of the Luvuvhu River) situated within the DDT application area, the Xikundu Weir, also within the Luvuvhu River, but downstream of the DDT application zone, and as reference the Albasini Dam located upstream of the area where DDT is applied.

Surface water samples were collected from the three waterbodies during 2005 and 2006 and screened for estrogenicity using the YES recombinant yeast RGA and the ER-CALUX mammalian cell RGA. Generally low estrogenic activity was detected in all three the waterbodies evaluated by both the YES (0 - 0.30 ng.L⁻¹ EEQs) and ER-CALUX (0.14 - 0.58 ng.L⁻¹ EEQs) screens. The Albasini (reference) Dam had EEQs of up to 0.30 ng.L⁻¹ (ER-CALUX). In addition, sharptooth catfish, *Clarias gariepinus*, and Mozambique tilapia, *Oreochromis mossambicus* were collected from the three aforementioned waterbodies during October 2005. The genital papillae of *C. gariepinus* were measured, gonadosomatic index (GSI) quantified and gonads of both species were evaluated both macroscopically and histologically. There was a 57% incidence of intersex in male *O. mossambicus*, whereas none of the *C. gariepinus* collected were intersex. The Albasini (reference) Dam was, however, characterised by the highest incidence of intersex (80%) compared to the DDT-impacted Nandoni Dam and Xikundu Weir (50% and 40% respectively). Chemical analyses indicated the presence of DDT in the fat of fish captured from the Nandoni Dam and Xikundu Weir, but not in Albasini Dam fish (Bornman *et al.*, 2009). The intersex may, therefore, have been the result of exposure to other estrogenic compound apart from DDT seeing that Albasini Dam surface water also exhibited estrogenicity.

A further, yet more in depth investigation was subsequently initiated by Bornman *et al.* (2010) evaluating the exact same waterbodies tested by Bornman *et al.* (2009). The study consisted of four surveys/collection events which occurred during October 2006, March 2007, October 2007 and February 2008. The *in vitro* investigation of surface water was expanded from Bornman *et al.* (2009) and samples were screened for estrogenicity, anti-estrogenicity (YES recombinant yeast screen and T47d-kbluc mammalian cell reporter gene assay) and anti-androgenicity (MDA-kb2 mammalian cell reporter gene assay) (Aneck-Hahn *et al.*, 2010). As in Bornman *et al.* (2009), *C. gariepinus* and *O. mossambicus* were applied as sentinels, and the endpoints evaluated included GSI, intersex (Barnhoorn *et al.*, 2010),

sperm motility, testis index (based on changes observed histopathologically (Marchand *et al.*, 2010) and testicular apoptosis, as well as various other toxicological endpoints not directly related to the endocrine system (Bornman *et al.*, 2010; Patrick *et al.*, 2010).

Generally low estrogenicity was detected with EEQs ranging between 0.04 and 0.57 ng.L⁻¹, being the highest in the Albasini (reference) Dam. No anti-estrogenicity or anti-androgenicity was detected in any of the waterbodies screened throughout the study period (Aneck-Hahn *et al.*, 2010).

Intersex was observed in *O. mossambicus* collected from the Albanisi Dam, Nandoni Dam and Xikundu Weir and the incidences were 48%, 63% and 58%, respectively (Barnhoorn *et al.*, 2010). Fish raised in the laboratory were applied as controls and a 0% intersex incidence was observed, suggesting that the intersex observed in *O. mossambicus* collected from the Limpopo Province was due to chemical exposure. However, the evidence is insufficient to link this anomaly with DDT exposure, considering the fact that intersex incidence was only slightly lower in the reference location where DDT is not applied compared to the fish collected from the DDT application zone or downstream (Barnhoorn *et al.*, 2009).

Sperm motility was evaluated in both *C. gariepinus* and *O. mossambicus* quantified as % motile sperm (%MOT), velocity curvilinear (VCL $\mu\text{m/s}$) and progression (PROG). The *O. mossambicus* collected from the Nandoni Dam had significantly higher VCL levels than those collected from the Albasini Dam and Xikundu Weir. Moreover, the *C. gariepinus* collected from Albasini Dam had significantly higher PROG than those collected from the Nandoni Dam and Xikundu Weir (Marchand *et al.*, 2010). Although these alterations in sperm motility may have been associated with chemical exposure, this prediction remains speculative because control fish from a pristine source or laboratory stock were not included in the investigation.

In a further study, the genitals of male freshwater molluscs, *Bulinus tropicus*, collected from a region where DDT is applied as well as a reference location, was evaluated (Bornman and Bouwman, 2012). The exact locations and date of specimen collection was not specified. The penis sheath–preputium length ratio (PSPLR) varied significantly among the two groups of molluscs. DDT was, however, also detected in the sediment of the reference location, yet at lower levels, and the difference could, therefore, not be directly linked to DDT exposure, but possibly other chemicals (Bornman and Bouwman, 2012).

In summary, the collection of studies performed in regions where DDT is continually sprayed for malaria prevention could not provide any conclusive evidence that the reproductive

systems of aquatic animals inhabiting the region are modulated by DDT or DDT metabolites. Potential disruption of thyroid or other endocrine pathways apart from reproduction, in fauna inhabiting DDT application zones, is, however, yet to be assessed.

2.4.3 Waterbodies in Gauteng Province

2.4.3.1 Rietvlei Nature Reserve

Rietvlei Nature Reserve (RNR) located in the City of Tshwane, Gauteng Province, is one of the best studied regions in South Africa regarding potential EDA in surface water, as well as effects in wildlife due to exposure to EDCs. The reserve is located downstream of agricultural lands, informal settlements and a large WWTP. The major contamination vector of the RNR is the Sesmyl stream which traverses the reserve and feeds the Marais and Rietvlei dams.

Initially, surface water collected from six locations within the reserve during 2001 were screened for estrogenicity using the YES recombinant yeast RGA, and EEQs between <LOD and 2.1 ng.L⁻¹ were detected (Aneck-Hahn *et al.*, 2008). A more detailed assessment of estrogenicity was subsequently performed, and in particular, surface water samples collected from the RNR at two month intervals over a two year period (2004-2005) were screened using both recombinant yeast (YES) and mammalian cell (ER-CALUX) RGAs (Aneck-Hahn *et al.*, 2007; Bornman *et al.*, 2007). The YES assay indicated EEQs of <LOD - 1.92 ng.L⁻¹, whereas the ER-CALUX assay indicated higher EEQs of <LOD - 16 ng.L⁻¹ (Aneck-Hahn *et al.*, 2007). The variation among the two test systems may have been due to cytotoxicity or the fact that the ER-CALUX assay can detect transactivation of both ESR1 and ESR2, whereas YES is limited to ESR1 (Aneck-Hahn *et al.*, 2007). In addition, as mentioned earlier in the present manuscript, Burger (2008) observed EEQs of up to 2.04 ng.L⁻¹ in Rietvlei Dam, and estrogenicity based on Vtg induction in frog liver slice cultures (winter 2004) as well as *in vivo* zebrafish exposure (autumn 2004).

Moreover, *C. gariepinus* were captured from the Marais and Rietvlei dams located in the RNR during the breeding season (collection date not specified), and gonads were evaluated macroscopically and histologically (Barnhoorn *et al.*, 2004). A 20% incidence of intersex was observed in RNR fish versus 0% in control individuals from a laboratory stock, suggesting that the condition was due to EDC exposure (Barnhoorn *et al.*, 2004). A more detailed histological evaluation of testes as well as ovaries of *C. gariepinus* from the Marais and Rietvlei dams was subsequently performed (Pieterse *et al.*, 2010). The fish ($N = 81$) were collected during the breeding season over a two year period (date not specified). Abnormalities were scored semi-quantitatively with endpoints including melano-macrophage

centres, cholesterol granulomas and intersex among others. The ovaries of RNR *C. gariepinus* were graded as “Class 1: normal”, and testis as “Class 2: normal tissue structure with moderate histological alterations” based on histopathological (Pieterse *et al.*, 2010). The intersex incidence was 7.8 % in males and 6.2 % in females, being lower than previously report for male *C. gariepinus* collected from the RNR (Barnhoorn *et al.*, 2004).

In a further study, *C. gariepinus* were collected over a two year period (Low-flow: September 2004 and 2005; high-flow: January 2005 and 2006) from the Marais and Rietvlei dams (Bornman *et al.*, 2007; Kruger *et al.*, 2013). In this case, the investigation focussed on male fish and in particular the urogenital papilla length (a secondary sexual characteristic), intersex and the association between these endpoints (Bornman *et al.*, 2007; Kruger *et al.*, 2013). Abnormalities in papillae were observed in 30.8% and 22.2% of the *C. gariepinus* captured from Marais and Rietvlei dams, respectively (Kruger *et al.*, 2013). 29.17% of the fish evaluated by Kruger *et al.* (2013) were classified as being intersex based on testis histology. Moreover, body mass normalised urogenital papilla length varied significantly among fish collected from the RNR, but not among intersex and normal males within the RNR population (Kruger *et al.*, 2013), suggesting that a papillae length index is not functional as indicator of intersex in *C. gariepinus*.

Intersex was furthermore investigated in *X. laevis* as amphibian model, and frogs were collected from the RNR as well as a reference location outside of the reserve (Du Preez, 2007). A 25% incidence of intersex was observed in the RNR male frogs compared to 13% in the reference frogs. The sample size from the RNR was, however, small ($N = 8$) and both the males in which oocytes could be identified were captured from a small pond that does not receive water from the Sesmyls stream (Du Preez, 2007).

Apoptosis in testicular tissues was described in *C. gariepinus* collected from Rietvlei Dam using the TUNEL assay and caspase-3 immunohistochemistry (McClusky *et al.*, 2008). The aim of the study was, however, to characterize a methodology to evaluate testicular apoptosis, and not necessarily assess the health and no reference or control fish were included to compare RNR fish with. A potential link between testicular apoptosis and EDC exposure in the RNR could, therefore, not be identified.

As representative of aquatic invertebrates in the RNR, the freshwater mollusc, *B. tropicus*, was studied, and in particular the lengths of penis sheaths and preputims were measured in individuals collected from the RNR and compared to snails collected from a reference location as well as museum specimens from the University of North West National Snail Collection (NSC). The penis sheath–preputium length ratio (PSPLR) of RNR molluscs was

significantly lower than both the reference site and NCS specimens indicating potential endocrine disruption in the RNR population (Wolmarans and De Kock, 2007).

The combined results of the laboratory bioassays and the wild collected animals clearly indicate endocrine disruption in the RNR. Intersex was confirmed in *C. gariepinus* in three seemingly independent studies performed in the RNR, and the fact that no intersex was observed in control fish (Barnhoorn *et al.*, 2004), as well as in wild *C. gariepinus* captured from other regions (Bornman *et al.*, 2009; Bornman *et al.*, 2010; Marchand *et al.*, 2012) advocates EDC exposure as causative factor. Genetic predisposition is, however, a factor to account for (Du Preez *et al.*, 2009) and may explain the variation in intersex incidence among RNR *C. gariepinus* and other populations.

2.4.3.2 Source water treated and distributed as drinking water

Three drinking water treatment works within the Gauteng Province was evaluated for estrogenic activity (Slabbert *et al.*, 2008; Du Preez and Slabbert, 2008). The raw feeds of the treatment works were surface water from two dams and a river respectively. The identities of the waterbodies were not disclosed. Surface water samples were collected at four instances between January 2005 and January 2006 and screened using a combination of *in vitro* and *in vivo* techniques namely: the YES recombinant yeast RGA, ER-CALUX mammalian cell RGA, Vtg production using trout hepatocyte culture, the *Xenopus* liver slice bioassay, Vtg induction in *D. rerio* (exposed *in vivo* for 14 days) quantified using ELISA or the Vtg/ALP screen.

Estrogenicity was detected in all the source surface water samples during at least one of the sampling events. There were, however, considerable variation in estrogenicity among the different biological assays applied. The YES assays indicated EEQs of 36 pg.L⁻¹ to 3.19 ng.L⁻¹, compared to the <LOD to 0.76 ng.L⁻¹ observed for the same samples using the ER-CALUX assay. In addition, none of the samples were estrogenic according to the frog liver slice bioassay or the zebrafish Vtg ELISA screen. Conversely, the trout hepatocyte screen showed induction factors of up to 2.81 (relative to the control) in surface water, whereas the zebrafish Vtg/ALP assay suggested increased Vtg levels (relative to the control) in three of the 12 water samples tested, and a decrease in Vtg in four of the 12 instances (Slabbert *et al.*, 2008).

The results of Slabbert *et al.* (2008) indicate the risk of EDC contamination in drinking water and, therefore, a hazard to human health. In fact, the YES assay indicated 66.67% of the treated wastewater samples as estrogenic, whereas the ER-CALUX indicated 33.33% to

estrogenic. The potencies of these treated samples were, however, low with the highest EEQ observed being 0.57 ng.L⁻¹.

2.4.4 Eerste and Kuils Rivers, Western Cape

The greater Eerste River catchment, which includes the Kuils River, has been evaluated for endocrine disruptive potential in various studies (Van Wyk *et al.*, 2005; Swart *et al.*, 2011; Van Wyk, 2013; Truter *et al.*, 2015). The land-use in the catchment is a combination of agriculture, urban and industries, and a number of WWTPs discharge treated effluent into the rivers.

Water collected during October 2003 from five locations within the Eerste and Kuils Rivers were screened using the 21 day XEMA assay in order to evaluate potential thyroid disruption (Van Wyk, 2013). The main impacts at the selected locations are WWTP effluents. The endpoints investigated included developmental stage, size and limb lengths. Significantly accelerated development was observed in tadpoles exposed to surface water collected from four of the five localities indicating thyroid disruption. In addition, tadpole lengths were significantly greater in three of the five locations sampled relative to the control (Van Wyk, 2013).

Surface water collected during January and April 2006 and 2007 from a pristine location (Jonkershoek Nature Reserve) and an impacted location (downstream of a WWTP, urban areas, agricultural lands and industries) in the Eerste River was tested for estrogenicity using a battery of *in vitro* and *in vivo* bioassays (Swart *et al.*, 2011). As *in vitro* screens, ESR1 production and cell proliferation was quantified using MCF-7 breast cancer cells, whereas Vtg production in juvenile *O. mossambicus*, exposed for five days, were quantified as *in vivo* endpoint. A significant increase in both MCF-7 ESR1 production and VTG induction in *O. mossambicus* was observed in surface water collected from the (anthropogenic) impacted location during April 2006 and January and April 2007. In addition, a significant induction of MCF-7 cell proliferation was observed in the April 2006 and April 2007 samples (Swart *et al.*, 2011). Surprisingly, a significant increase in Vtg was observed in fish exposed to surface water collected from the pristine location during January 2006 and 2007, whereas no estrogenic activity was detected *in vitro*. The estrogenic response in the fish was possibly the result of myco- and/or phytoestrogens present in the water, although further investigation is required to confirm this hypothesis (Swart *et al.*, 2011).

Surface water was collected from 11 localities in the Eerste River and its tributaries in the Stellenbosch district and screened for estrogenicity using the *Xenopus* liver slice bioassay (Van Wyk *et al.*, 2005). The dates of sample collection were not specified. The catchments

of the Eerste River at the sites sampled are subject to various sources of pollution including urban runoff, industrial effluents, agriculture and WWTP effluents. Five of the samples were found to be estrogenic, and in particular, water collected from two sites located downstream of urban and agricultural areas were estrogenic, one directly downstream of the Stellenbosch WWTP, one downstream of a tributary flowing through an urban and industrial area.

Moreover, the (anti)estrogenic and (anti)androgenic potential of surface water collected from the Eerste River Estuary during October 2012 was evaluated using the YES and YAS recombinant yeast assays (see section 2.4.9 of this chapter) (Truter *et al.*, 2015). The surface water tested negative for androgenicity, estrogenicity and anti-estrogenicity, whereas the anti-androgenic potency was $130.04 \mu\text{g.L}^{-1}$ FEQ. The findings of Truter *et al.* (2015) indicated low risk of endocrine disruption to fish inhabiting the Eerste River Estuary, a surprising finding considering the catchment represented by the estuary and the fact the Macassar WWTP releases treated waste water approximately 700 m upstream of the sampling location. A seasonal investigation will be of value to further research the risk of EDA in the Eerste River Estuary.

In general, the present literature indicate the risk of both reproductive and thyroid disruption in aquatic vertebrates inhabiting the Eerste River, and further investigations on wild captured fish and amphibians collected from this river system is required.

2.4.5 The Olifants River and Loskop Dam, Mpumalanga Province

The Olifants River has been described as one of the most polluted rivers in South Africa, and is subject to contamination by a diversity of point and non-point sources due to large urbanized, agricultural, industrial and mining areas in the upper catchment of the river (Oberholster *et al.*, 2012a; Dabrowski and de Klerk, 2013). Poorly maintained WWTPs are some of the most significant sources of pollution in the Olifants River. To my knowledge only two studies related to potential endocrine disruption have been performed to date on the Olifants River (excluding impoundments in the river). Genthe *et al.* (2013) evaluated estrogenicity in surface water collected from nine localities in the upper Olifants River catchment using the mammalian cell T47D-Kbluc RGA. The sampling dates and frequency of sampling was not described. Estradiol equivalents ranging between 0.04 and 8.88 ng.L^{-1} were detected, with the highest activity being present in surface water collected in the close proximity of a WWTP. A more in depth *in vitro* study was performed by Truter *et al.* (2016b) and surface water collected from five locations during the summer, autumn, winter and spring of 2011 were screened for (anti)estrogenicity and (anti)androgenicity using the YES

and YAS recombinant yeast RGAs. The sampling locations represented different land-use areas including agriculture, urban, and mining. Estrogenic activity was only detected at one of the sites sampled, located downstream of the Riverview WWTP, and alarmingly high EEQs of up to 43.01 ng.L^{-1} was detected. Anti-estrogenicity was not detected at any of the sites sampled, whereas androgenicity was detected solely at the Riverview WWTP-impacted location at a potency of 26.61 ng.L^{-1} dihydrotestosterone equivalents. Anti-androgenicity was more widespread and detected at four of the five localities evaluated during at least one of the seasons sampled. The highest potencies during winter, spring and summer was detected at the Riverview WWTP-impacted location with flutamide equivalents of up to $171.10 \text{ } \mu\text{g.L}^{-1}$ being observed.

Truter *et al.* (2016b) furthermore exposed 22 day old juvenile Mozambique tilapia, *O. mossambicus* to C18 extracts (non-polar and slightly polar compounds) of water collected during summer 2011, reconstituted in buffered reverse osmosis water, and described the expression (in whole body homogenates) of a number of genes as representatives of the reproductive, interrenal and thyroid endocrine axes. Surprisingly none of the genes representing the reproductive system were significantly altered in fish exposed to the C18 extracts representing the Riverview WWTP-impacted locality despite the high estrogenic and anti-androgenic potential detected in water from the said location. Exposure to a dosage of 45 ng.L^{-1} estradiol, however, similarly did not have a significant effect on the expression of genes representing the reproductive system suggesting that the (specific strain of) juvenile *O. mossambicus* were not sensitive models for the specific pathways investigated. The brain localised aromatase coding gene, *cytochrome p450, family 19 subfamily a, polypeptide 1b* (*cyp19a1b*), was significantly downregulated in fish exposed to C18 extracts of a site representing agriculture land-use. In addition, the thyroid linked *dio2* was significantly upregulated in fish representing three of the locations sampled.

In summary, fauna inhabiting the upper Olifants River is clearly at risk of endocrine disruption, and in fact, the highest EEQs observed in South African surface water to date was reported for this system. Intersex incidence and other endpoints for reproductive disorders in fish or amphibians captured from the upper Olifants River are yet to be evaluated and will be of value.

Loskop Dam is the second largest impoundment in the Olifants River, and is located directly downstream of the upper Olifants River catchment. Some of the fish species as well as Nile crocodiles, *Crocodylus niloticus*, and terrapins, *Pelusios signatus* inhabiting Loskop Dam have been diagnosed with a metabolic disorder known as pansteatitis (Oberholster *et al.*, 2012b). The organism with the most severe case of pansteatitis in Loskop Dam is *O.*

mossambicus, and these fish furthermore suffer from obesity. The potential link between pansteatitis, obesity and endocrine signalling was evaluated by Truter *et al.* (2016a). In particular, the expression of a number of genes representing the thyroid and interrenal systems as well as *pparg* was described in brain tissue of adult *O. mossambicus* collected from Loskop Dam as well as from a reference population. A significant increase in the expression of thyroid hormone receptor alpha (*thra*) and *dio2* was observed in Loskop Dam fish relative to fish from the alternative population, but gene expression did not vary among pansteatitis-suffering individuals and other individuals within the Loskop Dam population. In addition, *glucocorticoid receptor (gr)* and *mineralocorticoid receptor (mr)* expression did not vary among any of the groups evaluated. *In vivo* exposure of juvenile 30 dpf *O. mossambicus* were furthermore applied to test for potential thyroid disruption or interference with Ppar gamma signalling after exposure to surface water collected from Loskop Dam. The only significant differences relative to control treatments were in the expression of *thrb* and *pparg* in fish exposed to surface water collected from the transitional zone of the dam which contained microorganisms. The most likely explanation for the observed differences was that feeding on microorganisms, and not necessarily EDCs affected the HPT axis and Ppar gamma signalling (Truter *et al.*, 2016a).

The thyroid status of adult *O. mossambicus* collected from Loskop Dam were evaluated based on thyroid follicle histopathology and plasma triiodothyronine (T3) and thyroxine (T4) levels. Adult fish were collected during the autumn, winter, spring and summer of 2011 in Loskop Dam as well as Flag Boshielo Dam, 90 km downstream. Unaltered plasma T4, but increased plasma T3 levels were measured throughout the year in the Loskop Dam *O. mossambicus* relative to the reference fish (Dabrowski, 2014). In addition, the Loskop Dam fish were diagnosed with enlarged thyroid follicles (goiter) in combination with hypertrophy and hyperplasia of the thyroid follicular epithelium. The increased T3 may be associated with altered iodothyronine deiodinase activity, supported by the findings of Truter *et al.* (2016a) that reported increased *dio2* expression in Loskop Dam *O. mossambicus*. This, however, remains speculative and the exact cause of thyroid disruption as well as pansteatitis in Loskop Dam *O. mossambicus* remains unknown.

2.4.6 Eutrophic waterbodies

The eutrophication of rivers and dams in South Africa due to pollution is a concern (Oberholster *et al.*, 2009). Certain cyanotoxins released by phytoplankton species present in eutrophic systems are known to be EDCs (Sychrova *et al.*, 2012). Hartbeespoort Dam is a well-known eutrophic impoundment in South Africa, which has been screened for EDCs and associated biological effects. In particular, as mentioned earlier in the present manuscript,

estrogenicity was evaluated in Hartbeespoort Dam surface water during 2004 using a combination of *in vitro* and *in vivo* bioassays (Burger, 2008). Estrogenicity, measured using the YES recombinant screen, was observed during all four seasons sampled, but EEQs were relatively low, ranging between 0.2 and 0.65 ng.L⁻¹. Moreover, a single sample was androgenic (autumn 2004) with a potency of 22.02 ng.L⁻¹ TEQ indicated by the YAS recombinant yeast screen. Estrogenicity was furthermore shown using the Vtg/ALP *in vivo* zebrafish screen in surface water collected in the winter and summer of 2004, whereas the zebrafish Vtg (immunoassay) *in vivo* screen indicated estrogenicity during summer 2004 (Burger, 2008). Although the results of Burger (2008) confirmed estrogenicity and androgenicity in Hartbeespoort Dam, it remains unclear whether such biological activity was associated with the eutrophic condition of the dam and, therefore, cyanotoxins or other contaminants.

Sperm motility (quantified as: %MOT, VCL, velocity average path [VAP], velocity straight line [VSL] and PROG) and testicular histology (i.e., spermatogenic stage) was evaluated in *C. gariepinus* collected from Hartbeespoort Dam as well as a reference dam (Wagenaar *et al.*, 2012). The investigation included a high-flow (rainy season; October 2008) and low-flow (dry season; March 2009) sampling event. The VCL and PROG varied significantly among the two fish populations and within the Hartbeespoort Dam population among the high-flow and low-flow periods. Moreover, the interstitial tissues of the testes of Hartbeespoort Dam *C. gariepinus* appeared to be abnormal in comparison with those of the reference population. These altered interstitial tissues may have been related to proliferation of connective tissue (Wagenaar *et al.*, 2012).

In a further study featuring Hartbeespoort Dam, as well two other eutrophic impoundments namely the Klipvoor Dam and Bospoort Dam, *C. gariepinus* and common carp, *Cyprinus carpio* were collected in order to evaluate testes and ovaries for abnormalities (Wagenaar and Barnhoorn, 2015). The fish collection occurred during 2009 and 2010 and included individuals from a reference dam. No alterations were observed in the gonads of both fish species collected from the waterbodies investigated (Wagenaar and Barnhoorn, 2015).

Histology of the gonads of *O. mossambicus* and *C. gariepinus* was performed as indicator of endocrine disruption in Roodeplaat Dam, Gauteng Province (Marchand *et al.*, 2012). Adult fish were collected during the summer of 2007, and the ovaries were evaluated for testicular oocytes (i.e., intersex). In addition, both testes and ovaries were evaluated for various pathological abnormalities. Intersex was observed in 44% of the *O. mossambicus* but not in a single *C. gariepinus*. However, all the spermatogenic stages were present in the intersex fish, which Marchand *et al.* (2012) considered as evidence that spermatogenesis was not

affected. The only further alteration observed was melanomacrophage centres (MMCs), found to be present in the ovaries and testes of *C. gariepinus*. The age of the fish were, however, not determined and the MMCs may have been related to natural degenerative processes (Marchand *et al.*, 2012).

In summary, the limited number of studies describing potential endocrine disruption in fish inhabiting eutrophic South African waterbodies indicate subtle effects, and with no clear indication of adverse outcomes. Potential thyroid disruption in fish inhabiting eutrophic South African waterbodies is yet to be studied and will be of value, seeing that cyanotoxins have been shown to target the thyroid systems of certain fish (Yan *et al.*, 2012).

2.4.7 Farmed crocodiles maintained in contaminated river water

Only a single study evaluating endocrine disruption in reptiles in South Africa exists, and in particular Arukwe *et al.* (2016) evaluated potential impacts of EDC exposure in two year old farmed Nile crocodiles, *C. niloticus*. The animals were maintained in water extracted at a location downstream of the contaminated Hartbeespoort Dam (Burger, 2008), intense agricultural lands and the town of Brits, where a WWTP discharges into the river. The hatching success at the farm is poor ranging between 34 and 62% annually, and the impaired fecundity is suspected to be attributed to EDC exposure (Arukwe *et al.*, 2016). A selection of biomarkers describing endocrine disruptive activity related to reproductive impairment was evaluated. In particular, aromatase activity and testosterone oxidation (testosterone 6 β -hydroxylase activity) were quantified with the titrated water assay using microsomal fractions from gonad and liver tissue respectively. In addition, plasma estradiol, 11-ketotestosterone and testosterone levels, as well as the expression of a selection of genes representing the reproductive and thyroid systems were quantified in liver and gonad tissue collected from the same animals.

Plasma E₂ levels were higher in male than female animals, although not significantly. Similarly, *vgt* and zona radiata (eggshell) protein (*zp*) expression were higher in males than females, but also not significantly. Nonetheless, Arukwe *et al.* (2016) speculated that the aforementioned changes in males relative to females suggest EDC exposure, and in particular “estrogenic exposure of males”.

A major shortfall of the study by Arukwe *et al.* (2016) is the fact that no reference animals as an indication of the “normal” condition in the absence of EDC exposure, were evaluated. The authors, however, did measure the concentrations of various potential EDCs in the tissues of the animals investigated, and could, using principal component analysis (PCA), explore the associations between changes in the biomarkers evaluated and contaminant loads.

In summary, the findings of Arukwe *et al.* (2016) provide anecdotal evidence of endocrine disruption in farmed crocodiles based on selected molecular and biochemical endpoints. Further investigation is, however, needed, and in particular the evaluation of the endpoints described by Arukwe *et al.* (2016) in animals maintained in uncontaminated water as reference. In addition, histopathology of the gonads of crocodiles collected from the population described by Arukwe *et al.* (2016) as well as a reference population will be of value.

2.4.8 Neutralized acid mine drainage

Truter *et al.* (2014) evaluated the endocrine disruptive potential of neutralized acid mine drainage (AMD) by applying the expression of a selection of genes associated with thyroid, reproductive and interrenal signalling in juvenile *O. mossambicus* exposed *in vivo* as biomarkers. Surface water samples were collected during December 2012 from six localities in the Bloubank stream catchment as well as a reference location in a stream originating from an underground spring. Site 1 in the investigation was located directly downstream of an AMD (neutralization) treatment facility and the water, therefore, contained extremely high levels of certain metals including Mn and Al. The expression of *mineralocorticoid receptor* was significantly upregulated after exposure to treated AMD impacted water relative to exposure to water from the reference location. However, the expression of none of the other genes evaluated as biomarkers for endocrine disruption were significantly altered. The sites sampled further downstream of the AMD treatment plant is furthermore impacted by agriculture and a WWTP, and the gene expression biomarkers indicated more pronounced effects regarding potential EDA than sites solely impacted by neutralized AMD (Truter *et al.*, 2014). In summary, the data presented in Truter *et al.* (2014) suggest limited effects of neutralized AMD on endocrine signalling in *O. mossambicus* after short term exposure. Further investigations using other species and evaluating other mechanisms of action are, however, required.

2.4.9 The coastal and marine environment

Truter *et al.* (2015) evaluated the (anti)estrogenic and (anti)androgenic potential of surface water collected from harbours and river mouths in three major South African metropolises (i.e., eThekweni, Nelson Mandela and City of Cape Town) using the YES and YAS recombinant yeast assays. Estradiol equivalents (EEQs) of <LOD to 12.82 ng.L⁻¹ were detected with surface water collected from the Salt River Mouth in Cape Town being the most estrogenic. Moreover, FEQs of <LOD to 604.44 µg.L⁻¹ were detected, and the most potent androgen receptor antagonistic activity was observed in surface water collected from

the Umbilo River mouth which discharges into Durban Harbour. No anti-estrogenic or androgenic activity was detected. The biological activity described by Truter *et al.* (2015), with EEQs exceeding the PNEC for reproduction, as well as high FEQs at certain locations suggest that reproductive disorders may be prevalent in fish inhabiting some of the systems tested. No further published reports of EDA in South African marine and coastal environment describing potential effects in vertebrates exists, and such investigations are needed.

TBT exposure is known to cause imposex in molluscs (Matthiessen and Gibbs, 1998). Specimens of the neogastropod mollusc, *Nassarius kraussianus*, were collected from the Durban and Richards Bay harbours, as well as the Knysna Lagoon, between April and October 2002 (Marshall and Rajkumar, 2003). Gender and penis length was determined for 406 individuals. Imposex was confirmed at all the localities sampled, although the frequencies observed varied considerably at spatial scale. Richards Bay harbour was the most impacted, and 100% of the females collected from this waterway were found to have penises (i.e., imposex), compared to 29-100% in the Durban snails and 40.8-84.8% in the Knysna Lagoon. As was the case with imposex incidence, relative penis length (expressing the length of female penises relative to that of males) also varied significantly at spatial scale. The results of Marshall and Rajkumar (2003) confirm the prevalence of TBT contamination in the South African Coastal environment, although it was noted by the authors that the incidence was less severe than reported for many Northern Hemisphere harbours at the time. The variation in imposex observed within Durban Harbour and the Knysna Lagoon indicate spatial variability in TBT contamination, likely due to the relative proximity of pollution sources and flow dynamics at the location sampled (Marshall and Rajkumar, 2003). Tributyltin is a known obesogen, disrupting metabolic homeostasis in vertebrates (Grün and Blumberg, 2009). Further studies evaluating potential endpoints of TBT such as altered fat metabolism in fish collected from South African harbours will be of value.

2.5 Conclusions and future directions

Endocrine disruptive activity (EDA) and endocrine disruption in aquatic wildlife have been shown in a number of South African waterbodies including rivers and dams impacted by agriculture, WWTPs (representing domestic and industrial waste water) and urban run-off. There is, however, a clear bias in the literature towards endpoints relating to the reproductive system; whereas, potential disruption of the thyroid, adrenal, pancreatic and other metabolism linked endocrine pathways have received less or no attention (Figure 2.3).

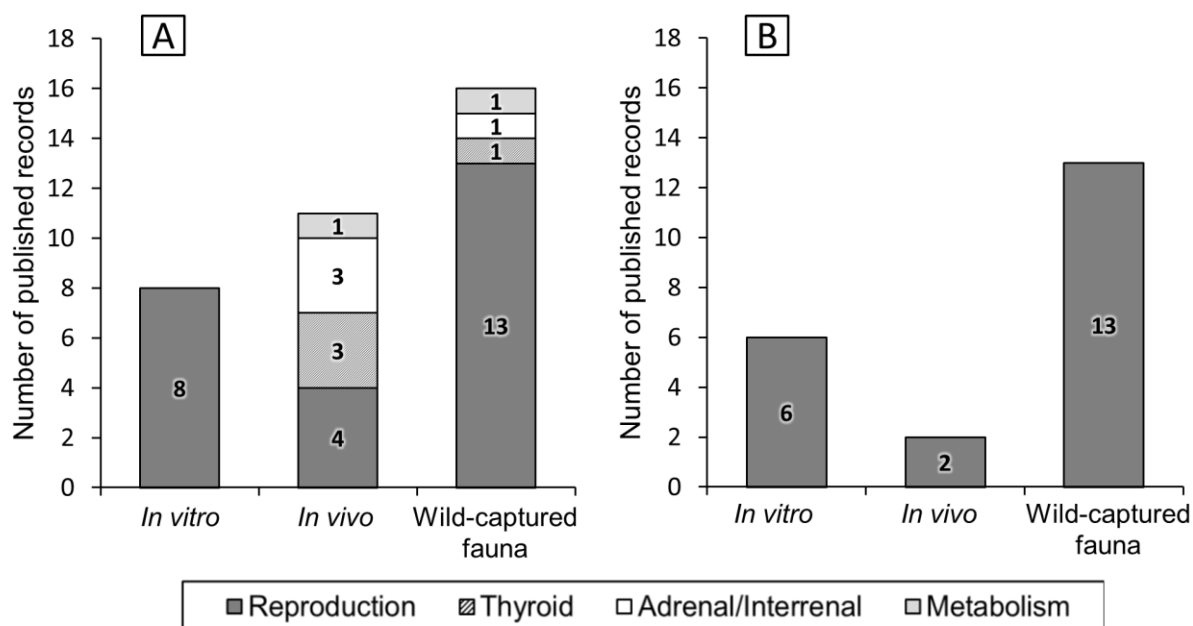


Figure 2.3: An overview of the peer reviewed publications evaluating endocrine disruptive activity in South African surface water using *in vitro* or *in vivo* biological assays, or animals captured from environmental waterbodies. “A” includes the publications which have emanated to date from this dissertation, whereas in “B” these studies were omitted. Only records published in peer reviewed scientific journals or conference proceedings are included in the figure.

Some of the main focus areas of EDC research in the South African aquatic environment include amphibians exposed to surface water potentially contaminated with agrochemicals, surface water and organisms collected from water bodies located in regions where DDT is applied for malaria control, the Rietvlei Nature Reserve, eutrophic dams, and the Eerste River in the Western Cape. Endocrine disruptive activity have, however, not been described (in the literature) for various economically important waterways including the Berg, Breede, Limpopo, mid and lower Olifants (Mpumalanga), Olifants (Western Cape), Orange, Tugela, and Vaal rivers. Moreover, the marine and coastal waters of South Africa (including surface water and aquatic organisms) require further assessment, especially specific impact zones such as WWTP outfalls and harbours. Waste water from the pulp and paper industry is a known contributor of EDA in environmental waters (Munkittrick *et al.*, 2013). However, no published study to date has evaluated the impact of factories associated with the paper and pulp industry on surface water and the endocrine systems of local fauna in South Africa, and such studies are needed.

The risk of hydraulic fracturing as source of EDCs in ground and surface water is now a known fact (Kassotis *et al.*, 2016). The envisioned shale gas mining in the South African

Karoo basin, therefore, has the potential of affecting the endocrine systems of humans and wildlife. The monitoring of ground and surface water bodies located in the proximity of shale gas mining operations for EDA, as well as baseline studies on the endocrine systems of local fauna prior to the initiation of gas extraction will be beneficial.

To date, the only aquatic vertebrate species that have been captured from the wild and evaluated for endocrine disruption in South Africa are *C. gariepinus*, *C. carpio*, *O. mossambicus*, *T. sparmanii* and *X. laevis*. These species are, however, not necessarily sensitive to EDCs, because of potential interspecific variability (Jorgenson *et al.*, 2015), and further investigations evaluating other fish species including morphologically smaller endemic species are needed. In addition, research on potential endocrine disruption in wild populations of aquatic reptiles such as crocodiles and terrapins is also required.

The River Eco-status Monitoring Programme (REMP, formerly River Health Programme) is an initiative of the South African Department of Water and Sanitation (DWS) aimed at the evaluation of the ecological status of riverine ecosystems (<https://www.dwa.gov.za/IWQS/rhp>). The REMP framework classifies the ecological status of a river as “natural, good, fair, poor and unacceptable” based on six indices including the “Fish Response Assessment Index (FRAI)”. The FRAI component of the REMP is a method described by Kleynhans (2007), which is based on the species diversity and abundance, as well as selected habitat characteristics at a particular locality, and does not include an actual physical assessment of fish health. The REMP can potentially be expanded, or used to motivate the investigation of causative links between low FRAI scores and contaminant induced endocrine disruption within river reaches. For example, histopathology of the gonads and thyroid glands can be performed on fish captured from waterbodies or sections of rivers with poor FRAI scores, to assess endocrine disruption.

A further nationwide initiative that can benefit from endpoints related to endocrine disruptive potential is the South African Green Drop Certification programme. Even though WWTPs are known to be a major source of EDCs in the aquatic environment, the testing of EDC loads or EDA does not feature in the Green Drop initiative’s screening framework. The inclusion of such endpoints related to EDA can provide valuable data regarding the chemical removal efficiency of a plant and risk to wildlife where waste water is released.

Omics (transcriptomics, lipidomics or proteomics) approaches are at the cutting edge of ecotoxicology. However, no study to date has applied omics approaches to evaluate the potential effects of contaminated surface water on the endocrine systems of aquatic organisms in South Africa. Similarly, studies evaluating endocrine disruption in wildlife

through chemical induced epigenetic changes (Skinner, 2016) have not been performed in the South African context.

Although as testified of by this manuscript, various approaches have been applied to screen for EDA and assess endocrine disruption in selected South African water bodies, many gaps still exist and promising opportunities for future research. It is evident that the present dissertation has made a significant contribution to the research of endocrine disruption in South African surface waters (Figure 2.3).

2.6 References

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Chapter 3: An *in vitro* and *in vivo* assessment of endocrine disruptive activity in a major South African river

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Declaration by the candidate

With regard to Chapter 3, the nature and scope of my contribution were as follows:

Nature of contribution Extent of contribution (%)

Nature of contribution	Extent of contribution
Conceptual design, experimental work, manuscript writing.	70%

The following co-authors have contributed to Chapter 3:

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Abstract

Endocrine disrupting compound (EDC) loads in rivers, and the associated risk to wildlife, may be linked to different anthropogenic stressors occupying river catchments. The aims of this study were to evaluate seasonal and spatial variation in steroid estrogen loads, and (anti)estrogenic and (anti)androgenic activity in a river catchment (upper Olifants River, South Africa), subject to a diversity of anthropogenic impacts. In addition, Mozambique tilapia, *Oreochromis mossambicus*, was applied as African-endemic sentinel and source of *in vivo* biomarkers. In particular, the expression of selected genes linked with reproductive, thyroid and adrenal signalling were determined in juveniles exposed to organic compounds extracted from surface water. Estradiol and ethinylestradiol were detected at all locations, yet the highest concentrations were observed during summer at a waste water treatment plant (WWTP)-impacted site (30.8 ng.L⁻¹ and 10.83 ng.L⁻¹ respectively). Moreover, *in vitro* estrogenic and androgenic activity was detected solely at the aforementioned locality. Anti-androgenic activity was more widespread, and detected at four of the localities sampled, with the highest potency at WWTP-impacted sites. The expression of the aromatase coding gene, *cyp19a1b*, was significantly downregulated in *O. mossambicus* representing a site dominated by agricultural land use. Moreover, the thyroid-linked *type 2 deiodinase (dio2)* was upregulated in fish representing three of the five localities; although with no clear link to a specific land use area. The present study suggests that different land cover areas contribute differentially to endocrine disruptive activity, and that a combinational approach (*in vitro* and *in vivo* biomarkers) is required to screen for EDC risk.

3.1 Introduction

Numerous industrial, domestic and agricultural chemicals are known to interfere with the endocrine systems of wildlife and humans (i.e., endocrine disruptive chemicals [EDCs]), potentially leading to adverse health effects (Colborn *et al.*, 1996; Casals-Casas and Desvergne, 2011; Jobling *et al.*, 2013). EDCs represent a diverse group of end chemicals, including hormones, pharmaceuticals, agrochemicals, flame retardants, disinfectants, food preservatives, phthalates, organohalogens and plasticizers (Casals-Casas and Desvergne, 2011) as well as a number of metals (Iavicoli *et al.*, 2009). Surface water represents a major sink of EDCs in the environment (Kloas *et al.*, 2009). EDCs may originate from a diversity of point and non-point sources, including mining- and manufacturing industries, agriculture and waste water treatment plants (WWTPs) (Diamanti-Kandarakis *et al.*, 2009). Ecosystem stability and health are key concerns related to EDCs, since the population viability of organisms can be directly affected. In particular, EDCs have been linked to effects such as

lowered sterility, feminization, masculinisation, altered sex ratios, developmental alterations and metabolism related disorders (Jobling *et al.*, 1998; Kime, 1998; Casals-Casas and Desvergne, 2011; Jobling *et al.*, 2013; Kidd *et al.*, 2013). It is, therefore, imperative that ecotoxicological assessments include screening for endocrine disruption as part of river/ecosystem health assessments in order to design and apply inclusive conservation and management plans.

Natural and synthetic steroid hormones, excreted by humans and animals, are the most biologically active endocrine disruptors known to be present in the aquatic environment (Sonneveld *et al.*, 2005). Human contraceptives contain high concentrations of the synthetic estrogen, ethinylestradiol and/or progesterone; whereas livestock growth stimulators applied in agriculture, represent a further source of estrogens and androgens (Matthiessen *et al.*, 2006; Liu *et al.*, 2012). One of the major contributors to steroid hormone contamination and overall EDC loads in rivers is WWTPs. Although WWTPs are able to remove a large portion of EDCs from waste water, many of these chemicals may still present in treated effluents released into rivers (Janex-Habibi *et al.*, 2009), and the removal efficiencies vary among treatment plants depending on the technologies applied (Koh *et al.*, 2008) as well as the management competence.

Chemical analyses are useful to describe contaminant loads in surface waters, and provide data for risk assessments in terms of endocrine disruption (Manickum and John, 2015). The sole use of chemical analyses may, however, not accurately describe risk due to chemical-chemical interactions (i.e., the mixture effect) (Kortenkamp, 2007), and the predicted activity is limited to the selection of chemicals screened for. Biological assays represent a more integrative approach, measuring biological activity and, therefore, the combined action of contaminants. However, bioassays cannot indicate the source of activity, and the combined use of chemical analyses and bioassays are, therefore, preferable.

Although many studies solely use *in vitro* bioassays to describe endocrine disruptive activity, whole animals will not necessarily be affected (Huggett *et al.*, 2003; Dobbins *et al.*, 2008). A combinational approach utilizing *in vitro* and *in vivo* bioassays is, therefore, favourable, allowing a more holistic evaluation of EDC risk (Dobbins *et al.*, 2008; Hermelink *et al.*, 2010).

Land use areas are known to contribute differentially to contaminant loads in river systems (Herlihy *et al.*, 1998; Miller *et al.*, 2011; Fucik *et al.*, 2014). The Olifants River is an important South African river and serves as source for agricultural irrigation schemes and drinking water for livestock and humans. The Olifants River has been described as one of South Africa's most polluted rivers (Oberholster *et al.*, 2010), flowing through a diversity of land

cover areas including highly industrialized as well as agricultural and residential regions in the Mpumalanga Province. Some of the known contributors to the pollution in the Olifants River are coal mining, coal-fired power generation, acid mine drainage from abandoned mines, manufacturing industries, WWTPs, agrochemicals applied to crops and feedlot waste (Driescher, 2007; Oberholster *et al.*, 2010; Dabrowski and De Klerk, 2013; Genthe *et al.*, 2013). All the aforementioned anthropogenic stressors present potential sources of EDCs. Dabrowski and De Klerk (2013) described the contribution of different land use activities in the upper Olifants River on metal, phosphate, nitrate, sulphate and basic water quality parameters. Associations between land use and EDC loads in the upper Olifants River are yet to be described. In fact, few reports on links between land use regions and endocrine disruptive activity in water bodies exist in the literature (Sellin Jeffries *et al.*, 2011; Mandiki *et al.*, 2014).

The aims of the current investigation were to evaluate the endocrine disruptive potential of surface waters from a river catchment impacted by a diversity of anthropogenic activities, and to explore associations between land use areas and the observed biological (endocrine) activity. The specific objectives were: (1) to investigate contamination of the upper Olifants River catchment with steroid estrogens, E₂ and EE₂, and assess the seasonal and spatial distribution of these contaminants; (2) to measure estrogenicity, anti-estrogenicity, androgenicity and anti-androgenicity in the upper Olifants River during four seasons using recombinant yeast bioassays; (3) to evaluate endocrine disruptive potential of surface water collected from the upper Olifants River using short-term *in vivo* juvenile fish exposures. The African-endemic Mozambique tilapia, *Oreochromis mossambicus*, was applied as sentinel, and source of gene expression based biomarkers, quantified in juveniles after 48 h exposure. The female hormone system was represented by *estrogen receptor 1 (esr1)*, *vitellogenin 1 (vtg1)* and *aromatase (cyp11b)*; the male hormone system by *androgen receptor 1 (ar1)*; the thyroid system by *thyroid hormone receptor alpha (thra)*, *thyroid hormone receptor beta (thrb)* and *type 2 deiodinase (dio2)*; and the adrenal endocrine axis by *glucocorticoid receptor 1 (gr1)* and *mineralocorticoid receptor (mr)*.

3.2 Materials and Methods

3.2.1 Study sites

Five study sites were selected based on their representation of different land use areas (Figure 3.1). The land use of site 1 (S 26.238575°; E 29.779634°) and site 2 (S 26.222718°; E 29.462552°) catchments are principally occupied by dry-land and irrigated agriculture as well as livestock farming. Site 3 (S 26.137503°; E 29.269979°) is downstream of agricultural

activity, coal mining and urbanised areas from where a single WWTP discharges into the river. Site 4 (S 25.841779°; E 29.266449°) is adjacent to the town of eMalahleni and in the proximity of the Riverview WWTP, which receives industrial and domestic wastewater. Site 5 (S 25.625199°; E 29.217785°) is surrounded by natural vegetation, but is located downstream of the confluence of the Klein-Olifants and Olifants rivers, therefore, potentially receiving contaminants from both the greater eMalahleni and Middelburg regions' catchments although both Middelburg and eMalahleni towns are at least 50 km upstream of this site. Moreover, dry-land agricultural practices are located within a 10 km radius of site 5 (Oberholster *et al.*, 2010; Dabrowski and De Klerk, 2013).

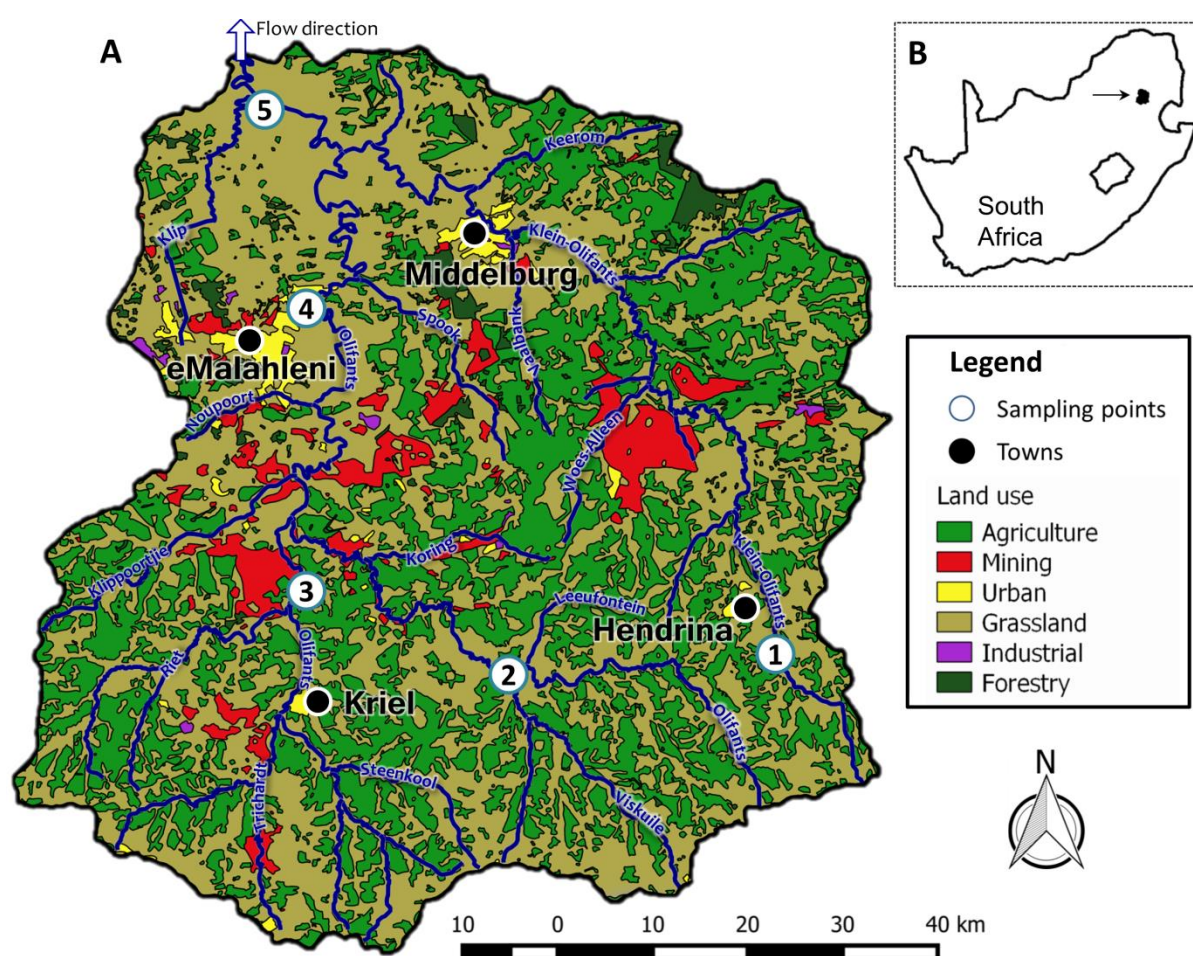


Figure 3.1: (A) Map of the upper Olifants River catchment with land use and the current sampling locations indicated. (B) Map insert indicating the location within South Africa. Map credits: QGIS Brighton 2.6, Open Source Geospatial Foundation; ENPAT 2001, SA Department of Environmental Affairs EGIS database.

3.2.2 Water collection and solid phase extraction

One 2.5 L surface water sample was collected in amber glass bottles from each of the five sampling locations (Figure 3.1) during April (autumn), June (winter), September (spring) and November (anti-estrogenic activity *in vitro*), 2011, and kept on ice or at 4 °C. The samples were subsequently passed through 0.5 µm glass fibre filters and the pH-adjusted to three using H₂SO₄. Organic compounds were extracted from 2 L of water within 96 h of collection through solid phase extraction (SPE) using 12 mL, 2 g DSC-18 columns (Supelco, Sigma-Aldrich, ZA). A Milli-Q water negative control was included in each extraction. Compounds were eluted from the SPE columns using a solvent mixture (40% hexane, 45% methanol and 15%, 2-propanol), air dried, reconstituted in dimethyl sulfoxide (DMSO) (Sigma, ZA) to a 1000x concentrated state and stored at -20 °C.

3.2.3 Enzyme-linked Immunosorbent Assays (ELISAs)

17β-Estradiol (E₂) and 17α-ethinylestradiol (EE₂) levels were determined in the C18 SPE extracts of water collected from the five localities across four seasons using commercially available ELISA kits (E₂: DRG International Inc., USA; EE₂: R-Biopharm, DE) according to the manufacturers' instructions. The extracted samples in DMSO (1000x concentrated) were diluted (E₂: 1/10; EE₂: 1/20) in a 0.1% w/v human serum albumin, 0.9% NaCl solution and assayed. The subsequent detection limits were 0.37 ng.L⁻¹ and 0.11 ng.L⁻¹ for E₂ and EE₂, respectively, after solvent blank correction.

3.2.4 In vitro recombinant yeast assay

Estrogenic, anti-estrogenic, androgenic and anti-androgenic activity of surface water C18 extracts were evaluated using, respectively, the Yeast Estrogen Screen (YES), Yeast Antiestrogen Screen (YAES), Yeast Androgen Screen (YAS) and Yeast Antiandrogen Screen (YAAS) recombinant yeast bioassays (Routledge and Sumpter 1996; Sohoni and Sumpter 1998). The two yeast strains stably transfected with human *estrogen receptor 1* (*ESR1*) and human androgen receptor (*AR*) respectively, in combination with a LacZ coupled reporter gene plasmid made available by JP Sumpter (Brunel University, UK). β-estradiol (E₂) (≥98% pure) as control for *ESRA* agonism, tamoxifen (TAM) (≥99% pure) as control for *ESRA* antagonism, dihydrotestosterone (DHT) (≥97.5% pure) as control for *AR* agonism and flutamide (FLU) as control for *AR* antagonism were purchased from Sigma.

3.2.4.1 Yeast culture

Minimal medium, growth medium and assay medium were prepared as described by Routledge and Sumpter (1996). Growth medium (50 mL) was inoculated with 125 µL

concentrated yeast stock and cultured for approximately 24 h (estrogen responsive strain) and 48 h (androgen responsive strain) on an orbital shaker at 155 rpm.

3.2.4.2 Yeast exposure

The surface water extracts and C18 (Milli-Q) negative controls were assayed in sterile 96-well flat-bottom plates (Costar, Corning, USA) at a 5× concentrated state ('1× concentrated' denotes the state in nature). The C18-extracts were diluted to the appropriate concentration in ethanol and 10 µL of these diluents were dispensed in duplicate per assay plate. Each plate furthermore contained a 12-point two-fold serial dilution of E₂ (2.72 µg.L⁻¹–1.33 ng.L⁻¹) (YES), TAM (185.76 µg.L⁻¹–0.09 µg.L⁻¹) (YAES), DHT (14.52 µg.L⁻¹–7.09 ng.L⁻¹) (YAS) or FLU (27.62 mg.L⁻¹–13.49 µg.L⁻¹) (YAES). The standards were introduced into duplicate wells using 10 µL of ethanol as a carrier

All wells in the YAES and YAAS assay plates were supplemented with 1.43 nM E₂ and 7.14 DHT nM (i.e., seven-fold dilution of the highest YES and YAS standard), respectively. A further six wells containing simply 1.43 nM E₂ or 7.14 nM DHT were included as positive blanks in the YAES and YAAS plates.

Once the ethanol was evaporated from assay plates in a laminar flow cabinet, 200 µL assay medium with yeast was dispensed per well (approximately 8 × 10⁶ cells). The lids of assay plates were subsequently sealed with autoclave tape and incubated at 32 °C in the dark for approximately 48 h (YAS and YAAS) or 72 h (YES and YAAS). YAS and YAAS plates were then incubated for a further 24 h at room temperature. The environmental samples were screened in two independent experiments. Estradiol, DHT, TAM and FLU equivalents values (expressing the relative potency of surface water samples) were calculated per assay plate using the plate-specific regression functions (Grover *et al.*, 2011). The intra- and inter-assay coefficients of variance for the yeast assays are described in Supporting material Table S3.1.

3.2.4.3 Calculations

Hormone receptor transactivation activity was quantified based on colour change at 570 nm. Absorbance measurements were corrected for cell density (determined spectrophotometrically at 620 nm) using the following equation (derived from Fent *et al.*, 2006):

$$\text{agonistic activity (AA)} = \text{Abs}_{540 \text{ nm (sample)}} - [\text{Abs}_{620 \text{ nm (sample)}} - \text{mean Abs}_{620 \text{ nm (solvent control)}}] \quad (1)$$

where $Abs_{540\text{ nm (sample)}}$ denotes the absorbance measurement at 570 nm for an individual sample or standard-containing well, $Abs_{620\text{ nm (sample)}}$ the absorbance measured at 620 nm, and mean $Abs_{620\text{ nm (solvent control)}}$ the mean 620 nm absorbance measured among the solvent blank wells. Estrogen and androgen receptor agonism was expressed as a percentage relative to the maximal AA of E₂ or DHT per assay plate as follows:

$$\text{relative AA (RAA)} = [AA_{(\text{sample})}/AA_{(\text{maximum})}] \times 100\% \quad (2)$$

where $AA_{(\text{sample})}$ denotes the AA determined per sample or standard-containing well, and $AA_{(\text{maximum})}$ the mean AA (among duplicate wells) of the E₂ or DHT concentration exhibiting the maximal AA per plate. Estrogen and androgen antagonistic activity was expressed as a percentage relative to the mean AA of the positive E₂ or DHT controls as follows:

$$\text{relative antagonistic activity (RAntiA)} = [AA_{(\text{sample})}/\text{mean } AA_{(\text{positive control})}] \times 100\% \quad (3)$$

where $AA_{(\text{sample})}$ denotes the AA determined per sample or standard-containing well, and $AA_{(\text{positive control})}$ the mean AA among the E₂ or DHT positive control replicates wells. The limit of detection (LOD) of RAA and RAntiA was calculated as follows:

$$\text{agonism LOD} = \text{mean } RAA_{(\text{solvent control})} + [3 \times \text{standard deviation (SD) } RAA_{(\text{solvent control})}] \quad (4)$$

$$\text{antagonism LOD} = \text{mean } RAntiA_{(\text{positive control})} - [3 \times \text{SD } RAntiA_{(\text{positive control})}] \quad (5)$$

where mean RAA (solvent control) and mean RAntiA (positive control) denotes the mean RAA and RAntiA of the wells representing the respective negative and positive controls. Estradiol, DHT, TAM and FLU equivalents were calculated per assay plate using the plate-specific regression function describing the relationship between RAA or RAntiA and standard dose (E₂, DHT, FLU or TAM).

3.2.5 In vivo fish exposures

3.2.5.1 Animal husbandry and exposures

Juvenile Mozambique tilapia fish (*Oreochromis mossambicus*) for short term exposure studies, at the 17-days post-fertilization (dpf) stage were obtained from a single breeding pair at the FeedTech aquaculture facility, Stellenbosch University, and maintained in aerated reverse osmosis (RO) water (containing 250 mg iodated marine salt, 80 mg NaHCO₃ per litre).

Fish were exposed to the C18-extracts of water collected from all sites, during the summer of 2011. Twenty fish (22 days post fertilization) were assigned per treatment in duplicate

glass tanks, 10 fish per replicate. The C18-extracts representing the five localities were reconstituted in 600 mL of buffered iodated RO water to the concentrated state as in nature (0.001% DMSO) and the pH was adjusted to 7 using HCl if needed. A DMSO solvent control exposure group was also included. The fish were acclimatized to the exposure containers and conditions for approximately 48 hours prior to exposures (i.e., constant aeration at 28 ± 1 °C with a 14:10 light:dark cycle, pH 7), and fed crushed tilapia pellets (AquaNutro, ZA) twice daily during this period. The fish were then transferred to the respective treatment groups and exposed without food for 48 h.

Juvenile *O. mossambicus* (22 dpf) were furthermore exposed to two concentrations of E₂ and a solvent control. The concentrations of E₂ in the tanks when the exposures commenced, were determined with ELISA (as described earlier in this manuscript) and was found to be 45.21 ± 6.59 ng.L⁻¹ and $8,754.0 \pm 419.57$ ng.L⁻¹ respectively. All fish were euthanized in 0.1% Benzocaine (Heynes Mathew, Ltd., ZA), snap frozen in liquid nitrogen or placed directly into 900 µL of TRIreagent (Sigma-Aldrich, ZA) and stored at -80 °C. Significant diurnal variation in thyroid corticosteroid hormones have been shown in fish (Laidley and Leatherland, 1988), and all individuals (exposed to surface water extracts) in which the expression of genes associated with thyroid and corticosteroid signalling was described, were therefore euthanized at 22h00 within a one hour window.

Animal husbandry, treatment and handling were performed according to the South African Standard: the care and use of animals for scientific purposes (SANS 10386:200X) and under the approval of the Stellenbosch University Research Ethics Committee (Protocol #: SU-ACUM12-00036).

3.2.5.2 RNA isolation and cDNA synthesis

Whole body homogenates of juvenile fish were prepared in TriReagent using an ultrasound sonicator (Omni-ruptor 400, Omni International Inc., USA). Total RNA was isolated according to the TriReagent technical bulletin. RNA integrity was assessed through agarose gel electrophoresis and Nanodrop (Thermo Scientific, USA). The RNA was subsequently DNase I (Sigma, ZA) treated, and complementary DNA (cDNA) was prepared from 2 µg of total RNA in 20 µL-, or 1 µg in 10 µL reaction volumes using Enhanced Avian HS RT-PCR kits (Sigma, ZA). The reverse transcription was performed using a combination of oligo(dT)₂₃ and random nonamers.

3.2.5.3 RT-qPCR

Messenger RNA expression of *esr1*, *vtg1*, *cyp19a1b*, *ar1*, *thra*, *thrb*, *dio2*, *gr1* and *mr* with *actin beta* (*actb*) as reference gene was evaluated using real-time RT-qPCR. The PCRs were performed as 15 μ L reactions containing 2 μ L cDNA as template (40 ng cDNA per reaction for *vtg1*; 100 ng/reaction *cyp19a1b*; 10 ng/reaction *thra*, *thrb*, *dio2*, *gr1*, *mr* and *esr1*; 5 ng/reaction *actb*), 7.5 μ L Jumpstart® SYBRgreen mix (Sigma, ZA), 0.33 μ M of each primer and nuclease free water. The PCR programs for all primer pairs included an enzyme activation step at 95 °C (9 minutes), followed by 40 cycles of denaturing at 95 °C (15 seconds), annealing at 58 - 63.5 °C (30 seconds) (Supporting information, Table S2.2) and elongation at 72 °C (45 seconds). Each PCR plate contained an internal non-template control (no cDNA). In order to determine plate-specific PCR efficiency, a six point two-fold or ten-fold (for *vtg1*) serial dilution of cDNA transcribed from the RNA of a negative control exposed individual (*thra*, *thrb*, *dio2*, *gr1*, *mr*, *ar1*, *actb*) or 8.75 μ g.L⁻¹ E₂ exposed individual (*esr1*, *vtg1*, *cyp19a1b*) were included per plate. All samples and controls were run in triplicate and amplicon quality was assessed through melting curve analyses.

Gene expression was quantified using the Pfaffl method (Pfaffl, 2001), relative to the solvent control treatment group. Amplification efficiencies were determined for each primer pair per PCR programme. Outliers were identified per treatment group through the Grubbs' test (Burns *et al.*, 2005) and removed.

The sources and sequences of primers are indicated in Table S3.2. Primers for *esr1* and *vtg1* were designed against the appropriate Genbank sequences (Supporting information, Table S3.2) using Primer Premier 6 (Premier Biosoft, USA). The *esr1* and *vtg1* primers' PCR products were sequenced by the Stellenbosch University Central Analytical Facility to validate the primers.

3.2.6 Statistical analyses

Normality and homogeneity of variance of the data was assessed using the respective Shapiro-Wilks and Levene's tests. The variance in parametric data was analysed using one-way ANOVA followed by Tukey's unequal *N* Spjotoll-Stoline corrected HSD *post hoc* test. Non-parametric gene expression data were rank transformed in order to comply with ANOVA assumptions and analysed using the aforementioned approach. Non-parametric steroid estrogen data were analysed using Kruskal-Wallis ANOVA in combination with a multiple comparison of mean ranks *post hoc* test. The relationship between estrogen concentrations, estrogenicity, anti-estrogenicity and the expression of *cyp19a1b* and *dio2*, were evaluated using principal component analysis (PCA) with land use among the five

sampling locations as supplementary variables. The land use data (Supporting information, Table S3.3) were obtained from Oberholster *et al.* (2012). The steroid hormone, yeast bioassay and gene expression data used in principal component analysis were log transformed, centered and standardized (Ter Braak and Smilauer, 2012). Probability values of < 0.05 were deemed significant. Statistical analyses were performed using Statistica 12 (Statsoft Inc., USA) and CANOCO version 5.1 (Microcomputer Power, USA).

3.3 Results

3.3.1 Steroid hormone analyses

17 β -Estradiol (E_2) and 17 α -ethinylestradiol (EE_2) was detected in the Upper Olifants River catchment at concentrations ranging from 0.72 to 30.8 ng.L⁻¹ and 0.45 to 10.83 ng.L⁻¹, respectively (Table 3.1). Locality (within the river) was a significant source of variation EE_2 concentrations measured across seasons ($H_{4,20} = 11.92$, $P = 0.02$), but not in E_2 ($H_{4,20} = 9.24$, $P = 0.06$, Kruskal-Wallis ANOVA). A stepwise seasonal increase of E_2 loads from autumn to summer is evident (Table 3.1). Although EE_2 was detected in the upper Olifants catchment throughout the year, only site 4 had detectable concentrations during all four seasons sampled (Table 3.1). The seasonal variation in EE_2 concentrations followed a similar trend to E_2 at site 4, being highest in summer (Table 3.1).

Table 3.1: (a) The mean of 17 β -estradiol (E₂) and (b) 17 α -ethinylestradiol (EE₂) levels (ng.L⁻¹) measured in water collected from five localities within the upper Olifants River catchment during autumn (May), winter (June), spring (September) and summer (November) 2011. Standard deviation among technical repeats is indicated in brackets. Measurements exceeding the predicted no effect concentration (PNEC) of E₂ (2 ng.L⁻¹) and EE₂ (0.1 ng.L⁻¹) for fish (Caldwell *et al.*, 2012) are indicated.

a)

Locality	17 β -estradiol (E ₂) (ng.L ⁻¹)			
	Autumn	Winter	Spring	Summer
Site 1	1.49 (0.08)	0.86 (0.02)	3.38 (0.14) ^a	11.37 (0.13) ^a
Site 2	0.72 (0.01)	0.69 (0.02)	1.01 (0.02)	5.36 (0.26) ^a
Site 3	8.08 (0.15) ^a	0.98 (0.02)	6.24 (0.49) ^a	10.14 (0.38) ^a
Site 4	3.90 (0.16) ^a	14.07 (0.64) ^a	13.51 (1.63) ^a	30.80 (3.72) ^a
Site 5	1.0 (0.01)	1.42 (0.05)	2.03 (0.05) ^a	3.60 (0.56) ^a

^a Exceeds the E₂ predicted no effect (PNEC) concentration for fish (Caldwell *et al.*, 2012)

b)

Locality	17 α -ethinylestradiol (EE ₂) (ng.L ⁻¹)			
	Autumn	Winter	Spring	Summer
Site 1	1.24 (0.24) ^b	<LOD	<LOD	0.70 (0.13) ^b
Site 2	0.45 (0.21) ^b	<LOD	<LOD	<LOD
Site 3	2.63 (1.05) ^b	<LOD	0.76 (0.10) ^b	0.87 (0.04) ^b
Site 4	1.36 (0.23) ^b	2.89 (0.33) ^b	3.26 (0.05) ^b	10.83 (0.78) ^b
Site 5	0.58 (0.26) ^b	<LOD	<LOD	0.58 (0.04) ^b

^b Exceeds the EE₂ predicted no effect (PNEC) concentration for fish (Caldwell *et al.*, 2012)

3.3.2 Yeast reporter gene assays

Estrogenic activity was limited to the WWTP impacted site 4 and during winter, spring and summer (Table 3.2). Similarly, androgenicity was limited to site 4 but only during winter, whereas anti-androgenic activity was observed in water collected from sites 1, 2, 3 and 4, with the highest activity at site 4 (Table 3.2). Anti-estrogenic activity was not observed in any of the samples tested.

Table 3.2: (a) Mean estrogen receptor agonism (expressed as 17β -estradiol equivalents), (b) androgen receptor agonism (expressed as dihydrotestosterone equivalents) and (c) androgen receptor antagonism (expressed as flutamide equivalents) observed in surface water collected from five localities within the upper Olifants River catchment. The standard deviations among yeast exposure plates are indicated in brackets.

a)

Locality	Estradiol equivalents (ng.L^{-1})			
	Autumn	Winter	Spring	Summer
Site 1	<LOD	<LOD	<LOD	<LOD
Site 2	<LOD	<LOD	<LOD	<LOD
Site 3	<LOD	<LOD	<LOD	<LOD
Site 4	<LOD	10.15 (0.90)	14.16 (0.80)	43.01 (1.73)
Site 5	<LOD	<LOD	<LOD	<LOD

b)

Locality	Dihydrotestosterone equivalents (ng.L^{-1})			
	Autumn	Winter	Spring	Summer
Site 1	<LOD	<LOD	<LOD	<LOD
Site 2	<LOD	<LOD	<LOD	<LOD
Site 3	<LOD	<LOD	<LOD	<LOD
Site 4	<LOD	26.61 (3.44)	<LOD	<LOD
Site 5	<LOD	<LOD	<LOD	<LOD

c)

Locality	Flutamide equivalents ($\mu\text{g.L}^{-1}$)			
	Autumn	Winter	Spring	Summer
Site 1	<LOD	39.50 (16.09)	<LOD	<LOD
Site 2	<LOD	<LOD	56.78 (17.72)	<LOD
Site 3	<LOD	21.97 (10.86)	93.20 (28.72)	<LOD
Site 4	<LOD	133.87 (11.71)	171.10 (29.89)	88.75 (14.71)
Site 5	<LOD	<LOD	<LOD	<LOD

3.3.3 Gene expression

The expression of *cyp19a1b* varied significantly among the treatment groups ($F_{5,23} = 3.38$, $P = 0.02$) and was significantly lower in juvenile *O. mossambicus* exposed to site 2 water than to the solvent control ($P = 0.01$) (Figure 3.2). In contrast, no significant variance was observed in the expression of the reproductive linked *vtg1* ($F_{5,13} = 0.87$, $P = 0.52$), *esr1* ($F_{5,23} = 0.43$, $P = 0.82$) (Figure 3.2) or *ar1* ($F_{5,32} = 0.16$, $P = 0.97$) (Figure 3.3) among the treatments representing the upper Olifants River and solvent control. Exposure to E_2 resulted in increased *cyp19a1b* and *vtg1* expression, although only significantly so in the high dosage treatment (i.e., 8754 ng.L^{-1}) ($P < 0.01$) (Figure 3.2). The expression of *esr1* was unchanged in response to E_2 exposure ($F_{2,12} = 0.11$; $P = 0.89$) (Figure 3.2).

The expression of *type 2 deiodinase*, linked to thyroid signalling, varied significantly among the treatment groups ($F_{5,23} = 9.58$, $P < 0.001$). In particular, *dio2* expression was higher in the site 1 ($P = 0.001$), site 3 ($P < 0.001$) and site 5 ($P = 0.001$) treatments relative to the solvent control (Figure 3.3). Conversely, no significant variance was observed among treatments in the expression of *thra* ($F_{5,32} = 0.77$, $P = 0.57$), *thrb* ($F_{5,32} = 0.62$, $P = 0.68$), *gr1* ($F_{5,32} = 0.52$, $P = 0.76$) and *mr* ($F_{5,32} = 1.07$, $P = 0.39$) (Figure 3.3).

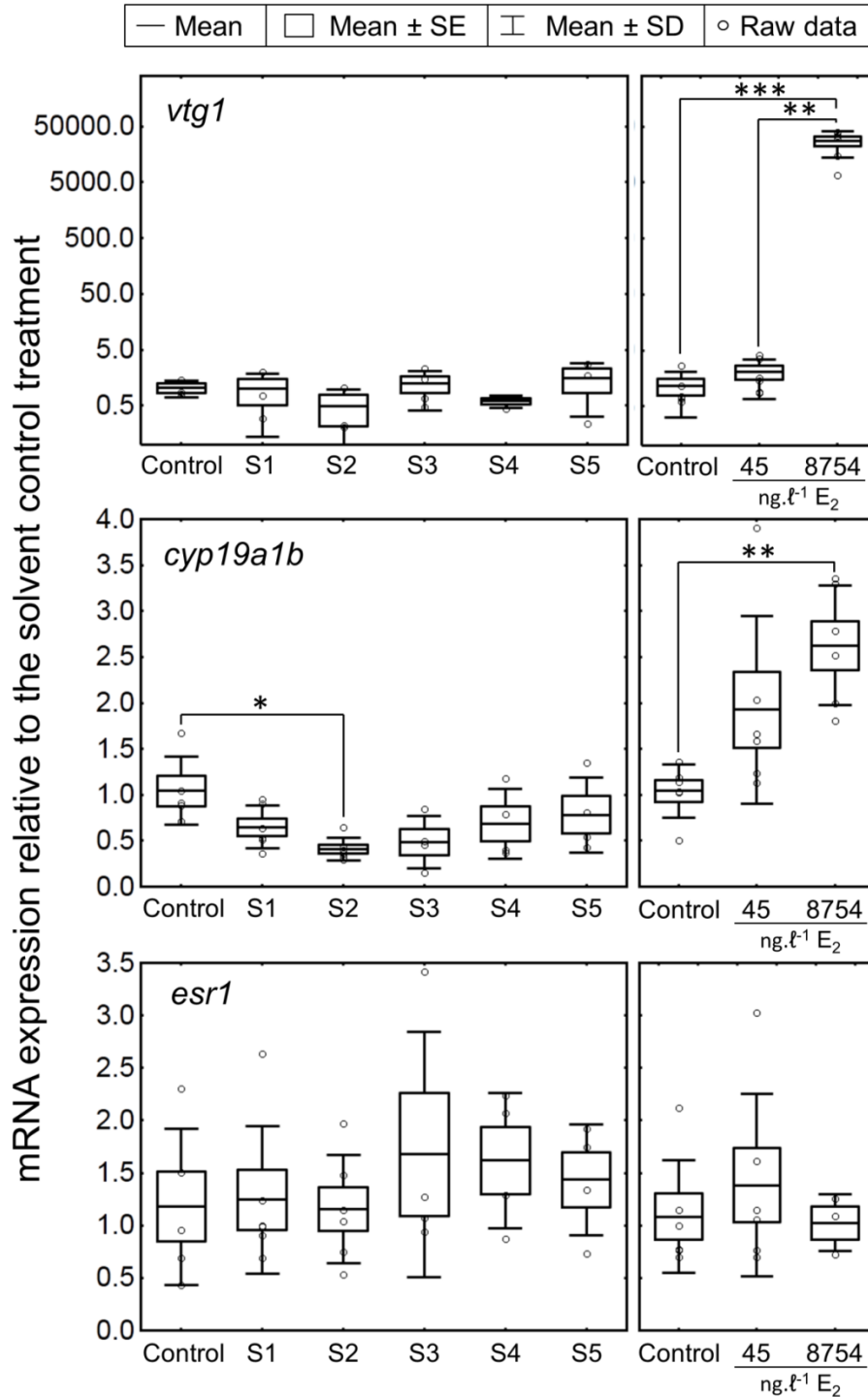


Figure 3.2: Expression of *vitellogenin 1* (*vtg1*), *aromatase* (*cyp19a1b*) and *estrogen receptor 1* (*esr1*) in juvenile *Oreochromis mossambicus* (24 dpf) exposed for 48 h to C18 solid phase extracts of water collected from five localities (S1 to S5) within the upper Olifants River catchment and a solvent control (0.001% DMSO). Significant differences are indicated with asterisks (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$).

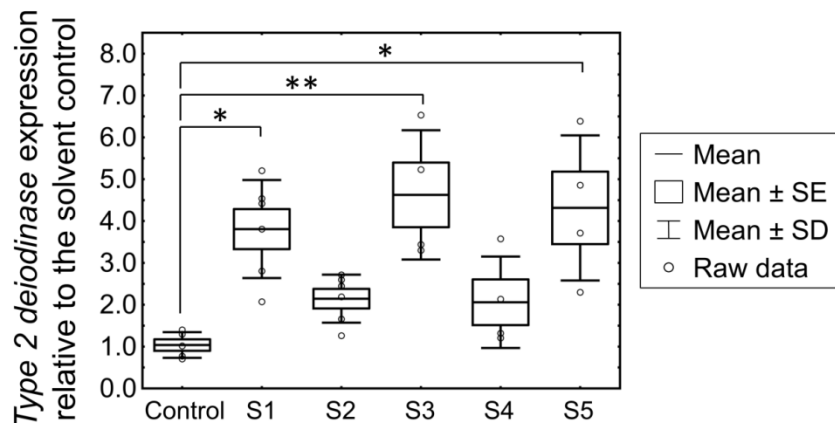


Figure 3.3: Expression of *type 2 iodothyronine deiodinase (dio2)* in juvenile *Oreochromis mossambicus* (24 dpf) exposed for 48 h to C18 solid phase extracts of water collected from five localities (S1 to S5) within the upper Olifants River catchment and a solvent control (0.001% DMSO). Significant differences are indicated with asterisks (* $P < 0.05$, ** $P < 0.001$).

3.3.4 Associations of bioassay data relative to land use

A PCA biplot indicates a positive correlation between E_2 and EE_2 levels, estrogenicity and anti-androgenicity, and an association between these variables and the WWTP impacted site 4 (Figure 3.4). In addition, the expression of *cyp19a1b* correlated with agricultural land use and site 4 associated with these parameters (Figure 3.4), whereas *dio2* expression associated more with natural land use and forestry, and sites 1, 3 and 5 (Figure 3.4).

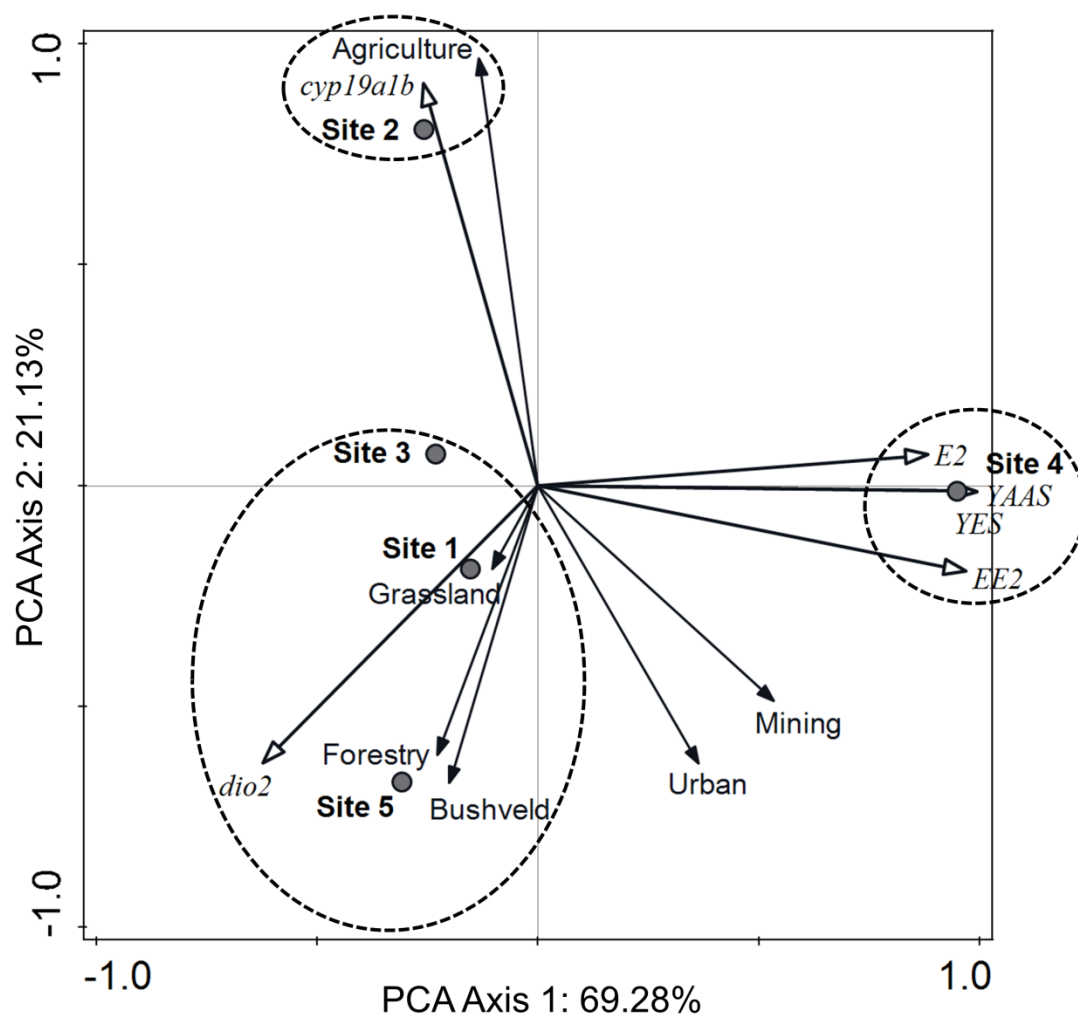


Figure 3.4: A principal component biplot indicating the association among five sampling locations within the upper Olifants River catchment with regard to the expression of *O. mossambicus* type 2 iodothyronine deiodinase (*dio2*) and aromatase (*cyp19a1b*) genes in combination with 17β -estradiol, ethinylestradiol concentrations, estrogenicity (YES) and anti-androgenicity (YAAS). The mean change in gene expression relative to a solvent control treatment group was applied for *dio2* and *cyp19a1b*. Land use types were treated as supplementary variables in the PCA.

3.4 Discussion

The present study describes E_2 and EE_2 loads together with *in vitro* determined (anti)estrogenic and (anti)androgenic activity in surface waters collected from a highly (anthropogenic) impacted river catchment in South Africa. Gene expression biomarkers in juvenile *O. mossambicus*, exposed *in vivo*, were furthermore applied to evaluate the endocrine disruptive potential of surface water collected from the studied river catchment.

Although E_2 and EE_2 were detected in water from all the sites sampled, as expected, water collected from the WWTP impacted site 4 (~120 m downstream of the WWTP outlet) contained the highest steroid estrogen loads. In particular, the E_2 and EE_2 loads recorded at site 4 during winter, spring and summer equalled or exceeded previous reported concentrations for surface waters (see Table 3.4) and even WWTP effluents (Pawlowski *et al.*, 2004; Swart and Pool, 2007).

The EE_2 detected at sites 1 and 2 impacted by agricultural activities may have originated from homesteads in the close vicinity of the river in those regions. Growth stimulators applied in cattle farming contain high concentrations of E_2 of which substantial concentrations are excreted by the supplemented animals and together with natural estrogens may end up in river systems (Khan *et al.*, 2008). Cattle and swine excrete more natural estrogens than humans, and have been shown to contribute significantly to steroid hormone contamination of water bodies (Johnson *et al.*, 2006). The E_2 detected in water collected from sites 1 and 2 may, therefore, originate from natural hormones excreted by human or animals, or livestock dosed with growth promoters.

The seasonal variation in steroid hormone concentrations may be related to river levels, being a result of a concentration effect. Although summer is the rainy season in the study region, 2011 was a dry year and the river levels were in fact lower during the summer sampling than autumn, winter and spring (South African Department of Water and Sanitation, 2015).

Table 3.4. Selected reports of steroid estrogen concentrations and estradiol equivalents (EEQs) measured in surface water globally.

Country	Detected range (ng.L ⁻¹)			Reference
	E ₂	EE ₂	EEQ	
Portugal	8.9 - 11.5	<3.15		Rocha <i>et al.</i> , 2013
France	1.4 - 3.2	1.1 - 2.9	0.30 - 4.52	Cargouet <i>et al.</i> , 2004
Germany	0.15 - 3.6	0.1 - 5.1		Kuch and Ballschmitter 2001
Netherlands	0.4	1	< 0.17	Vethaak <i>et al.</i> , 2005
Italy	0.11	0.04		Baronti <i>et al.</i> , 2000
South Korea	1.15 - 10.70	0.23 - 1.76	0.38 - 6.27	Ra <i>et al.</i> , 2011
Japan	2.6 - 14.7			Furuichi <i>et al.</i> , 2004
Israel		0.2 - 19.4		Barel-Cohen <i>et al.</i> , 2006
China	ND - 1.78	ND - 2.67	0.08 - 2.4	Jiang <i>et al.</i> , 2012
South Africa	ND - 30.8	ND - 10.83		Present study

The *in vitro* yeast screen data confirmed estrogenic and anti-androgenic activity at the WWTP impacted site 4 during winter, spring and summer, as well as androgenic activity during winter. The present study suggests that among the anthropogenic stressors represented (mining, agriculture, WWTP), WWTP effluents could be regarded as a major contributor to EDC contamination in the upper Olifants River. The WWTP located upstream of site 4 was audited in 2012, shortly after the samples applied in the present study were collected, and reportedly, the plant's operational capacity exceeded the design capacity by 43% at the time (South African Department of Water Affairs, 2012). The WWTP adjacent to site 4 has been shown to impact water quality (i.e., nitrate and phosphate levels) and modify algae assemblages for up to 40 km downstream, and possibly even further (Oberholster *et al.*, 2013). The present study provides further evidence of the detrimental impact of the Riverview WWTP on the Olifants River. A diversity of EDCs have previously been detected in the influents of WWTPs, including alkylphenols, phthalates, flame retardants such as polybrominated diphenyl ethers (PBDEs), polycyclic aromatic hydrocarbons (PAHs), pharmaceuticals, personal care products, polychlorinated biphenyls (PCBs) and other persistent organic pollutants (Deblonde *et al.*, 2011; Olujimi *et al.*, 2012). Although functional WWTPs can remove the majority of the organic (biologically active) contaminants during treatment (Deblonde *et al.*, 2011; Zhou *et al.*, 2012), poorly maintained plants may not

(Johnson *et al.*, 2007), resulting in the release of a diversity of organic contaminants into the aquatic environment, which is likely the case at the plant located upstream of site 4.

In a recent study, nine localities within the upper Olifants River catchment were screened for the presence of estrogenic activity using the T47D-KBluc mammalian cell reporter gene assay (Genthe *et al.*, 2013). Estrogenicity was observed in all nine localities investigated, with estradiol equivalent values ranging from 0.04 to 8.88 ng.L⁻¹, indicating the presence of estrogen agonists in the system. Only one of the localities sampled by Genthe *et al.* (2013) were sampled in the present study, site 5 located downstream of the Klein Olifants-Olifants confluence, and 1.42 ng.L⁻¹ EEQ was observed, whereas no activity was detected in the present study. The T47D-KBluc assay is more sensitive than the YES assay and further tests for estrogen receptor beta (ESR2) agonism, whereas YES is limited to ESR1 (Leusch *et al.*, 2010). Genthe *et al.* (2013) did not measure estrogenicity at the location described as “site 4” in the present study.

Disruption of the male hormone-, thyroid- and adrenal endocrine systems remains unexplored in the Olifants, and in reality very few studies investigating these targets of endocrine disruptors exists for African rivers (Barnhoorn *et al.*, 2010; Truter *et al.*, 2014). Anti-androgenic activity was detected in the samples representing all the sites apart from the “downstream” site 5. The most pronounced anti-androgenicity was observed during spring (Site 4 > Site 3 > Site 2). The anti-androgenic activity presently observed in the upper Olifants River (0 – 171.10 µg FEQ.L⁻¹) corresponds to the levels detected in other surface waters: United Kingdom, 5 – 250 µg FEQ.L⁻¹ (Grover *et al.*, 2011); Italy, 438.15 µg FEQ.L⁻¹ (Urbatzka *et al.*, 2007); China, 26 – 935 µg FEQ.L⁻¹ (Zhao *et al.*, 2011); South Africa (coastal waters) 0 – 596.94 µg FEQ.L⁻¹ (Truter *et al.*, 2015). A diversity of compounds have been identified as anti-androgenic including certain pesticides (Orton *et al.*, 2011; Archer and Van Wyk 2015), crude oil (Vrabie *et al.*, 2010), bisphenol A (Sohoni and Sumpter, 1998), flame retardants (Kojima *et al.*, 2009), phthalates (Takeuchi *et al.*, 2005) and PCBs (Takeuchi *et al.*, 2011). It is, however, impossible to establish which contaminants were responsible for the anti-androgenic activity observed in the present study based on the data available. An evaluation of pesticides, industrial chemicals and other pollutants such as PAHs will be of great value to assess the quality of the upper Olifants River.

Several compounds that inhibit AR mediated transcription in mammals, have similar action in fish (Ankley *et al.*, 2004; Wilson *et al.*, 2007). For example, *ar* expression is altered in the ovaries of female fathead minnows exposed to the pharmaceutical, flutamide (320 µg.L⁻¹) (Filby *et al.*, 2007). Similarly, in the tilapia species, *Sciaenochromis fryeri*, flutamide was shown to antagonise androgen receptor transactivation (Golan and Levavi-Sivan, 2014).

Although, in the present study, anti-androgenic activity was widespread in the upper Olifants River and at a potency of up to $171.1 \mu\text{g.L}^{-1}$ FEQ, no significant variation was observed among sampling locations in the expression of *ar1* in juvenile *O. mossambicus*. It is, however, known that anti-androgenic *in vitro* responses (based on human androgen receptor interaction) does not necessarily predict changes in androgen receptor gene expression in amphibians or fish (Hermelink *et al.*, 2010). Nonetheless, the results of the present study provide anecdotal evidence that *ar1* expression in whole body homogenates of juvenile *O. mossambicus* is not a sensitive biomarker for anti-androgenic activity in environmental water.

Reproductive abnormalities have been observed in fish exposed to E_2 and EE_2 concentrations as low as 8.7 ng.L^{-1} (Seki *et al.*, 2005) and 0.1 ng.L^{-1} , respectively (Zha *et al.*, 2008). Caldwell *et al.* (2012) described E_2 - and EE_2 “predicted no effect concentrations” (PNEC) for fish based on the literature available at the time and assigned concentrations of 2 ng.L^{-1} and 0.1 ng.L^{-1} to E_2 and EE_2 respectively. In the current investigation, all five localities exceeded the E_2 PNEC during summer, whereas site 4 exceeded this benchmark throughout the year, site 3 during autumn spring and summer, and sites 1 and 5 during spring and summer (Table 3.2). Moreover, EE_2 concentrations exceeded the PNEC throughout the year at site 4, during summer, autumn and spring at site 3, summer and autumn at sites 1 and 5, and in autumn at site 2 (Table 3.2). However, no significant changes in the expression of the estrogen responsive *vtg1*, *esr1* or *cyp19a1b* were observed in juvenile fish exposed to organic compounds extracted from the site 4 surface water, despite the 10.83 ng.L^{-1} EE_2 , 30.80 ng.L^{-1} E_2 and 43.01 ng.L^{-1} estradiol equivalents (yeast screen) observed for the exact same samples that the fish were exposed to. Similarly, exposure to 45 ng.L^{-1} E_2 did not induce significant changes in the aforementioned genes, and the present data, therefore, suggests that juvenile *O. mossambicus* exhibits a low sensitivity in terms of estrogen induced changes in *vtg1*, *esr1* or *cyp19a1b* expression. There are, however, potential confounding factors that may have contributed to the fact that no changes in *vtg1*, *esr1* or *cyp19a1b* expression were observed, including the specific strain of *O. mossambicus* that was used (strain-effect), or the fact that whole body homogenates were evaluated and not liver, gonad or brain tissue. Esterhuysen *et al.* (2009) reported a significant increase in *vtg1* expression in 20 dpf *O. mossambicus* exposed for 24 h to 500 ng.L^{-1} E_2 . The same dosage, however, had no effect after 12 h, whereas a higher dose ($1 \mu\text{g.L}^{-1}$) did. In a further study, a significant increase in plasma Vtg was observed in 35 dpf *O. mossambicus* exposed to a high dosage of the synthetic estrogen, diethylstilbestrol (DES) ($100 \mu\text{g.L}^{-1}$) for five days, whereas a lower dose (200 ng.L^{-1}) only had an effect after 10 days (Swart 2009). Exposure time, therefore, seems to be an important

determinant of sensitivity Vtg levels or *vtg1* expression as biomarker for estrogenicity (Takemura and Kim, 2001; Davis *et al.*, 2007). In the present study, the absence of significant variation in *vtg1* expression despite high environmental estrogen concentrations may, therefore, be due to the short exposure period (i.e., 48 h).

The age of the fish exposed in the present study may also have contributed to the low sensitivity observed in response to estrogen. Swart (2009) observed a stepwise increase of Vtg concentrations proportionate to increased age (15 to 50 dpf) in juvenile *O. mossambicus* exposed to DES. The fish in the present study were exposed at 22 dpf, and this age was chosen based on a previous study indicating that *vtg1* expression is upregulated in 20 dpf *O. mossambicus* exposed to 500 ng.L⁻¹ E₂ (Esterhuyse *et al.*, 2009).

The expression of *cyp19a1b* was significantly higher in 24 dpf *O. mossambicus* exposed to a high dosage of E₂ (i.e., 8,754 ± 419.57 ng.L⁻¹) corresponding to previous reports in the literature for the species (Tsai *et al.*, 2000), whereas a low dosage of 45.21 ± 6.59 ng.L⁻¹ did not induce a significant change in *cyp19a1b* expression (Figure 3.3). The reduced expression of *cyp19a1b* in fish exposed to organic extracts representing site 2, yet absence of anti-estrogenic activity *in vitro*, suggests that altered *cyp19a1b* expression was not associated with estrogen receptor antagonists.

Even though site 4 was characterized by estrogenic and anti-androgenic activity during summer, no significant variation in the expression of the estrogen responsive *vtg1*, *cyp19a1b* or *esr1*, as well as *ar1* was observed in the juvenile fish exposed to organic compounds extracted from site 4 surface water. As was the case in fish exposed to surface water extracts from the agriculture dominated site 2 in the present study, Truter *et al.* (2014) observed a significant decrease in *cyp19a1b* expression in juvenile *O. mossambicus* exposed to water collected from a site located downstream of agricultural lands and a WWTP. Decreases in *cyp19a1b* expression (i.e., brain localized aromatase gene) and aromatase inhibition in general have been linked with reproductive disorders in fish (Hecker *et al.*, 2007; Cheshenko *et al.*, 2008; Diotel *et al.*, 2010), and the results of the present study, therefore, suggests that the viability of fish communities in the proximity of site 2 may be compromised. Further investigation, such as histopathology of gonads, is, however, essential to assess the incidence and extent of reproductive disruption in fish populations (Hutchinson *et al.*, 2006). Various triazole and imadazole fungicides are known aromatase (translated in fish from *cyp19a1a* and *cyp19a1b* mRNAs) inhibitors (Ankley *et al.*, 2005; Hinfray *et al.*, 2006). The catchment area of site 2 is dominated by agricultural lands (Figure 3.4), and the *cyp19a1b* inhibition observed in fish exposed to organic extracts representing site 2 may, therefore, be linked to fungicide exposure. Further investigation is, however,

required, and, for example, compound-specific chemical analyses may provide insight regarding the potential aromatase inhibitors present in Olifants River waters.

In the present study, an increased expression of *dio2* (gene coding for DIO2, a critical counterpart in the regulation of thyroid action at central and peripheral level, Drigo *et al.*, 2013) was observed in fish exposed to organic extracts representing three of the five sites, suggesting a degree of thyroid disruptive potential. The exact cause of the increased *dio2* expression is, however, unknown, and no distinct association between *dio2* expression and any particular land use type was observed (Figure 3.4). Increased *dio2* expression was also reported for adult *O. mossambicus* inhabiting Loskop Dam, an impoundment located approximately 30 km downstream of the present site 5 (Truter *et al.*, 2016). However, this observation in Loskop Dam fish may have been related to metabolic disorders, and not short term exposure to chemicals (as in the present study), seeing that *dio2* expression was unchanged juvenile *O. mossambicus* exposed to water from the dam for 48 h (Truter *et al.*, 2016). Various compounds have been shown to affect *dio2* expression such as certain PCBs (Song *et al.*, 2013), pesticides (Yu *et al.*, 2013) and flame retardants (Noyes *et al.*, 2013). Moreover, Pickard-Aitken *et al.* (2007) reported higher *dio2* expression in walleye, *Sander vitreus* captured downstream of a paper mill and WWTP outfall relative to fish captured from an upstream reference and downstream location. Chemical screening for known thyroid disruptors in combination with thyroid histopathology in fish captured from upper Olifants River will be of value and help to determine the cause and extent of thyroid disruption in the system.

3.5 Conclusions

This study provides evidence of the nature of endocrine disruption associated with different land use areas in a river catchment subject to a diversity of anthropogenic stressors. Steroid estrogen concentrations exceeding the predicted no effect concentration for fish reproduction were detected at all sites sampled during at least one of the four seasons. *In vitro* screens for (anti)estrogenicity and (anti)androgenicity indicated that of all the land use regions represented, a site located in the proximity of a WWTP in an urban area as the most impacted. In contrast, the *in vivo* gene expression based biomarkers suggested water from the aforementioned WWTP impacted location would not cause endocrine disruption. These biomarkers, however, indicated the potential for disruption of aromatase (an enzyme involved with steroidogenesis) in a location within agricultural lands, and type 2 deiodinase (linked to the thyroid cascade) disruption at three locations, yet with no distinct land use type as potential source. The lack of significant changes in *vtg1* and *esr1* expression in fish representing WWTP impacted sites is surprising, because of the high estrogen

concentrations present. Age and the time of exposure can affect the sensitivity of *O. mossambicus* to estrogen exposure, and the age of fish (22 dpf) and duration of exposure (48 h) in the present study may have contributed to the lack of significant changes in the expression of genes linked to the reproductive system. Although the *in vitro* assays predicted modulation of the reproductive system for certain locations, the gene expression based biomarkers in juvenile *O. mossambicus* did not. The sole use of either *in vitro* hormone receptor screens or *in vivo* gene expression biomarkers screens would, therefore, have given different estimates of risk at the locations tested. The present study, therefore, advocates the need for the use of a combination of *in vitro* and *in vivo* bioassays to screen surface waters to assess endocrine disruptive potential for risk assessments.

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3.7 Supporting information

Table S3.1: The intra- and inter-assay coefficients of variance observed in the present study for the Yeast Estrogen Screen (YES), Yeast Anti-Estrogen Screen (YAES), Yeast Androgen Screen (YAS) and Yeast Anti-Androgen Screen (YAAS).

Test	Sample ¹	Coefficient of variance (%)	
		Intra-assay	Inter-assay
YES	1.25 x 10 ⁻⁹ M E ₂	3.79	2.55
YAES	1.25 x 10 ⁻⁷ M TAM	2.94	8.63
YAS	3.13 x 10 ⁻⁹ M DHT	3.69	6.85
YAAS	3.13 x 10 ⁻⁶ M FLU	1.95	5.37

¹Compound and concentration evaluated within and among assay plates.

Table S3.2: Primer sequences in the 5' to 3' direction applied for RT-qPCR.

Target	Primer Sequence	Ta (°C)	Source	PCR efficiency (%) ¹
<i>thra</i>	F: GCTCAGGGCTCACAGTGGAA R: AACGACACGGGTGATGGC	63.5	Shiao <i>et al.</i> , 2007	102.57±1.12
<i>thrb</i>	F: AATGTGTTATTGACAAAGTG R: GATCGGATGAAAGCAGGATA	63.5	Shiao <i>et al.</i> , 2007	100.07±3.74
<i>dio2</i>	F: TACAACAGAGAAAGATTGCCTACC R: TTCAAGACTCCTACCGTTTACCA	57	Truter <i>et al.</i> , 2014	94.72±6.41
<i>gr1</i>	F: CCAGCAAGCGCAAAATAACA R: GAAAATGAAAGGAAAGGGAGATCT	63.5	Aruna <i>et al.</i> , 2012	101.11±3.56
<i>mr</i>	F: TGGTACGCATGGTCAAATGG R: TCAGGGTGATTTGGTCCTCAAT	65	Aruna <i>et al.</i> , 2012	104.64±0.48
<i>ar1</i>	F: CTATCAAGAGTGGGCCTTCGG R: GCGCCTTAAACTGCGATCTG	65	Ijiri <i>et al.</i> , 2008	102.79±1.49
<i>esr1</i>	F: TGGTACGCATGGTCAAATGG R: TCAGGGTGATTTGGTCCTCAAT	62.5	AM284390.1 ²	98.22±4.71
<i>vtg1</i>	F: TATGGAGGCTGTGCTAG R: CGGTGAACATAGGTGTATCT	62.5	AJ889835.1 ²	99.22±1.86
<i>cyp19a1b</i>	F: GAGCGTCAGAAGTCACTGC R: GCTCAAATCAGGGTCTCCC	60	Esterhuysen <i>et al.</i> , 2008	99.36±3.47
<i>actb</i>	F: TGTGATGGTGGGTATGGG R: CTGTGGTGGTGAAGGAGTAG	63.5	Esterhuysen <i>et al.</i> , 2008	93.64±3.31

¹Efficiency (E) was calculated per PCR plate based on a serial dilution containing at least four concentrations of juvenile *O. mossambicus* cDNA. The error in E calculated among assay plates is expressed as standard deviation.

²Genbank accession number

Table S3.3: Land use contribution of the localities investigated. The data was obtained from Oberholster *et al.* (2012).

Locality	Land use contribution (%)					
	Agriculture	Bushveld	Forestry	Grassland	Mining	Urban
Site 1	43.0	0.0	0.0	57.0	0.0	<0.1
Site 2	48.0	0.1	0.1	51.6	0.2	<0.1
Site 3	43.1	<0.1	<0.1	50.6	3.8	1.0
Site 4	42.9	0.2	0.1	51.6	5.4	1.3
Site 5	40.9	1.2	0.6	51.5	4.4	1.5

3.7.1 References

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Chapter 4: The expression of selected genes linked to metabolic homeostasis in obese pansteatitis-suffering Mozambique tilapia, *Oreochromis mossambicus* (Peters)

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Declaration by the candidate

With regard to Chapter 4, the nature and scope of my contribution were as follows:

Nature of contribution Extent of contribution (%)

Nature of contribution	Extent of contribution
Conceptual design, experimental work, manuscript writing.	70%

The following co-authors have contributed to Chapter 4:

Name	Email address and institutional affiliation	Nature of contribution	Extent of contribution
Prof JH van Wyk	jhvw@sun.ac.za Department of Botany and Zoology, Stellenbosch University	Conceptual design, manuscript editing.	30%
Dr PJ Oberholster	poberholster@csir.co.za Natural Resources and the Environment, CSIR	Conceptual design, algae identification, manuscript editing.	
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Abstract

The *Oreochromis mossambicus* (Peters) population inhabiting Loskop Dam, South Africa is characterized by a high incidence of obesity and pansteatitis. Potential links between the impaired health of Loskop Dam *O. mossambicus* and the endocrine system were investigated by assessing the expression of selected genes associated with the thyroid and adrenal endocrine axes as well as *peroxisome proliferator-activated receptor gamma* (*pparg*). Moreover, contaminant induced thyroid and/or metabolic modulation in Loskop Dam water was evaluated using juvenile *O. mossambicus* in laboratory exposures. The expression of *thyroid hormone receptor alpha* (*thra*) and *type 2 deiodinase* (*dio2*) was higher in Loskop *O. mossambicus* than fish from another population, suggesting a degree of thyroid disruption. The altered gene expression may be a consequence, rather than cause of obesity. Expression of *dio2* and *pparg* was higher in juvenile *O. mossambicus* exposed to un-filtered compared to filtered dam water, and my data suggests fasting as causative factor. Micro-organism abundance can, therefore, be a confounding factor in studies applying molecular markers to test for thyroid modulation by environmental waters. Pansteatitis was not a significant source of variance in the expression of any of the genes investigated, suggesting that the disease is not associated with disrupted endocrine signalling.

4.1 Introduction

The hypothalamus pituitary thyroid (HPT) and hypothalamus pituitary adrenal (HPA) endocrine axes are critical counterparts in the maintenance of ionic-, thermal and energy homeostasis in vertebrates. Thyroid- and corticosteroid hormone action occurs predominantly via hormone receptor mediated transcriptional activation in peripheral tissues (Stahn and Buttgerit, 2008; Cheng *et al.*, 2010). Energy balance regulation is likely the most eminent cooperative function of the HPT and HPA axes, even though this cooperation occurs through independent action.

Various compounds are known to potentially modulate the vertebrate thyroid and adrenal systems including pesticides, industrial chemicals, metals and pharmaceuticals, of which many are present in the aquatic environment (Brown *et al.*, 2004; Odermatt and Gumy, 2008). Metabolism as target of endocrine disrupting compounds (EDCs) has received increased attention in recent years, partly due to the global proliferation of obesity and associated metabolism related disorders such as type-2 diabetes in humans (Newbold 2010; Casals-Casas and Desvergne, 2011; Migliarini *et al.*, 2011; Regnier and Sargis, 2014). For example, certain EDCs such as tributyltin (TBT), perfluoro alkyl compounds (PFCs) and bisphenol A have been shown to drive a positive energy balance and have hence been

classified as obesogens (Grün *et al.*, 2006; Grün and Blumberg, 2009). Exogenous metabolism modulators may act directly by targeting biochemical pathways, or through epigenetic modification (Newbold, 2010; Janesick and Blumberg, 2012; Regnier and Sargis, 2014). Peroxisome proliferator-activated receptor gamma (Pparg) is recognised as one of the principal targets of adipogenesis modulation, since various EDCs have been shown to be agonists of the said transcription factor (Hurst and Waxman, 2003; Pereira-Fernandes *et al.*, 2013; Regnier and Sargis, 2014). Glucocorticoid receptor activation is a further important potential mechanism of EDC mediated adipogenesis, yet less well-studied than Pparg (Sargis *et al.*, 2010).

A number of researchers have investigated links between environmental chemical exposure and metabolic disorders, through mammalian models or human epidemiological studies (Casals-Casas and Desvergne, 2011; Jin *et al.*, 2014; Regnier and Sargis, 2014). Very little is, however, known regarding metabolic disruption in wildlife, such as fish inhabiting waters contaminated by anthropogenic activity. Studies evaluating the potential of environmental waters to disrupt metabolism in model organisms exposed *in vivo* may provide valuable insight regarding the pathology of metabolic disorders and links with pollutants in the environment.

The Olifants River is an important trans-boundary southern African river, subject to anthropogenic impacts associated with the mining, agriculture, manufacturing and coal fired power generation industries as well as urbanized regions (Dabrowski and de Klerk, 2013). The upper catchment is likely the most pronounced source of contamination to the greater Olifants River (Oberholster *et al.*, 2012a). Loskop Dam is the second largest impoundment in the Olifants River and is located on the border of the upper and middle catchments. A high proportion of Loskop Dam Mozambique tilapia *Oreochromis mossambicus* (Peters, 1852) are obese (Oberholster *et al.*, 2012a), and elevated levels of serum cholesterol and triglycerides have been observed compared to fish from a reference dam (Flag Boshielo Dam) within the same watercourse, approximately 90 km downstream (Oberholster *et al.*, 2012a). In addition, individuals from the Loskop Dam *O. mossambicus* population have been reported to suffer from pansteatitis (Oberholster *et al.*, 2012b), a health disorder associated with oxidative damage of fat tissue, characterized by the deposition of ceroid pigment and is consequently referred to as “yellow fat disease” (Niza *et al.*, 2003; Huchzermeyer *et al.*, 2011). Mozambique tilapia (*O. mossambicus*) specimens collected from Loskop Dam were found to be severely affected, showing evidence of pansteatitis within the mesenteric fat as well as the brain cavity and eye sockets (J.W. Luus-Powell, unpublished data). It has been suggested that pansteatitis is caused by a vitamin E deficiency, in most cases, as a

consequence of high dietary levels of polyunsaturated fats (Fytianou *et al.*, 2006) and/or oxidized (rancid) fat intake (Huchzermeyer, 2003). However, it has been postulated that pansteatitis pathogenesis may also be linked to contaminants such as cyanotoxins (Rattner and McGowan 2007; Neagari *et al.*, 2011), polychlorinated biphenyls (PCBs) (Oros *et al.*, 2013) and metals (Oberholster *et al.*, 2012b). The aforementioned studies, however, did not provide conclusive evidence and simply speculated a causative link between contaminant exposure and pansteatitis. The fact that cyanotoxins, PCBs and certain metals are known to cause lipid peroxidation (Waraho *et al.*, 2011; Paskerova *et al.*, 2012) provides support for a contaminant induced pansteatitis hypothesis.

One of the most significant pansteatitis-linked mortality events observed to date was the death of approximately 275 Nile crocodiles, *Crocodylus niloticus* Laurenti, from 2008 to 2009 in the Olifants River gorge within the Kruger National Park (KNP), in South Africa (Ferreira and Pienaar 2011). The uncertainty regarding the cause of pansteatitis in the crocodiles led to the examination of local fish, which uncovered a high prevalence of pansteatitis in the sharp tooth catfish, *Clarias gariepinus* (Burchell), population (Huchzermeyer *et al.*, 2011; Huchzermeyer, 2012).

Loskop Dam is located approximately 530 km upstream of the region in the KNP where the reported crocodile deaths occurred. Interestingly, in Loskop Dam, pansteatitis is not limited to *O. mossambicus*, but has also been observed in *C. niloticus* (Botha *et al.*, 2011), serrated hinged terrapins, *Pelusios sinuatus* (Smith), (Oberholster *et al.*, 2012b) and red nose labeo, *Labeo rosae* Steindachner, (J.W. Luus-Powell, unpublished data). Botha *et al.* (2011) speculated that the pansteatitis observed in the Loskop Dam crocodiles (*C. niloticus*) was associated with the consumption of rancid fish after a number of fish die-off events. Although numerous reports of pansteatitis in farmed fish exists (Roberts and Richards, 1978; Goodwin, 2006; Roberts and Agius, 2008), reports of pansteatitis infected wild fish populations such as has been observed in Loskop Dam and in the KNP are extremely limited in the literature and the only record we could locate was for the common dab, *Limanda limanda* (Linnaeus), captured from the North Sea (Bruno *et al.*, 1991; Begg *et al.*, 2000) and the long rough dab, *Hippoglossoides platessoides* (Fabricius) (Begg *et al.*, 2000).

Although energy homeostasis (including adipogenesis) is regulated in peripheral tissues, these processes are also centrally controlled (Bantubungi *et al.*, 2012). The aims of the current investigation were firstly: to evaluate the expression of selected thyroid- and corticosteroid-linked genes as well as an adipogenesis-linked gene, *pparg* in brain tissue of adult *O. mossambicus* collected from Loskop Dam representing a population characterized by a high prevalence of obesity and pansteatitis in comparison to individuals from non-obese

pansteatitis-free populations. The genes investigated included *thyroid hormone receptor alpha* (*thra*), *thyroid hormone receptor beta* (*thrb*), *type 2 iodothyronine deiodinase* (*dio2*), *thyroid stimulating hormone beta* (*tshb*), *glucocorticoid receptor-1* (*gr1*), *gr2*, *mineralocorticoid receptor* (*mr*) and the adipogenesis-linked *pparg*. Secondly, the expression of *thra*, *thrb*, *dio2* and *pparg* was quantified in juvenile *O. mossambicus* after short-term laboratory exposure to water collected from three localities within Loskop Dam. Thirdly, the association between pansteatitis incidence and thyroid- and interrenal signalling was evaluated by comparing the expression of *thrb*, *thrb*, *dio2*, *tshb*, *pparg*, *gr1*, *gr2* and *mr* among *O. mossambicus* diagnosed with pansteatitis and individuals with no visible sign of the disease within the Loskop Dam population.

4.2 Materials and Methods

4.2.1 Fish populations

Loskop Dam is located within the Loskop Nature Reserve in Mpumalanga Province, South Africa (Figure 4.1). Coal mines, agriculture, steel smelters, waste water treatment plants and abandoned mines are likely the most significant contaminant vectors of Loskop Dam via the upper Olifants River (Dabrowski and de Klerk, 2013). Loskop Dam is of commercial importance and supplies irrigation water to approximately 25 600 hectares of agricultural lands of which a considerable portion of the produce is sourced to foreign markets (Oberholster and Botha, 2011; de Lange *et al.*, 2012). The dam is an esteemed angling destination, partly due to the large *O. mossambicus* present; in fact, the current world record *O. mossambicus* was captured in the dam (IGFA, 2014).

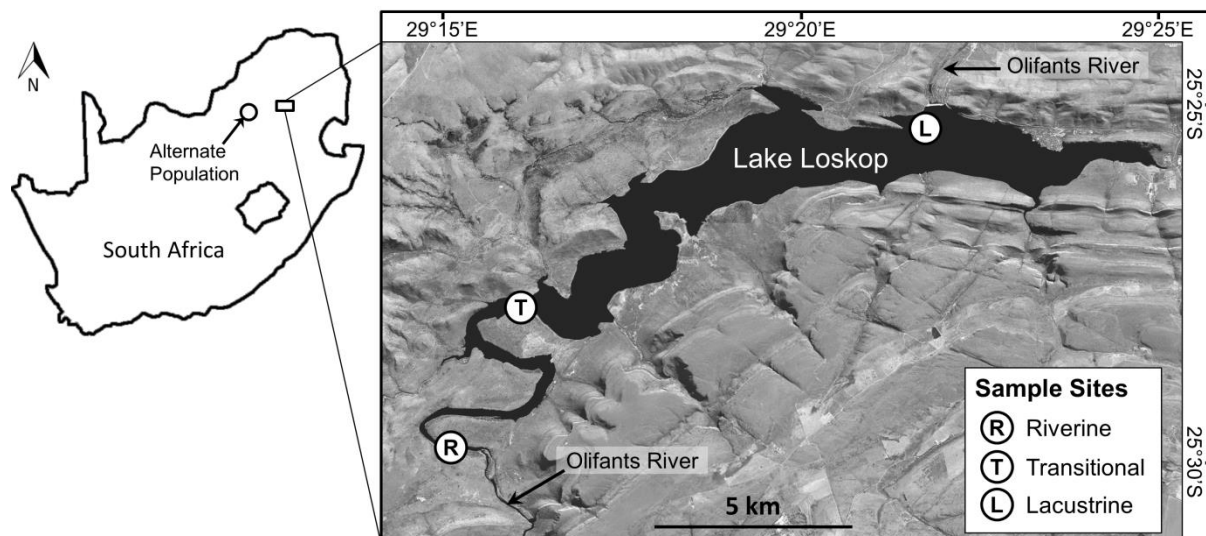


Figure 4.1: Loskop Dam and its position within South Africa, as well as the location of an alternate population from which adult *Oreochromis mossambicus* were collected. The three localities within Loskop Dam from where water applied in fish exposures and chemical analysis was collected are indicated. Map credits: Google Inc. Alternate population location: S25 37 54.7 E 27 39 58.8.

An alternative population of *O. mossambicus* inhabiting small dams on a farm approximately 200 km east of Loskop Dam were also studied for comparative purposes (Figure 4.1). Neither obesity nor pancreatitis have been observed in the said population (Figure 4.2). The Loskop Dam and alternative population occur within the same climatic and bioclimatic regions (Conradie, 2012). The farm has two subdivisions of dams supplied, respectively, with water from a borehole and from Hartbeespoort Dam via an irrigation canal. Hartbeespoort Dam is known to be impacted by agricultural run-off, waste water treatment plant effluents, domestic waste from informal settlements, mine water from its upper catchment (Greichus *et al.*, 1977) and furthermore contains high numbers of the cyanotoxin releasing cyanobacterium, *Microcystis aeruginosa* (Kützing, 1846) (Oberholster and Botha, 2010; Ballot *et al.*, 2014). The fish captured from the Hartbeespoort Dam canal-supplied dams are, therefore, likely to be exposed to contaminants albeit being approximately 28 km downstream of the dam.

4.2.2 Adult fish collection

Adult *O. mossambicus* were obtained from Loskop Dam (Figure 4.1) on the 6th of April 2013 during an annual angling event. The organising committee requested live fish to be entered,

and anglers, therefore, maintained the fish in dam water until they were brought on land. The fish that could not be dissected immediately were kept in a tank containing approximately 500 L of tap water.

Adult *O. mossambicus* from the alternative population were captured (Figure 4.1) using dragnets (4 cm mesh size). The nets were reeled in from the land and fish were then immediately transferred to a 500 L tank containing tap water. The fish of both Loskop Dam and the alternative population were mixed sexes and the Loskop Dam fish were on average slightly larger (standard lengths: Loskop Dam, 33.29 ± 4.10 cm; borehole-supplied dams, 29.55 ± 2.98 cm irrigation-canal-supplied dams, 28.25 ± 23.54 cm).

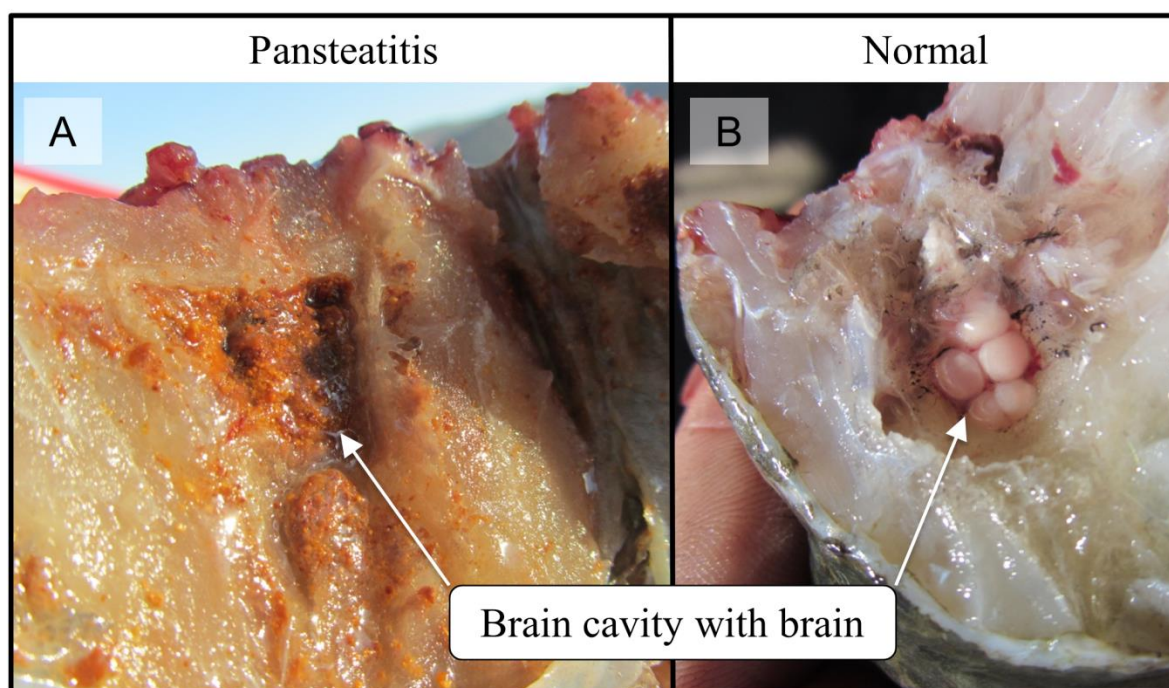


Figure 4.2: Examples of the brain cavities of pansteatitis suffering *Oreochromis mossambicus* captured from Loskop Dam (A) and pansteatitis free fish from an alternative population (B).

All the fish were killed through decapitation on the day of capture and dissected immediately. The Loskop Dam fish were processed between 15h00 and 19h00, borehole-supplied dam fish between 14h00 to 19h00 and irrigation-canal-supplied water fish 18h00 and 22h00. Whole brains were carefully removed and placed in 1 mL TriReagent (Sigma, ZA) on ice (Loskop Dam: $N = 10$; Irrigation-canal-supplied dams: $N = 8$; Borehole-supplied dams: $N = 8$). The gender of each fish was determined through gross morphological investigation of the gonads. Pansteatitis lesions were identified macroscopically in the mesenteric fat. The body

mass and standard length (snout-to-fork) of each fish was recorded and applied to calculate Fulton's Condition Factor ($CF = W/L^3$) (Ricker, 1975).

4.2.3 Juvenile fish exposures

4.2.3.1 Water collection

Water was collected from the lacustrine, transitional and riverine zones of Loskop Dam (Figure 4.1) on the 8th of December 2012. The samples were collected from the photic zone (1.5 m integrated sample), and transported to the laboratory in acid cleaned PTFE capped amber glass bottles, kept on ice packs or ice.

4.2.3.2 Exposures

Each locality was represented by three exposure groups namely: (1) water as collected from the dam (containing algae and other micro-organisms); (2) filtered water (1.2 μm glass fibre MGC filters [Munktell, DE]); (3) filtered water containing 50 $\mu\text{g}\cdot\text{L}^{-1}$ triiodothyronine (Sigma, ZA). Juvenile *O. mossambicus* (13 days post fertilization [dpf]) were obtained from a single breeding pair (Rivendell Hatchery, Grahamstown, ZA) prior to the exposure, and maintained in buffered reverse osmosis (RO) water (containing 250 mg marine salt, 80 mg NaHCO_3 per litre) at 28 ± 1 °C subject to a 14h:10h light:dark cycle. Seven fish (30 dpf) were assigned per treatment group representing the three localities as well as a buffered RO water (pH 7) negative control. The fish were exposed to 800 mL of liquid in 1 L glass containers for 48 h, without food. All the fish were acclimatised for at least 48 h prior to the exposure to similar containers and volumes as applied during exposures, and fed crushed tilapia pellets (AquaNutro, ZA) twice a day. The fish were euthanized in 0.1% benzocaine at exposure termination and either transferred to TriReagent (Sigma, ZA) or snap frozen and stored at -80 °C. The fish exposures were approved by the Stellenbosch University Committee for Animal Care and Use (Protocol No. SU-ACUM12-00036).

4.2.4 Chemical analysis

Aliquots (50 mL) of the water collected from Loskop Dam as well as an ultrapure water blank were 1.2 μm glass-microfibre filtered and applied for chemical analysis. The concentrations of Ca, K, Mg, Na, P and Si were measured using a Thermo ICAP 6300 ICP-AES (Thermo Scientific, USA), and Al, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, V, Zn using an Agilent 7700x ICP-MS (Agilent Technologies, USA) by the Stellenbosch University Central Analytical Facility.

4.2.5 Phytoplankton identification

The phytoplankton was identified in each of the samples collected from Loskop Dam in order to evaluate the potential influence of the phytoplankton assemblage and abundance on *thra*, *thrb*, *dio2* and *pparg* expression in the juvenile fish exposed to the unfiltered water samples. Aliquots (50 mL) of the water collected from Loskop Dam were applied for phytoplankton identification using a compound microscope at 1250x magnification (Van Vuuren *et al.*, 2006). The samples were analysed using the strip-count method, after being sedimented in an algae chamber (APHA, AWWA and WPCF 1992).

4.2.6 RNA isolation and cDNA synthesis

Juvenile fish and brain tissue were homogenized in TriReagent (Sigma, ZA) using an ultrasound sonicator (Omni-ruptor 400, Omni International Inc., USA). Total RNA was isolated according to the TriReagent technical bulletin. RNA integrity was assessed spectrophotometrically using a Nanodrop (Thermo Scientific, USA) and through agarose gel electrophoresis. The RNA of suitable integrity was subsequently DNase I (Sigma, ZA) treated. Complementary DNA (cDNA) was synthesized from brain RNA using Enhanced Avian HSRT kits (Sigma, ZA) (1 µg RNA per 10 µL reaction volume) and juvenile whole body homogenates using Maxima H-minus cDNA synthesis kits (Thermo Scientific, USA) (2 µg RNA per 10 µL reaction volume) according to the manufacturers' instructions.

4.2.7 RT-qPCR

Messenger RNA expression of *thra*, *thrb*, *dio2*, *tshb*, *gr1*, *gr2*, *mr* and *pparg* with *actin beta* (*actb*) as reference gene was evaluated using real-time RT-qPCR. The PCRs were performed as 15 µL reactions containing 2 µL cDNA (whole body homogenate samples, 10 ng per reaction; brain samples, 20 ng per reaction for *dio2* and 5 ng per reaction for the other genes), 7.5 µL Jumpstart® SYBRgreen mix (Sigma, ZA), 0.33 µM of each primer and nuclease free water. The PCR programs for all primer pairs included an enzyme activation step at 95 °C (9 minutes), followed by 40 cycles of denaturing at 95 °C (15 seconds), annealing at 57 - 63.5 °C (Table 4.1) (30 seconds) and elongation at 72 °C (45 seconds). Each PCR plate contained an internal non-template control (no cDNA) as well as a five point two-fold serial dilution, "theoretical" cDNA concentrations ranging from 80 ng to 5 ng for *dio2* and 25 ng to 1.56 ng for the other genes, assuming 1 µg of RNA yielded 1 µg of cDNA during the cDNA synthesis reactions. All samples and controls were run in triplicate and dissociation curves were applied to confirm single-fragment amplification. Gene expression was quantified using the Pfaffl method (Pfaffl, 2001). Amplification efficiencies were

determined for each primer pair per PCR programme. Outliers were identified per treatment group with the Grubbs' test and removed (Burns *et al.*, 2005).

Primers for *dio2*, *tshb* and *pparg* were designed against the appropriate Genbank sequence of *Siganus guttatus* (Bloch) (Table 4.1), using PRIMER 3 (Rozen and Skaletsky, 2000). The *dio2*, *tshb* and *pparg* primers' PCR products were sequenced by the Stellenbosch University Central Analytical Facility to confirm primer validity.

Table 4.1: Primer sequences applied in the current investigation, displayed in the 5' to 3' direction.

Target	Primer Sequence	Ta (°C)	Source
<i>thra</i>	F: GCTCAGGGCTCACAGTGGAA R: AACGACACGGGTGATGGC	63.5	Shiao <i>et al.</i> , 2008
<i>thrb</i>	F: AATGTGTTATTGACAAAGTG R: GATCGGATGAAAGCAGGATA	63.5	Shiao <i>et al.</i> , 2008
<i>dio2</i>	F: TACAACAGAGAAAGATTGCCTACC R: TTCAAGACTCCTACCGTTTACCA	57	GU372962.1 ¹
<i>tshb</i>	F: AGGGACAGCAACATGAGGGGA R: GGACAGCCAGGCAGAATAGC	59.3	XM003453648.1 ¹
<i>gr1</i>	F: CCAGCAAGCGCAAATAACA R: GAAAATGAAAGGAAAGGGAGATCT	63.5	Aruna <i>et al.</i> , 2012
<i>gr2</i>	F: CCAGCAAGCGCAAATAACA R: GAAAATGAAAGGAAAGGGAGATCT	60	Aruna <i>et al.</i> , 2012
<i>mr</i>	F: TGGTACGCATGGTGAAATGG R: TCAGGGTGATTTGGTCCTCAAT	65	Aruna <i>et al.</i> , 2012
<i>pparg</i>	F: TGCGAGGGCTGTAAGGGTTT R: ACTTGTTGCGGGACTTCTTGTG	59	AY590304.1 ¹
<i>actb</i>	F: TGTGATGGTGGGTATGGG R: CTGTGGTGGTGAAGGAGTAG	63.5	Esterhuyse <i>et al.</i> , 2008

¹Genbank accession number

4.2.8 Statistical analysis

Normality and homogeneity of variance was assessed in the data using the respective Shapiro-Wilk's *W* test, normal probability plots and Levene's test. The Student's *t*-test and Man-Whitney *U*-test were applied for pairwise comparisons in gene specific mRNA abundance. The Variance Estimation and Precision (VEPAC) module was applied for Generalized Linear Model Analysis of Covariance (GLM ANCOVA) in combination with the LSD *post hoc* test (Statistica 11, Statsoft, USA). Probability values (*P*) of less than 0.05 were accepted as significant.

4.3 Results

4.3.1 Adult fish

The effect of condition factor (CF), gender, locality and the gender-locality interaction on the expression of *thra*, *thrb*, *dio2*, *tshb*, *mr*, *gr1*, *gr2* and *pparg* observed in the Loskop Dam fish and fish from the other dams were evaluated using GLM ANCOVA. No significant association between CF and the expression of any of the genes investigated were observed. Gender was, however, a significant source of variation in the expression of *thrb* and *gr2*, in both cases being higher in males than females (Table 4.2). The sample sizes were, however, small for the gender analysis, and the results, therefore, anecdotal. In addition, *thra* varied significantly among localities (Table 4.2a). In particular, *thra* expression was significantly higher in Loskop Dam fish than both the borehole ($P = 0.01$) and irrigation-canal fish ($P < 0.01$) (Figure 4.3). Although there was a clear trend for higher *dio2* expression in both the Loskop Dam and irrigation-canal water fish relative to those collected from borehole- water supplied dams (Figure 4.3), the GLM ANCOVA indicated no significant effect by CF, gender, locality and the gender-locality interaction (Table 4.2a). However, when the variation of *dio2* expression was re-analysed using a one way ANOVA, location was indicated as a significant source of variation and *dio2* expression was significantly lower in fish captured from dams supplied by borehole water than both the irrigation-canal water fish ($P = 0.02$) and Loskop Dam fish ($P = 0.01$) (Figure 4.3).

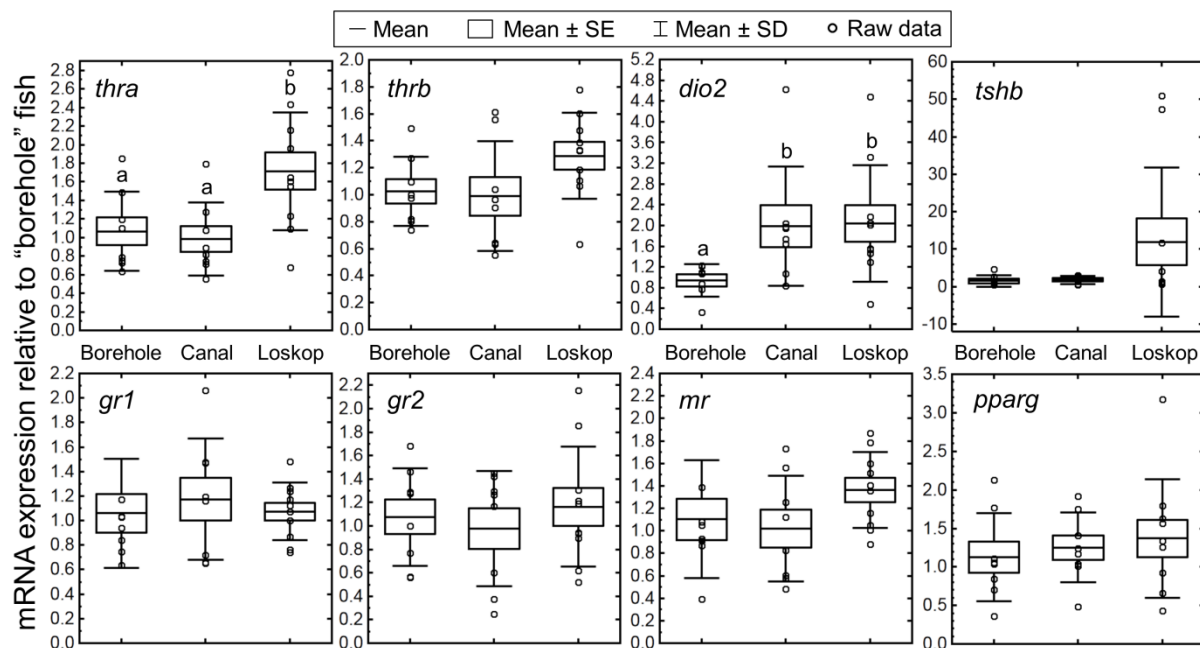


Figure 4.3: The mRNA expression of *thra*, *thrb*, *dio2*, *tshb*, *mr*, *gr1*, *gr2* and *pparg* in the (total) brain tissue of adult *Oreochromis mossambicus* captured from Loskop Dam ($N = 10$)

and reference fish maintained in small dams supplied by borehole water ($N = 8$) and irrigation canal water ($N = 8$), respectively. Dissimilar characters indicate statistically significant differences (Fisher's LSD *post hoc* test, $\alpha = 0.05$).

Condition factor (CF) varied significantly among the localities ($F_{2,20} = 13.15$; $P < 0.01$), and was significantly higher in the Loskop Dam fish ($38.0 \pm 4.7 \text{ mg.cm}^{-3}$, mean \pm SD) than both the borehole ($31.0 \pm 2.4 \text{ mg.cm}^{-3}$) ($P < 0.01$) and irrigation-canal water ($30.0 \pm 5.7 \text{ mg.cm}^{-3}$) ($P < 0.01$) fish.

Table 4.2: Generalized Linear Model Analysis of Covariance (GLM ANCOVA) of the expression of (a) *thra*, *thrb* and *dio2*, (b) *pparg* and *tshb*, (c) *mr*, *gr1* and *gr2* in the (total) brain tissue of adult *Oreochromis mossambicus* captured from Loskop Dam ($N = 10$) and reference fish maintained in small dams supplied by borehole water ($N = 8$) and irrigation canal water ($N = 8$), respectively. Fulton's condition factor was applied as covariate. Asterisks indicate statistically significant effects.

a)

Source of Variance	df	<i>thra</i>		<i>thrb</i>		df	<i>dio2</i>	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>		<i>F</i>	<i>P</i>
Condition Factor	1,19	1.24	0.28	0.00	0.95	1,18	0.18	0.68
Locality	2,19	4.80	0.02*	0.62	0.55	2,18	2.01	0.16
Gender	1,19	0.04	0.85	4.92	0.04*	1,18	0.16	0.69
Locality*Gender	2,19	2.02	0.16	1.98	0.17	2,18	0.25	0.78

b)

Source of Variance	df	<i>pparg</i>		df	<i>tshb</i>	
		<i>F</i>	<i>P</i>		<i>F</i>	<i>P</i>
Condition Factor	1,19	0.59	0.45	1,17	0.18	0.68
Locality	2,19	0.17	0.84	2,17	0.98	0.40
Gender	1,19	2.46	0.13	1,17	0.86	0.37
Locality*Gender	2,19	0.12	0.89	2,17	0.27	0.77

c)

Source of Variance	df	<i>gr1</i>		<i>gr2</i>		<i>mr</i>		<i>pparg</i>	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Condition Factor	1,19	1.46	0.24	0.07	0.80	0.58	0.45	0.59	0.45
Locality	2,19	0.54	0.59	0.11	0.90	0.92	0.42	0.17	0.84
Gender	1,19	0.56	0.46	7.94	0.01*	0.49	0.49	2.46	0.13
Locality*Gender	2,19	1.61	0.23	0.58	0.57	1.27	0.30	0.12	0.89

The expression of *thra*, *thrb*, *dio2*, *tshb*, *gr1*, *gr2*, *mr* and *pparg* in fish diagnosed with pansteatitis was evaluated using GLM ANCOVA using pansteatitis and gender as fixed effects and CF as covariate. The presence of pansteatitis was not associated with significant variation in the expression of any of the genes investigated. Gender was, however, a significant source of variation in the expression of *gr2* (Table 4.3; Figure 4.4). Moreover, CF had a significant effect on *thrb* expression (Table 4.3; Figure 4.4). When the association between CF and *thrb* expression within the Loskop Dam population was, however, evaluated with linear regression analysis, no significant association between *thrb* expression and CF was observed ($R^2 = 0.21$, $P = 0.18$).

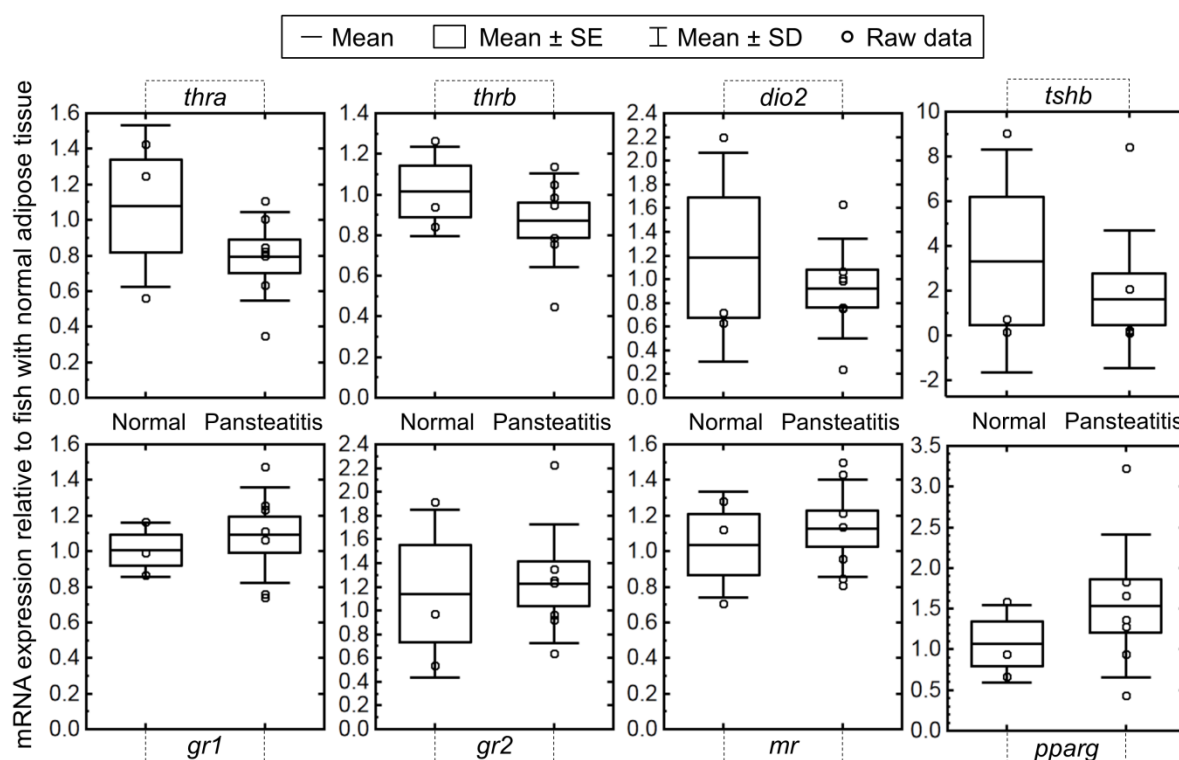


Figure 4.4: The mRNA expression of *thra*, *thrb*, *dio2*, *tshb*, *mr*, *gr1*, *gr2* and *pparg* in the (total) brain tissue of *Oreochromis mossambicus* with macroscopically visible pansteatitis ($N = 7$) or without visible pansteatitis (“normal”) ($N = 3$). No significant differences were observed among the “normal” and fish diagnosed with pansteatitis (Student’s t-test, $\alpha = 0.05$).

Table 4.3: Generalized Linear Model Analysis of Covariance (GLM ANCOVA) of the expression of (a) *thra*, *thrb*, *dio2* and *tshb*, (b) *mr*, *gr1*, *gr2* and *pparg* in the (total) brain tissue of *Oreochromis mossambicus* with macroscopically visible pansteatitis ($N = 7$) or without visible pansteatitis ($N = 3$). Fulton's condition factor was applied as covariate. Asterisks indicate statistically significant effects.

a)

Source of Variance	df	<i>thra</i>		<i>thrb</i>		<i>dio2</i>		<i>tshb</i>	
		F	P	F	P	F	P	F	P
Condition Factor	1,5	0.38	0.57	7.09	0.04*	1.06	0.35	1.06	0.35
Pansteatitis	1,5	1.00	0.36	3.89	0.11	1.72	0.25	1.72	0.25
Gender	1,5	1.07	0.35	0.90	0.39	0.14	0.72	0.14	0.72
Panst*Gender	1,5	0.08	0.79	2.67	0.16	1.24	0.32	1.24	0.32

b)

Source of Variance	df	<i>gr1</i>		<i>gr2</i>		<i>mr</i>		<i>pparg</i>	
		F	P	F	P	F	P	F	P
Condition Factor	1,5	0.22	0.66	6.04	0.06	0.84	0.40	1.06	0.35
Pansteatitis	1,5	1.83	0.23	12.43	0.02*	3.51	0.12	1.72	0.25
Gender	1,5	0.09	0.78	0.05	0.83	0.39	0.56	0.14	0.72
Panst*Gender	1,5	0.00	0.99	1.43	0.29	1.06	0.35	1.24	0.32

4.3.2 Juvenile exposures

The expression of *thra* in the juvenile fish did not vary significantly among the control and environmental water treatments and no significant pairwise differences were observed (Figure 4.5a).

The expression of *thrb* did not vary significantly among the Loskop Dam riverine, transitional and lacustrine zone treatment groups and both the negative and positive (T_3) control treatments (Figure 4.5b). The only significant difference in *thrb* expression was between the negative control and the $5 \mu\text{g.L}^{-1} T_3$ control ($P < 0.01$), riverine zone water, supplemented with T_3 ($P = 0.04$) and transitional zone water, supplemented with T_3 ($P = 0.05$) (Figure 4.5b). There was, however, a trend for *thrb* to be higher in the total and filtered transitional zone treatments than the control (Figure 4.5b) (total: $P = 0.08$; filtered: $P = 0.06$).

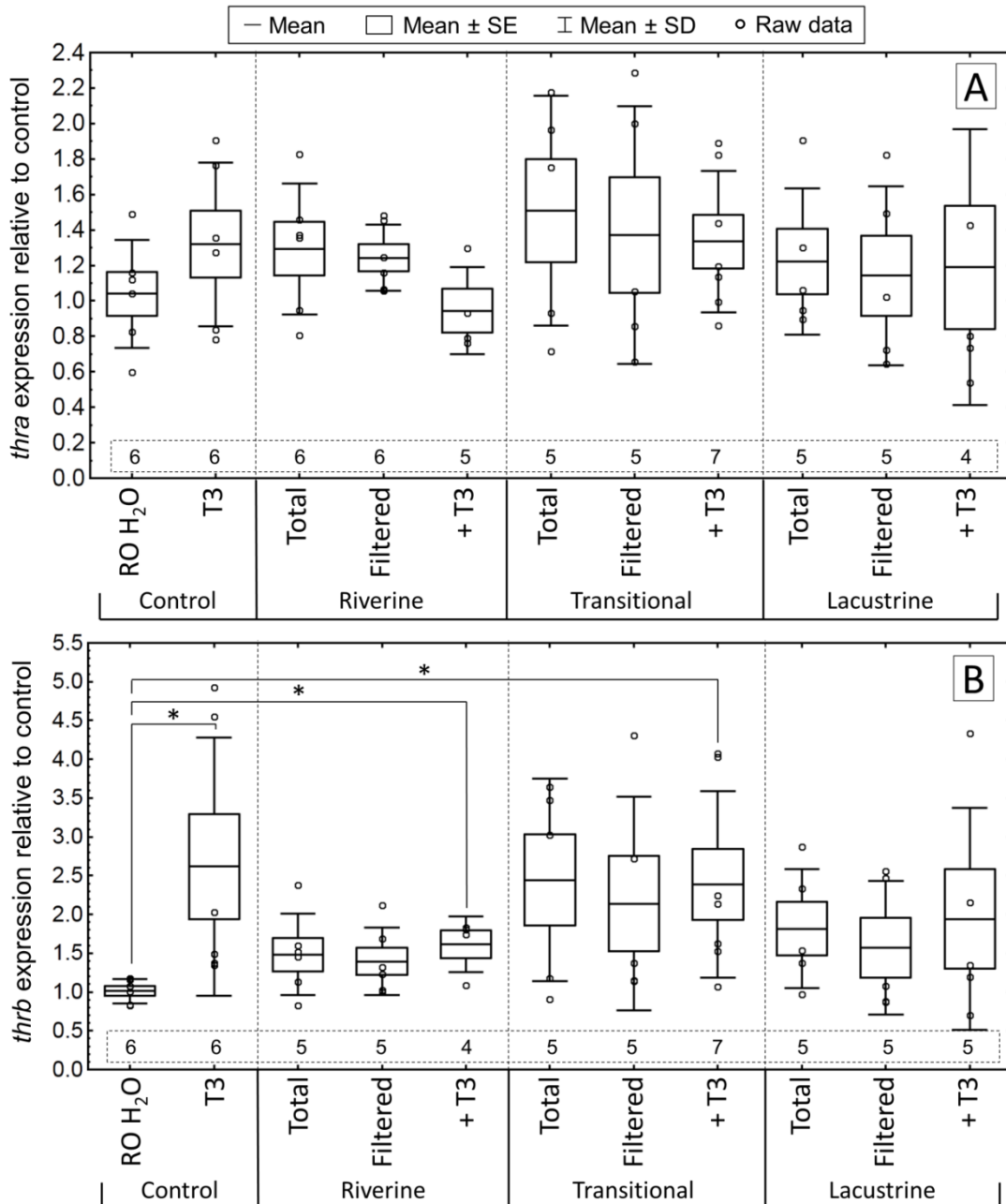


Figure 4.5: Expression of (A) *thra* and (B) *thrb* in juvenile *Oreochromis mossambicus* (32 dpf, whole body homogenates) exposed for 48 h to water from the lacustrine, transitional and riverine sections of Loskop Dam. Each locality within the dam was represented by three treatment groups namely: a total sample (hence including algae and bacteria); 1.2 μm filtered; filtered and supplemented with 5 $\mu\text{g}\cdot\text{L}^{-1}$ of triiodothyronine (T₃). Significant differences are indicated by asterisks (Student's t-test or Man-Whitney U-test). The numbers within the horizontal boxes indicate the number of fish representing each treatment.

Type 2 deiodinase expression varied significantly among the control, T₃ supplemented, riverine, transitional and lacustrine zone treatment groups ($F_{10,43} = 3.23$, $P < 0.01$) (Figure 4.6a). In particular, *dio2* expression was significantly higher in the unfiltered (micro-organism containing) transitional zone treatment than the control ($P = 0.01$), as well as both the filtered- and T₃ supplemented transitional zone treatments ($P = 0.01$ and $P < 0.01$ respectively) (Figure 4.6a). Moreover, *dio2* expression in the unfiltered transitional zone treatment was significantly higher than the filtered ($P = 0.03$) and -T₃ supplemented ($P < 0.001$) lacustrine zone water, filtered ($P < 0.001$) and -T₃ supplemented ($P < 0.001$) riverine zone water, and the 5 µg.L⁻¹ T₃ control treatments ($P < 0.01$) (Figure 4.6a).

Similar to the trend observed in the transitional zone treatments, *dio2* expression was significantly higher in the unfiltered riverine zone treatment than both the filtered ($P = 0.04$) and T₃ supplemented ($P < 0.01$) groups. Furthermore, the *dio2* expression was significantly higher in the unfiltered lacustrine zone treatment than the T₃ supplemented treatment ($P = 0.04$) (Figure 4.6a). Finally, *dio2* expression was significantly higher in the unfiltered lacustrine zone treatment than both the filtered riverine ($P = 0.02$) and T₃ supplemented riverine zone ($P < 0.01$) treatments (Figure 4.6a).

Peroxisome proliferator-activated receptor gamma (pparg) expression was significantly higher in the unfiltered lacustrine zone treatment than the control ($P < 0.01$) (Figure 4.6b). Moreover, *pparg* expression was significantly lower in the filtered- than unfiltered transitional zone treatments ($P = 0.03$; Figure 4.6b).

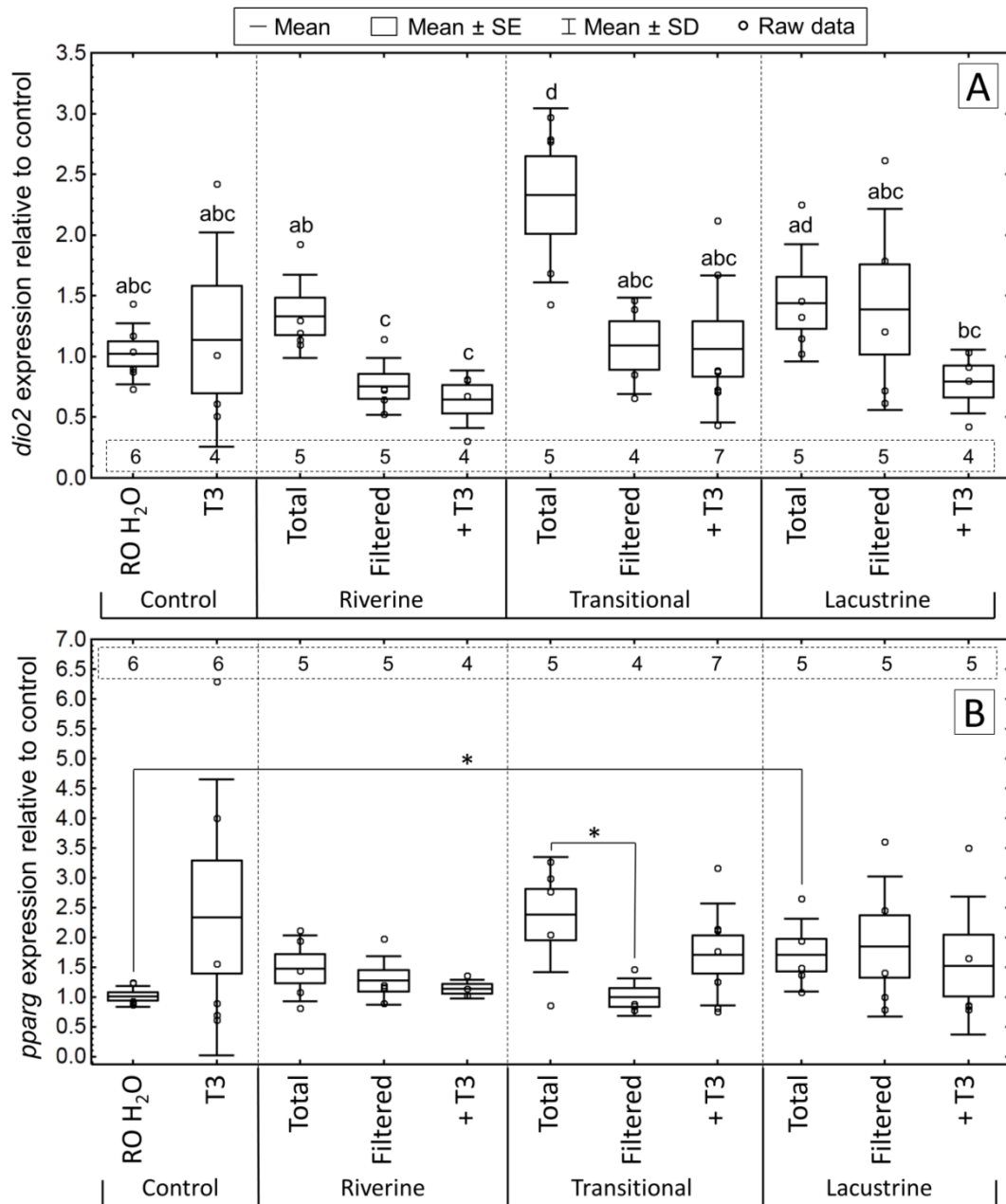


Figure 4.6: Expression of (A) *dio2* and (B) *pparg* in juvenile *Oreochromis mossambicus* (32 dpf, whole body homogenates) exposed for 48 h to water from the lacustrine, transitional and riverine sections of Loskop Dam. Each locality within the dam was represented by three treatment groups namely: a total sample (hence including algae and bacteria); 1.2 μm filtered; filtered and supplemented with 5 $\mu\text{g}\cdot\text{L}^{-1}$ of triiodothyronine (T₃). Significant differences are indicated by dissimilar characters (Fisher's LSD *post hoc* test) or asterisks (Student's t-test or Man-Whitney U-test). The numbers within the horizontal boxes indicate the number of fish representing each treatment.

4.3.3 Metal analysis

Moderate to low concentrations of metals were detected in the water collected from Loskop Dam in light of the South African Department of Water Affairs guidelines for aquatic ecosystems and aquaculture, respectively (DWAF, 1996) (Table 4.4). Iron was the only element that exceeded the South African Water Affairs Guideline for aquaculture ($10 \mu\text{g}\cdot\text{L}^{-1}$) (DWAF 1996) in water collected from the riverine and transitional zone localities of Loskop Dam as well as the irrigation-canal supplied waters (Table 4.4). Moreover, Al, Cu, and Zn exceeded the SA DWAF aquatic ecosystems guideline in Loskop Dam as well as both the bore hole- and irrigation-canal-supplied waters (Table 4.4). In general, the metal analysis data indicates that the quality of the irrigation-canal-supplied dam waters was poor, characterized by high levels of Al, Cu, Fe, Pb and Zn relative to Loskop Dam and the borehole-supplied waters (Table 4.4). Moreover, the borehole-supplied dam waters had high levels of Pb and Zn, relative to the Loskop Dam waters (Table 4.4).

Table 4.4: The concentrations ($\mu\text{g.L}^{-1}$) of selected metals and other elements in water collected from three locations within Loskop Dam, as well as dams inhabited by the alternative population evaluated in the current study (i.e., borehole- and irrigation canal supplied dams respectively).
 “a” Superscript: Exceeds the South African Department of Water Affairs and Forestry (SA DWAF) aquatic ecosystems guideline (DWAF, 1996).
 “b” Superscript: Exceeds the SA DWAF aquaculture guideline (DWAF, 1996).

Element	Loskop Dam Dec 2012			Loskop Dam Apr 2013			Alt Population Apr 2013	
	Lacustrine	Transitional	Riverine	Lacustrine	Transitional	Riverine	Borehole	Canal
Al	3.51	6.67 ^a	11.17 ^a	0.41	6.42 ^a	2.55	11.63 ^a	71.67 ^a
As	0.54	0.55	0.33	0.59	0.93	1.57	0.6	0.55
B	28.60	31.71	28.11	31.37	56.43	74.95	122.2	164.81
Ba	51.77	58.55	29.12	22.58	47.34	34.89	197.82	334.05
Ca	25,040	21,460	20,010	24,300	23,670	29,720	96,830	35,000
Cd	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	0.06
Co	0.11	0.34	0.57	0.05	0.13	0.56	0.27	0.64
Cr	<L.O.D.	<L.O.D.	0.31	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.
Cu	0.81 ^a	1.03 ^a	1.16 ^a	0.43 ^a	0.40 ^a	1.33 ^a	3.18 ^a	6.96 ^a
Fe	5.38	12.96 ^b	19.48 ^b	7.26	15.14 ^b	5.62	8.96	44.65 ^b
Hg	<L.O.D.	<L.O.D.	<L.O.D.	0.04	0.03	0.03	0.04	0.01
K	4,610	3,707	3,317	4,427	4,577	6,947	880	7,250
Li	11.82	13.55	11.14	12.45	11.33	17.02	0.94	0.79
Mg	17,577.5	15,177.5	15,177.5	17,010	17,560	21,950	63,790	23,150
Mn	0.86	2.79	4.47	0.83	0.36	1.59	1.08	4.3
Mo	0.31	0.15	0.15	5.10	2.53	2.33	1.26	2.34
Na	24,110	22,290	21,160	23,980	26,160	47,510	53,500	53,020
Ni	2.09	3.05	4.59	1.29	2.01	4.36	2.81	5.9
P	<L.O.D.	8.50	14.10	4.2	47.1	16.8	10	30
Pb	0.01	0.01	<L.O.D.	<L.O.D.	0.01	0.05	0.21 ^a	0.52 ^a
Sb	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	0.2	0.37
Se	0.06	<L.O.D.	<L.O.D.	0.01	0.04	1.08	0.9	0.24
Si	300	1,725.1	1,946.1	234.4	965.7	2,686	26,230	6,620
Sn	<L.O.D.	<L.O.D.	<L.O.D.	0.08	0.08	0.10	<L.O.D.	<L.O.D.
Sr	145.99	119.97	113.68	102.74	102.96	133.54	299.68	158.67
V	0.52	1.68	1.53	<L.O.D.	0.04	0.23	36.27	14.82
Zn	4.11 ^a	4.56 ^a	8.63 ^a	1.74	3.02 ^a	2.17 ^a	64.22 ^a	97.44 ^a

Limit of detection (L.O.D.) Cd 0.05 $\mu\text{g.L}^{-1}$; Cr 0.3 $\mu\text{g.L}^{-1}$; Hg 0.01 $\mu\text{g.L}^{-1}$; Pb 0.01 $\mu\text{g.L}^{-1}$; Sn 0.08 $\mu\text{g.L}^{-1}$; Sb 0.1 $\mu\text{g.L}^{-1}$; V 0.04 $\mu\text{g.L}^{-1}$

4.3.4 Phytoplankton classification

The lacustrine zone of Loskop Dam had the highest phytoplankton diversity, and eight species could be identified which, however, all occurred at low numbers (Table 4.5). The transitional zone phytoplankton community was dominated by the dinoflagellate, *Ceratium hirundinella* (Müller) (57 cells.mL⁻¹). In addition, a low number of the cyanobacterium, *Microcystis aeruginosa* (Kützing, 1846) (9 cells.mL⁻¹) was present in the water collected from the transitional zone, whereas a higher concentration of this species was present in the riverine zone sample (14 cells.mL⁻¹) (Table 4.5).

Table 4.5: The phytoplankton species composition and relative abundance in water collected from three locations within Loskop Dam during December 2012. The water samples containing algae were applied in juvenile fish exposures.

Algal group or genus and species	Locality		
	Lacustrine	Transitional (Cells.mL ⁻¹)	Riverine
Bacillariophyceae			
<i>Melosira varians</i>	-	-	23
<i>Fragillaria crotonensis</i>	21	13	-
<i>Aulacoseira granulata</i>	-	3	6
<i>Diatoma vulgare</i>	-	-	11
<i>Pinnularia viridis</i>	-	-	4
Chlorophyceae			
<i>Coelastrum reticulatum</i>	2	-	-
<i>Pandorina morum</i>	8	-	-
<i>Straurastrum anatum</i>	3	-	-
Cyanophyceae			
<i>Microcystis aeruginosa</i>	3	9	14
Dinophyceae			
<i>Ceratium hirundinella</i>	5	57	-
<i>Peridinium bipes</i>	-	23	-
Euglenophyceae			
<i>Trachelomonas volvocina</i>	7	-	4
Cryptophyceae			
<i>Cryptomonas ovata</i>	9	-	-

4.4 Discussion

The significant increase in *thra* and *dio2* expression observed in the brain tissue of adult *O. mossambicus* from Loskop Dam relative to the alternative population fish maintained in borehole water suggests a degree of thyroid disruption in these fish. A similar expression signature has been observed in fish exposed to pesticides and flame retardants. In particular, Jin *et al.* (2011) observed increased expression of *thra* and *dio2* in the brains of juvenile female Japanese medaka, *Oryzias latipes* (Temminck and Schlegel), exposed to 100 µg.L⁻¹ metolachlor herbicide. Noyes *et al.* (2013) reported increased expression of both *thra* and *dio2* in brain tissues of adult male fathead minnows, *Pimephales promelas* Rafinesque, in response to the flame retardant, BDE-209. A further study showed increased expression of *thra* in adult rare minnow, *Gobiocypris rarus* Ye and Fu, brains, but not in juveniles exposed to a low dosage of 20 ng.L⁻¹ acetochlor herbicide (Li *et al.*, 2009). Recent studies reporting on zebrafish, *Danio rerio* (Hamilton), larvae exposures have shown increased *thra* expression in response to the flame retardants tris(1,3-dichloro-2-propyl) phosphate (TDCPP) (0.2 mg.L⁻¹) and triphenyl phosphate (TPP) (2 mg.L⁻¹) (Liu *et al.*, 2013), and increases in the expression of both *thra* and *dio2* in response to the fungicide, hexaconazole (Yu *et al.*, 2013). Moreover, Zhang *et al.* (2013) observed significant increases in *dio2* expression in the brain tissue of goldfish, *Carassius auratus* (Linnaeus), brains exposed to a low dosage of the organophosphate insecticide, monocrotophos (10 µg.L⁻¹).

Metal contaminants present a further potential source of thyroid disruption in fish (Brown *et al.*, 2004). Studies linking altered expression of thyroid linked genes and metal exposure in fish are, however, limited (Eyckmans *et al.*, 2010; Li *et al.*, 2014; Truter *et al.*, 2014). Cd, Sn and Hg have been shown to modify de-iodination in fish, and in general, the said enzymatic process seems to present a metal sensitive segment of the teleost fish thyroid cascade (Brown *et al.*, 2004). However, we could not locate any studies empirically testing associations between *dio2* or *thra* expression and specific metals in fish, apart from Li *et al.* (2014) who observed down-regulation of *thra* expression in rare minnows *G. rarus* exposed to Cu.

The catchment of Loskop Dam hosts industrial, domestic and agricultural compartments as well as abandoned mines emanating acid mine drainage (Dabrowski and de Klerk, 2013). In reality, members of the Loskop Dam *O. mossambicus* population are chronically exposed to a cocktail of metals and organic contaminants through dermal- and gill epithelium contact as well as feeding (bioaccumulation), and these inputs are likely variable over time. Although the metal concentrations observed in the current study were found to be moderate to low (in

light of South African Department of Water Affairs guideline for aquatic ecosystems [DWAF 1996]), up to 1.22 mg.L^{-1} Fe and 1.61 mg.L^{-1} Al has been recorded in the dam (Oberholster *et al.*, 2010). It is, therefore, not surprising that Oberholster *et al.* (2012b) measured median concentrations of 2580 mg.kg^{-1} Al and $10\ 679 \text{ mg.kg}^{-1}$ Fe in the intestines of *O. mossambicus*. Little is known regarding the organic contaminant loads in Loskop Dam apart from Grobler (1994) who tested for a number of persistent organic pollutants including PCBs and organochlorine pesticides of which none could be detected in the water, yet moderate concentrations of heptachlor ($1 \text{ }\mu\text{g.kg}^{-1}$) and DDE ($8.4 \text{ }\mu\text{g.kg}^{-1}$) were measured in *O. mossambicus* muscle tissue. More detailed studies on pesticide, industrial chemical and heavy metal concentrations in *O. mossambicus* tissues and the dam water will be of value to identify links between contaminant exposures and the impaired health of these fish. The absence of significant variation in both *thra* and *dio2* among the control and filtered Loskop Dam treatments in the current study's juvenile exposures provides evidence that at the time of sampling, the expression of *thra* and *dio2* would not have been altered in *O. mossambicus* by exogenous substances in the water.

Oreochromis mossambicus from Loskop Dam was found to have high plasma T_3 concentrations yet corresponding T_4 concentrations relative to fish from a downstream dam consistently during autumn, winter and spring (Oberholster *et al.*, 2012a). Although plasma T_3 was not measured in the present study, the (inter-seasonal) results of Oberholster *et al.* (2012a) suggest the likelihood of high plasma T_3 levels. Type 2 deiodinase facilitates the conversion of T_4 to the biologically more active T_3 , and *dio2* is typically down-regulated in response to increased plasma T_3 levels, and up-regulated when plasma T_3 is low (Mol *et al.*, 1999; Darras and Van Herck, 2012). High *dio2* expression in combination with high plasma T_3 levels (hyperthyroid) is, therefore, unexpected. Iodine deficiency is known to cause increased *dio2* expression in mammals, facilitating increased T_3/T_4 ratios. Although both T_3 and T_4 decrease during severe iodine deficiency, T_4 decreases pronouncedly during mild deficiency (McLanahan *et al.*, 2008; Lavado-Autric *et al.*, 2013). The corresponding T_4 concentrations in Loskop Dam relative to the downstream Flag Boshielo Dam (Oberholster *et al.*, 2012a) suggest that the increased *dio2* expression observed in the present study may not be related to an iodine deficiency phenomenon. There may be a link between chronic exposure to contaminants and the increased *dio2* despite high plasma T_3 (Picard-Aitken *et al.*, 2007). Several chemicals have been implicated as Dio2 disruptors including the flame retardant metabolite 6-OH-BDE-47 (Dong *et al.*, 2013), diethylstilbestrol (DES), the herbicide ioxynil (Morgado *et al.*, 2009), 2,3,7,8-tetrachlorodibenzo-p-dioxin, polychlorinated biphenyls (PCBs), amiodarone, iodoacetic acid (reviewed in Song *et al.*, 2013), and as previously mentioned metolachlor (Jin *et al.*, 2011), hexaconazole (Yu *et al.*, 2013) and BDE-209

(Noyes *et al.*, 2013). It is, however, difficult to determine the mechanism of action through which Dio2 disruption occurs seeing that *dio2* regulation *per se* is complex, *dio2* being a cyclic adenosine monophosphate (cAMP) responsive gene further influenced by factors such as the hedgehog pathway and endoplasmic reticulum stress (Drigo *et al.*, 2013). Although the increased plasma T₃ levels observed by Oberholster *et al.* (2012a) may have been attributed to increased *dio2* activity potentially induced by *dio2* targeted contaminants, further investigation is required to test this hypothesis.

Although increased T₃ levels are commonly observed in association with obesity (Bray *et al.*, 1976; Kitahara *et al.*, 2012), the high T₃ is believed to be a consequence rather than a cause of obesity (Reinehr, 2010; Longhi and Radetti, 2013). In particular high T₃ in association with obesity is likely attributed to leptin, which counteracts a positive energy balance (and progression of obesity) by activating the HPT axis (Reinehr 2010; Pearce 2012), or by altering deiodinase activity (Cettour-Rose *et al.*, 2002; Zimmermann-Belsing *et al.*, 2003). Leptin was shown to increase *dio2* expression in rat peripheral tissues (Cettour-Rose *et al.*, 2002; Araujo and Carvalho, 2011), and increase *thra* expression in chondrocytes *in vitro* (Wang *et al.*, 2011). The altered *thra* and *dio2* expression and higher plasma T₃ observed in the Loskop Dam *O. mossambicus* (Oberholster *et al.*, 2012a) may be compensatory in response to the obese condition of the fish and, therefore, not directly induced by thyroid disrupting compounds. Although fat percentages were not recorded in the present study, CF was significantly higher in Loskop Dam fish than those from the alternative population. However, when CF was applied as covariate in GLM ANCOVAs, CF was not a significant source of variation in the expression of any of the eight genes investigated among Loskop Dam *O. mossambicus* and the alternative population fish, suggesting that the altered gene expression was not related to variation in CF and, therefore, possibly fat content. This prediction is, however, speculative seeing that CF is not necessarily a good indicator of fat content (Naeem *et al.*, 2011).

The obesity in Loskop Dam fish may be related to the disturbance of other adipogenic pathways and potentially through obesogens (Grün and Blumberg, 2009; Regnier and Sargis, 2014) and even epigenetic changes such as developmental programming, which may also be associated with obesogens (Janesick and Blumberg, 2012). Obesity is an emerging global epidemic in humans, and some researchers have recently commenced the use of fish models to study the condition (Tingaud-Sequeira *et al.*, 2011; Hasumura *et al.*, 2012; Ichimura *et al.*, 2013). The *O. mossambicus* of Loskop Dam presents a promising opportunity to study the potential links between obesity and contaminant exposures through direct or epigenetic modifications in a wild population.

The higher *dio2* and *pparg* expression in juvenile *O. mossambicus* in response to non-filtered relative to filtered Loskop Dam transitional zone water was likely caused by the biotic content (i.e., micro-organisms). The significant difference in *dio2* expression among the unfiltered transitional zone treatment and negative (RO water) control is, therefore, likely also attributed to the biotic content of the water and not thyroid disruptors.

Hepatic Dio2 activity was found to decrease in adult Nile tilapia, *Oreochromis niloticus* (Linnaeus), after 48 h of fasting (Van der Geyten *et al.*, 1998). In the present study, juveniles were not fed during the 48 h exposure. The lower *dio2* expression observed in the filtered transitional and riverine zone treatments relative to the non-filtered treatments may, therefore, be associated with fasting in the filtered samples seeing that fish in the non-filtered treatments were able to forage (de Moor *et al.*, 1986; Van der Geyten *et al.*, 1998; Oberholster *et al.*, 2012b).

Although the response of Pparg to fasting in fish has not been described in the literature to date, *pparg* expression was shown to decrease in rat adipose tissues after 39 h of fasting (Kajita *et al.*, 2008). The decrease in *pparg* expression in filtered relative to non-filtered transitional zone water may, therefore, also be the result of fasting.

Aquatic animals are commonly fasted during short-term exposures in studies applying gene expression based biomarkers for thyroid disruption (Helbing *et al.*, 2003; Opitz *et al.*, 2006). The present data indicates that fasting may, however, be a confounding factor when unfiltered environmental samples are screened, especially in model organisms such as small fish or tadpoles.

The current investigation attempted to identify potential links between the thyroid and interrenal endocrine system and pansteatitis in adult *O. mossambicus* inhabiting Loskop Dam. The lack of any significant differences in the expression of key metabolism-linked genes, *thra*, *thrb*, *dio2*, *tshb*, *gr1*, *gr2* and *mr* among pansteatitis suffering and pansteatitis free fish provides evidence that the ailment may not be directly linked to thyroid disruption nor associated with activation of corticosteroid pathways. The exact cause of pansteatitis in Loskop Dam *O. mossambicus*, therefore, remains unresolved. Further work on the said *O. mossambicus* population focused on the more typical pansteatitis pathogenesis (i.e., vitamin E deficiency as a consequence of high dietary levels of polyunsaturated fats and/or oxidised fat ingestion [Huchzermeyer, 2003; Fytianou *et al.*, 2006]) will be of value.

4.5 References

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**Chapter 5: The impacts of neutralized acid mine drainage
contaminated water on the expression of selected
endocrine-linked genes in juvenile Mozambique tilapia
Oreochromis mossambicus exposed *in vivo***

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Declaration by the candidate

With regard to Chapter 5, the nature and scope of my contribution were as follows:

Nature of contribution Extent of contribution (%)

Nature of contribution	Extent of contribution
Conceptual design, experimental work, manuscript writing.	75%

The following co-authors have contributed to Chapter 5:

Name	Email address and institutional affiliation	Nature of contribution	Extent of contribution
Prof JH van Wyk	jhvw@sun.ac.za Department of Botany and Zoology, Stellenbosch University	Conceptual design, manuscript editing.	25%
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Abstract

Acid mine drainage (AMD) is a global environmental concern due to detrimental impacts on river ecosystems. Little is, however, known regarding the biological impacts of neutralized AMD on aquatic vertebrates despite excessive discharge into watercourses. The aim of this investigation was to evaluate the endocrine modulatory potential of neutralized AMD, using molecular biomarkers in the teleost fish *Oreochromis mossambicus* in exposure studies. Surface water was collected from six locations downstream of a high density sludge (HDS) AMD treatment plant and a reference site unimpacted by AMD. The concentrations of 28 elements, including 22 metals, were quantified in the exposure water in order to identify potential links to altered gene expression. Relatively high concentrations of manganese ($\sim 10 \text{ mg.L}^{-1}$), nickel ($\sim 0.1 \text{ mg.L}^{-1}$) and cobalt ($\sim 0.03 \text{ mg.L}^{-1}$) were detected downstream of the HDS plant. The expression of *thyroid hormone receptor alpha (thra)*, *thyroid hormone receptor alpha (thrb)*, *androgen receptor-1 (ar1)*, *ar2*, *glucocorticoid receptor-1 (gr1)*, *gr2*, *mineralocorticoid receptor (mr)* and *aromatase (cyp19a1b)* was quantified in juvenile fish after 48 h exposure. Slight but significant changes were observed in the expression of *gr1* and *mr* in fish exposed to water collected directly downstream of the HDS plant, consisting of approximately 95% neutralized AMD. The most pronounced alterations in gene expression (i.e., *thra*, *thrb*, *gr1*, *gr2*, *ar1* and *mr*) was associated with water collected further downstream at a location with no other apparent contamination vectors apart from the neutralised AMD. The altered gene expression associated with the “downstream” locality coincided with higher concentrations of certain metals relative to the locality adjacent to the HDS plant which may indicate a causative link. The current study provides evidence of endocrine disruptive activity associated with neutralized AMD contamination in regard to alterations in the expression of key genes linked to the thyroid, interrenal and gonadal endocrine axes of a teleost fish species.

5.1 Introduction

Acid mine drainage is one of the major environmental risks associated with the mining industry (Akcil and Koldas, 2006), disturbing natural ecosystems by lowering the pH and by releasing contaminants such as metals and other toxic substances into water bodies. In particular, AMD is generally characterized by high levels of manganese, nickel, cobalt, iron and aluminium and other metals known to be harmful to wildlife (Akcil and Koldas, 2006). A further negative impact of AMD is salinization and the high sulphate content (Tutu *et al.*, 2008). Not surprisingly, numerous studies have demonstrated detrimental effects of AMD on wildlife at organismal as well as ecosystem level, including overall declines in macro

invertebrate diversity (Janssens de Bisthoven *et al.*, 2006; van Dam *et al.*, 2008). Moreover, highly AMD impacted waterways are reported to be devoid of fish and other organisms (Parsons, 1977).

In many cases, AMD cannot be prevented or controlled and has to be treated. The most common approach to AMD treatment is pH control, but although such remedying action is capable of increasing the pH to near-neutral and subsequent precipitation of a large fraction of elements from the solution, not all metals are removed (Cravotta and Trahan, 1999).

Certain metals have been identified as endocrine disrupting compounds (EDCs) (Reviewed in Iavicoli *et al.*, 2009). For example, reference has been made to different metals including Al, As, Ba, Cd, Co, Cr (II), Cu, Hg, Ni, Pb, Sn, Sb as “metalloestrogens” (Darbre, 2006), whereas Cd has been implicated as an androgen receptor agonist *in vitro* (Martin *et al.*, 2002). Moreover, certain metals have been shown to disrupt the teleost fish reproductive- (Kime, 1998), thyroid- (Carr and Patino, 2011) and interrenal (Hontela 1998) endocrine systems. Treated AMD may, therefore, exhibit endocrine disruptive potency due to metal loads.

Although reports exist indicating that the treatment of acidic waters reduces or even eliminates the endocrine disruptive effects in certain fish (Brown *et al.*, 1990; Sangalang *et al.*, 1990), these works were performed on acidic river or lake water, in most cases acidified by atmospheric deposition and not AMD (Baker *et al.*, 1991). Even though endocrine disruptive activity has been investigated in a number of water bodies, to my knowledge no study to date has explicitly investigated the endocrine disruptive potential of neutralized AMD contamination in fish. The biological activities of individual chemicals are known to be altered when present in mixtures of other chemicals or elements, an occurrence referred to as the “cocktail effect”, wherein which inter-chemical interactive effects such as potentiation or antagonism may occur (Celander, 2011). Neutralized AMD present an opportunity to investigate the effects of complex contaminant mixtures, consisting of metals and other elements, on the vertebrate endocrine system, without the toxic acid mediated effects associated with untreated AMD.

Alterations in the expression of certain genes have been shown as sensitive biomarkers for endocrine disruption after exposure periods as short as 12 h (Helbing *et al.*, 2003; Opitz *et al.*, 2006). Although changes in mRNA transcript abundance will not necessarily result in physiological impairment, these toxicogenomic biomarkers can be applied as signposts prompting further investigation based on chronic testing including more concrete endpoints such as impaired fecundity or development (Hutchinson *et al.*, 2006).

Acid mine drainage (AMD) is a concern in certain regions in South Africa, due to decades of intensive mining activity (McCarthy, 2011). The Western Basin of the Witwatersrand has been identified as one of the problem areas in relation to AMD in South Africa, where a number of streams are subject to contaminated water from abandoned gold mines (McCarthy, 2011). The objective of this investigation was to evaluate aspects of water quality and the subsequent endocrine disruptive potencies of surface water collected from a river catchment receiving high volumes of neutralized (treated) AMD, released by a high density sludge plant, in the Witwatersrand Western Basin, South Africa. The specific aims were firstly to collect water samples from six localities and a reference location uncontaminated by AMD, record basic water quality parameters and determine the concentrations of selected metals and other elements. Secondly to expose juvenile *O. mossambicus* for a short term (48 h) and quantify altered expression of *thyroid hormone receptor alpha (thra)*, *thyroid hormone receptor beta (thrb)*, *androgen receptor-1 (ar1)*, *ar2*, *glucocorticoid receptor-1 (gr1)*, *gr2*, *mineralocorticoid receptor* and *aromatase (cyp19a1b)* as representative of the endocrine system.

5.2 Materials and Methods

5.2.1 Study sites

Water was collected from seven localities in the Bloubank stream catchment near Krugersdorp, South Africa (Figure 5.1). The Bloubank stream is a tributary of the Crocodile River, and at times receive AMD emanating from flooded defunct gold mines associated within the West Rand Gold Field. The Tweelopie stream originates in close proximity of the Randfontein Estates Gold Mine and represents the most direct route for AMD into the Bloubank drainage. A high density sludge AMD treatment plant discharges into the Tweelopie stream (approximately 18 ML/d at the time of sampling) and the majority of water in the headwaters of this stream consists of treated AMD.

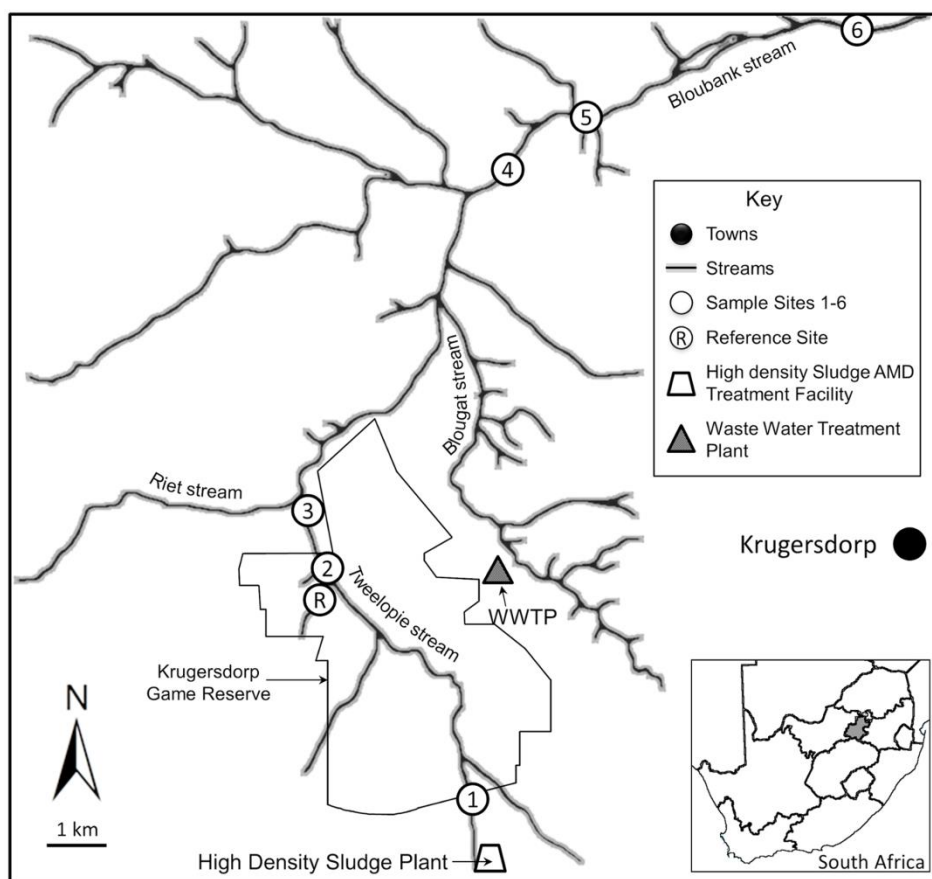


Figure 5.1: Map indicating the seven sampling locations within the Bloubank stream drainage. The inset indicates the approximate location of the study area in South Africa.

Site 1 is located 800 meters downstream of the point where neutralised AMD released from a HDS facility enters the Tweelopie stream (Figure 5.1), and the water consisted of approximately 95% neutralized AMD when the current study was performed. Sites 2 and 3 are located downstream within the Tweelopie stream, whereas sites 4, 5 and 6 are further downstream and in the Bloubank stream. Water at the latter sites are subject to further anthropogenic impacts including agricultural runoff and spray drift via the Riet stream and waste water treatment plant (WWTP) effluent via the Blougat stream (Figure 5.1). A reference site uncontaminated by AMD was also investigated. The reference site is located within the Krugersdorp Game Reserve in a tributary of the Tweelopie stream with corresponding geology as all six the other sites (i.e., dolomitic strata from the Malmani Subgroup within the Transvaal Supergroup [Hobbs and Cobbing, 2007]) (Figure 5.1).

5.2.2 Water collection

Surface water samples were collected on the 6th of December 2012 in PTFE capped glass bottles and kept on ice packs or ice in the dark. The samples were filtered through 11 μ m

nitral cellulose stacked on 1.2 µm glass-microfibre filters (Munktell, DE). 50 mL of each filtered sample including a filter blank was used for metal analyses.

5.2.3 Chemical analysis

The concentrations of Ca, K, Mg, Na, P, Rb and Si was measured using a Thermo ICAP 6300 ICP-AES (Thermo Scientific, USA), and Al, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Ti, V, Zn using an Agilent 7700x ICP-MS (Agilent Technologies, USA).

5.2.4 Fish exposure

Juvenile *O. mossambicus* (13 days post fertilization [dpf]) were obtained from a single breeding pair (Rivendell Hatchery, Grahamstown, ZA) prior to the exposure, and maintained in buffered reverse osmosis (RO) water (containing 250 mg iodine containing marine salt, 80 mg NaHCO₃ per litre) at 28 ± 1 °C subject to a 14h:10h light:dark cycle. Seven fish (30 dpf) were assigned per treatment group representing the seven localities as well as a buffered RO water (pH 7) negative control. The fish were exposed to 800 mL of liquid in 1 L glass containers for 48 h, without food. All the fish were acclimatised for at least 48 h prior to the exposure to similar containers and volumes as applied during exposures, being fed crushed tilapia pellets (AquaNutro, ZA) twice a day. The fish were euthanized in 0.1% benzocaine at exposure termination and either transferred to TriReagent (Sigma, DE) or snap frozen and stored at -80 °C. The current study was approved by the Stellenbosch University Committee for Animal Care and Use (Protocol No. SU-ACUM12-00036).

5.2.5 RNA isolation and cDNA synthesis

Whole body homogenates of juvenile fish were prepared in TriReagent (Sigma, DE) using an ultrasound sonicator (Omni-ruptor 400, Omni International Inc., USA). Total RNA was isolated according to the TriReagent technical bulletin. RNA integrity was assessed through agarose gel electrophoresis. The RNA was subsequently DNase I (Sigma, DE) treated, and complementary DNA (cDNA) was prepared from 4 µg of total RNA in 20 µL-, or 2 µg in 10 µL reaction volumes using Maxima H-minus cDNA synthesis kits (Thermo Scientific, USA) according to manufacturer's instructions.

5.2.6 RT-qPCR

Messenger RNA expression of *thyroid receptor alpha (thra)*, *thrb*, *glucocorticoid receptor-1 (gr1)*, *gr2*, *androgen receptor-1 (ar1)*, *ar2*, mineralocorticoid receptor (*mr*) and aromatase *cyp19a1b* with β-actin as reference gene was evaluated using real-time RT-qPCR. The

PCRs were performed as 15 μ L reactions containing 2 μ L cDNA as template (200 ng cDNA per reaction for *cyp19a1b* and 20 ng/reaction for all the other genes), 7.5 μ L Jumpstart® SYBRgreen mix (Sigma, DE), 0.33 μ M of each primer and nuclease free water. The PCR programs for all primer pairs included an enzyme activation step at 95 °C (9 min), followed by 40 cycles of denaturing at 95 °C (15 sec), annealing at 58 - 63.5 °C (30 sec) and elongation at 72 °C (45 sec). The primer sequences and annealing temperatures (T_a) of *thra*, *thrb*, *gr1*, *gr2*, *mr*, *ar1*, *cyp19a1b* and *actb* are described earlier in this dissertation (Tables S3.2 and 4.1), whereas *ar2* had a T_a of 58 °C with primer sequences being (in the 5' to 3' direction) AGGGTGAGGTCGGCGAAT (forward) and TGGACTCAAACCTGGTGTCGT (reverse) (Ijiri *et al.*, 2008). Each PCR plate contained an internal non-template control (no cDNA) as well as a five point two-fold serial dilution “theoretical” cDNA concentrations ranging from 25 ng to 1.56 ng, assuming 1 μ g of total RNA yielded 1 μ g of cDNA during the cDNA synthesis reactions. All samples and controls were run in triplicate and dissociation curves were applied to confirm single-fragment amplification. Gene expression was quantified using the Pfaffl method (Pfaffl 2001), with β -*actin* as normalizer, relative to the buffered RO water control treatment. Amplification efficiencies were determined for each primer pair per PCR programme. Outliers were identified per treatment group through the Grubbs' test and removed (Burns *et al.*, 2005).

5.2.7 Statistical analysis

Normality and homogeneity of variance was assessed in the data using the respective Shapiro-Wilk's W test, normal probability plots and Levene's test. Variation in gene-specific mRNA abundance was evaluated using one-way or Kruskal-Wallis ANOVA for parametric and non-parametric data respectively. Pairwise differences were assessed with Duncan's test or multiple comparisons of mean ranks. Probability values (P) of less than 0.05 were accepted as significant.

The relationships of metal, water quality and gene expression data among sites were explored using multivariate analyses (Shaw, 2003). Principal Component Analysis (PCA) biplots were applied to describe the spatial variability (among localities) in element concentrations and basic water chemistry, as well as the expression of selected genes associated with the endocrine system. The angles among arrows depict the degree of correlation between the individual variables, and the smaller the angle, the larger the correlation.

A Redundancy Analysis (RDA) triplot was used to describe the among-site gene expression signatures with the basic water quality and element concentrations overlaid. The data

applied in both the PCAs and RDA were log transformed. Statistical analyses were performed using Statistica version 11 (Statsoft Inc., USA) and CANOCO version 4.5 (Ter Braak and Smilauer, 2002). Ordination biplots and triplots were created using CanoDraw 4.12 (Ter Braak and Smilauer, 2002).

5.3 Results

5.3.1 Water chemistry

Near neutral pH values were observed for water from most of the locations (Table 5.1). The water collected from sites 1, 2 and 3 contained high concentrations of Ni, Co and Mn (Table 5.1). The observed Mn concentration exceeded the WHO drinking water standards at sites 1, 2, 3, 5 and 6, and Ni at sites 1, 2, 3 and 4 (Table 5.1) (WHO, 2005; Kim *et al.*, 2006; WHO, 2011). Salinity, total dissolved solids (TDS) and electrical conductivity (EC) was markedly higher at sites 1, 2 and 3, relative to sites 4, 5, 6 and the reference site (Table 5.1). Phosphate concentrations were below the current detection limit at sites 1, 2, 3 and the reference site, whereas P concentrations ranging between 0.43 mg.L⁻¹ and 1.03 mg.L⁻¹ was observed at sites 4, 5 and 6 which are located downstream of a WWTP and agricultural land-use area. Electrical conductivity, TDS, Fe, Ca, Mg, Li, Na and Sr showed a longitudinal decrease from the Tweelapie stream headwaters downstream across the six study locations within the Bloubank drainage (Table 5.1).

Table 5.1: The pH, electrical conductivity (EC), salinity, total dissolved solids (TDS) and element concentrations (in parts per billion) of water collected from seven locations within the AMD impacted Bloubank stream catchment, Krugersdorp, South Africa during December 2012.

Parameter	Locality						
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Ref
pH	6.98	6.58	6.77	7.55	7.49	7.81	7.88
EC ($\mu\text{S}/\text{cm}$)	3030	2660	2650	604	714	846	232
Salinity (‰)	1.56	1.36	1.39	0.29	0.35	0.42	0.11
TDS ($\text{mg}\cdot\text{L}^{-1}$)	1551	1354	1362	289	350	415	110.3
Al	N.D.	2.30	1.51	4.37	2.43	1.24	1.24
As	1.22	0.67	0.77	1.86	1.21	0.81	N.D.
B	50.85	44.64	42.57	73.23	46.61	40.24	3.32
Ba	20.51	22.23	26.28	35.89	26.90	24.19	16.21
Ca	960600	537100	390200	24380	43630	45520	17850
Cd	0.04	0.14	0.16	0.03	N.D.	N.D.	N.D.
Co	18.56	35.31	35.41	0.67	1.02	1.32	1.51
Cr	N.D.	N.D.	0.25	0.20	0.63	1.36	0.73
Cu	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Fe	171.42	122.13	60.69	29.35	11.48	N.D.	N.D.
Hg	<0.01	ND	ND	0.01	0.01	N.D.	<0.01
K	6109	5103	4327	4927	4082	2300	316.8
Li	124.70	76.56	71.99	19.29	12.72	11.51	1.25
Mg	112092.7	88192.7	82332.7	7878.7	22222.7	31772.7	13192.7
Mn	9919.66	12379.27	12553.63	35.09	69.54	71.40	12.95
Mo	ND	ND	0.12	1.79	0.69	0.43	N.D.
Na	87380	72410	66210	50300	47650	40360	2660
Ni	102.04	139.88	141.94	27.57	16.71	9.02	0.10
P	N.D.	N.D.	N.D.	1033	708.4	426	N.D.
Pb	0.05	0.07	0.13	0.33	0.14	0.05	0.01
Rb	21.6	26	13.3	13.3	N.D.	N.D.	N.D.
Sb	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Se	0.41	0.23	0.39	0.30	0.07	0.06	0.36
Si	1019.4	2945.4	2858.4	4443.4	5137.4	5462.4	5343.4
Sn	<0.01	0.01	N.D.	0.39	0.29	0.15	N.D.
Sr	509.49	364.61	372.52	109.85	87.98	71.58	13.19
Ti	N.D.	N.D.	0.11	0.20	0.29	N.D.	N.D.
V	N.D.	N.D.	<0.01	N.D.	N.D.	N.D.	N.D.
Zn	37.83	153.75	137.99	21.41	18.08	7.49	1.97

A principal component analysis (PCA) biplot of the observed element concentrations together with the basic water chemistry variables indicates a strong grouping of sites 1, 2 and 3, and correlation with Cd, Ca, Mn, Mg, Co and salinity (Figure 5.2). Moreover, the PCA biplot indicates a grouping of sites 4, 5 and 6 and correlation with Al, P, Sn, Mo, Hg, whereas the reference site is segregated from both the aforementioned groupings (Figure 5. 2).

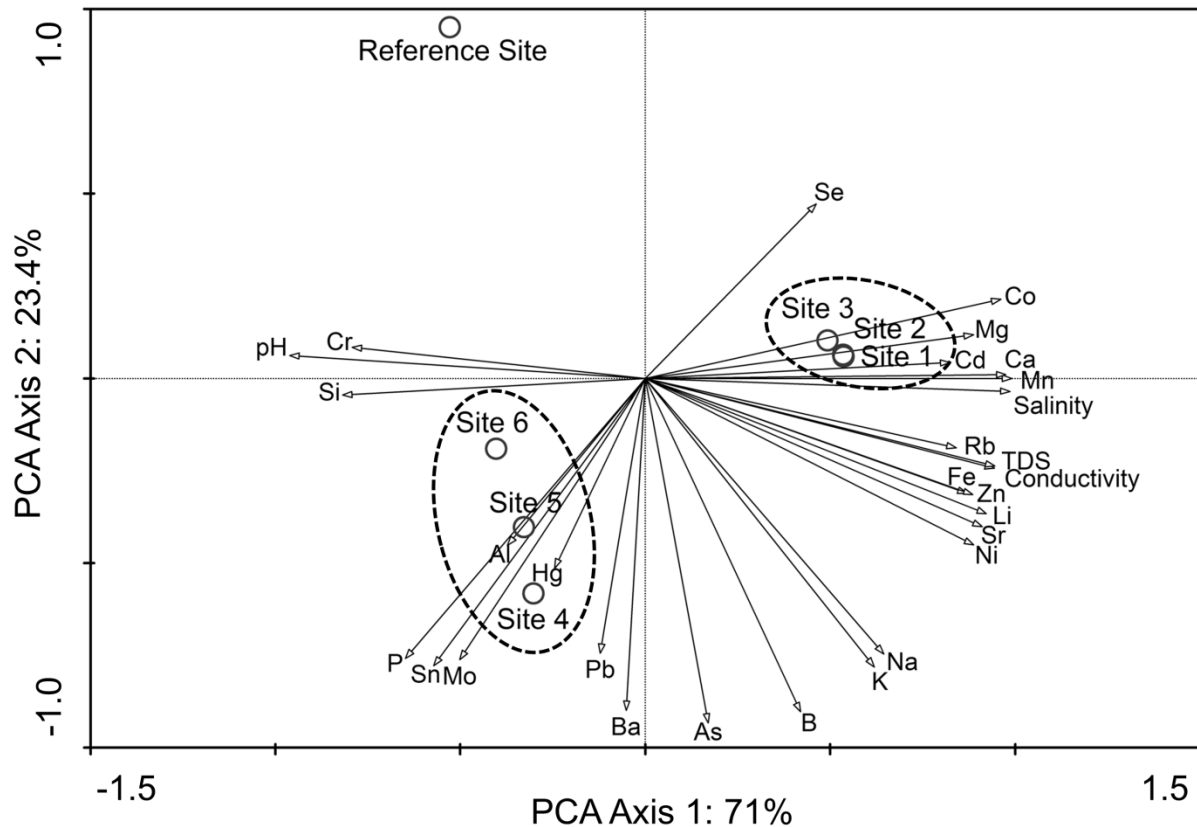


Figure 5.2: A Principal Component Analysis (PCA) biplot indicating the (dis)similarities among seven sampling localities within the Bloubank stream catchment in relation to salinity, total dissolved solids, electrical conductivity, pH and the concentrations of 23 elements.

5.3.2 mRNA expression

There was significant variation among treatments in the expression of *thra* ($F_{7,46} = 2.95$, $P = 0.01$), *thrb* ($F_{7,46} = 3.17$, $P < 0.01$), *gr2* ($F_{7,45} = 4.95$, $P < 0.001$), *mr* ($F_{7,46} = 2.72$, $P = 0.02$), *ar1* ($F_{7,46} =$, $P = 0.02$) and *cyp19a1b* ($F_{7,46} = 2.87$, $P = 0.01$) but not in *gr1* ($F_{7,45} = 2.05$, $P = 0.07$) and *ar2* ($F_{7,45} = 1.37$, $P = 0.24$). There were, however, significant differences in *gr1* expression among the control treatment and both the site 1 ($t_{12} = -2.61$, $P = 0.02$, Student's t-test) and site 3 ($t_{12} = -3.29$, $P = 0.006$, Student's t-test) treatments when these pairwise differences were considered independently. Similarly *gr1* expression varied significantly

among the reference site treatment and the site 1 ($t_{12} = -2.39$, $P = 0.03$, Student's t-test) and site 3 ($t_{12} = -3.03$, $P = 0.01$, Student's t-test) treatments.

The expression of *gr2* was significantly higher in the fish representing sites 2, 3, 4, 5, and 6 than both the reference site and buffered RO water treatments (Figure 5.3). Moreover, *mr* expression was significantly higher in the fish exposed to water collected from site 1 than both the reference site and buffered RO control treatments (Figure 5.3). In addition, the expression of *ar1* was significantly higher in the site 3 treatment relative to the fish exposed to buffered RO water and reference site treatments (Figure 5.3).

The expression of *thra* was significantly higher in fish exposed to water from sites 1 and 3 relative to those exposed to buffered RO water, and significantly higher in fish representing site 3 than the reference site treatment (Figure 5.3). *thrb* expression was significantly higher in the reference site, and sites 1, 2, 3, 4 and 6 treatments relative to the fish exposed to buffered RO water (Figure 5.3). Site 3 was the only treatment which exhibited significantly higher *thrb* expression than the reference site treatment (Figure 5.3).

Aromatase (*cyp19a1b*) expression was significantly lower in fish exposed to water collected from site 4 than buffered RO water as well as the site 1, 3, 5 and 6 treatments (Figure 5.3).

Although a number of these aforementioned differences were shown to be statistically significant, the effects were in most cases minor, and the mean fold mRNA expression relative to the control treatment rarely exceeded two, as was the case for *thrb* and *gr2* in the site 3 treatment group (Figure 5.3).

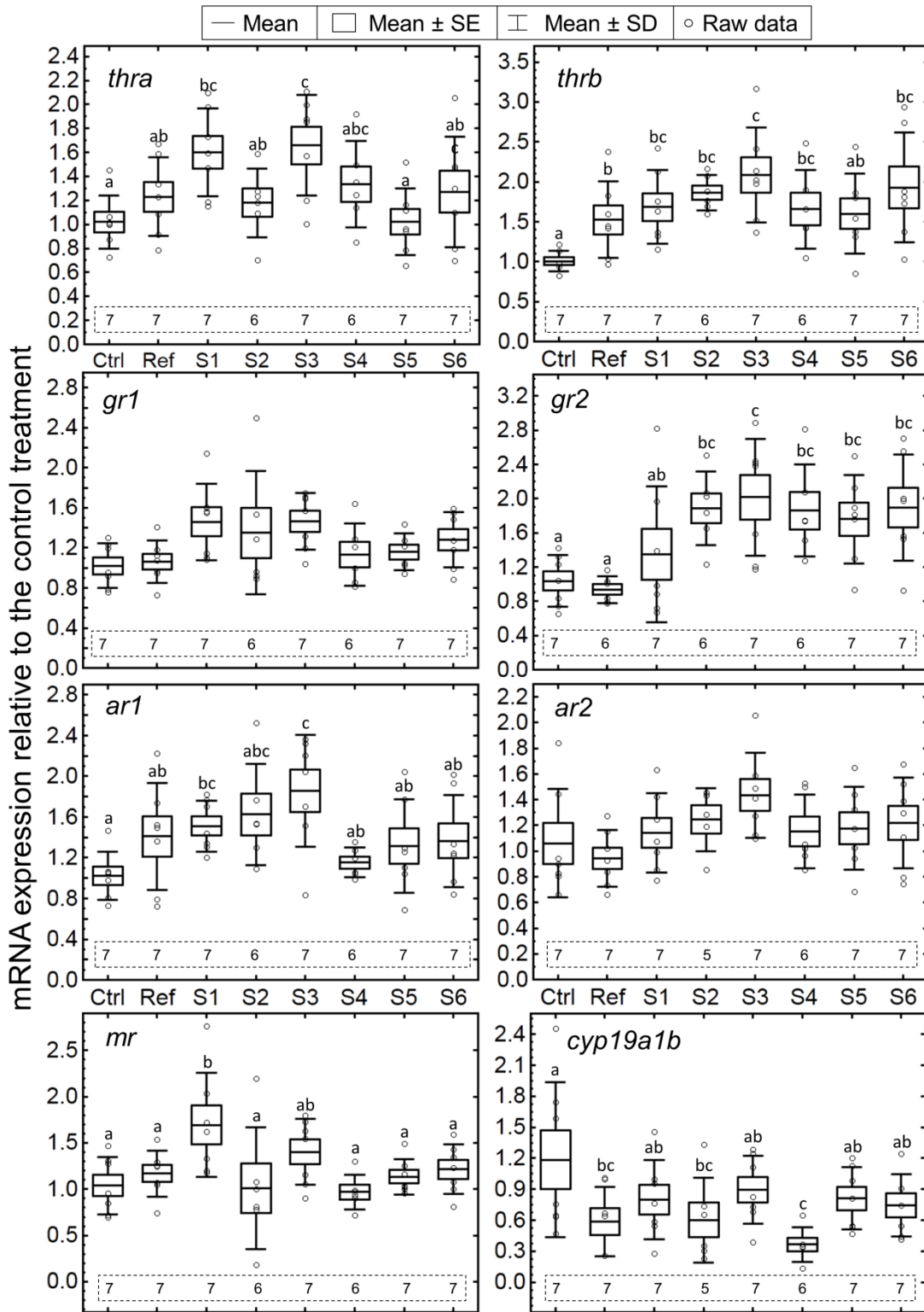


Figure 5.3: Expression of *thra*, *thrb*, *gr1*, *gr2*, *ar1*, *ar2*, *mr* and *cyp19a1b* in *Oreochromis mossambicus* (32 dpf) exposed to surface water collected from six acid mine drainage impacted locations, a reference location not impacted by AMD, and buffered iodated RO water. Significant differences are indicated by dissimilar characters above figure bars. The numbers within the horizontal boxes indicate the number of fish representing each treatment.

The expression signatures of *thra*, *thra*, *ar1*, *ar2*, *gr1*, *gr2* and *mr* were compared among the eight treatment groups using a principal component analysis (Figure 5.4). The results were illustrated using a PCA biplot where each point represents an individual fish (Figure 5.4). Interestingly, *gr1* expression was closely correlated to *mr* and *ar2*, as illustrated by the small angle among arrows, but not with *gr2* (Figure 5.4).

Site 4 was the only treatment group that was totally segregated from the reference site treatment in ordinal space, whereas both sites 2, 3 and 4 were segregated from the control treatment group (Figure 5.4).

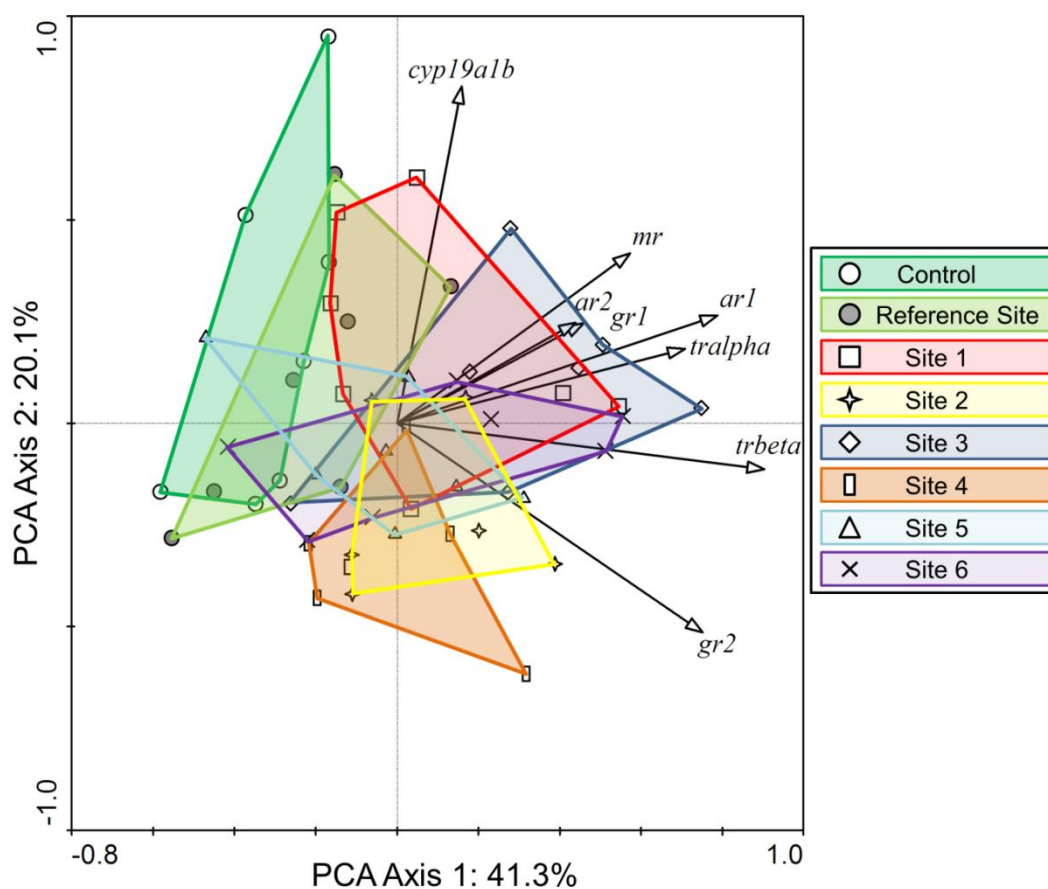


Figure 5.4: A Principal Component Analysis (PCA) biplot indicating the among-treatment (dis)similarities in the expression of selected genes associated with the endocrine system in juvenile *O. mossambicus* exposed to water from seven locations within the Bloubank stream catchment as well as a buffered RO water control. Each point represents an individual fish, whereas the arrows represent *thyroid hormone receptor alpha* (*tralpha*), *thyroid hormone receptor beta* (*trbeta*), *androgen receptor-1* (*ar1*), *ar2*, *glucocorticoid receptor-1* (*gr1*), *gr2*, *mineralocorticoid receptor* (*mr*) and *aromatase* (*cyp19a1b*) respectively.

5.3.3 Redundancy analysis

A redundancy analysis (RDA) was performed to explore the correspondence in gene expression in the exposed fish, water quality and element concentrations among the seven localities sampled (Figure 5.5). The RDA was expressed through an ordination triplot which indicated two abstract groupings, particularly; sites 1 and 3 are segregated from sites 2, 4, 5, 6. Both the aforementioned groups are segregated from the reference site (Figure 5.5).

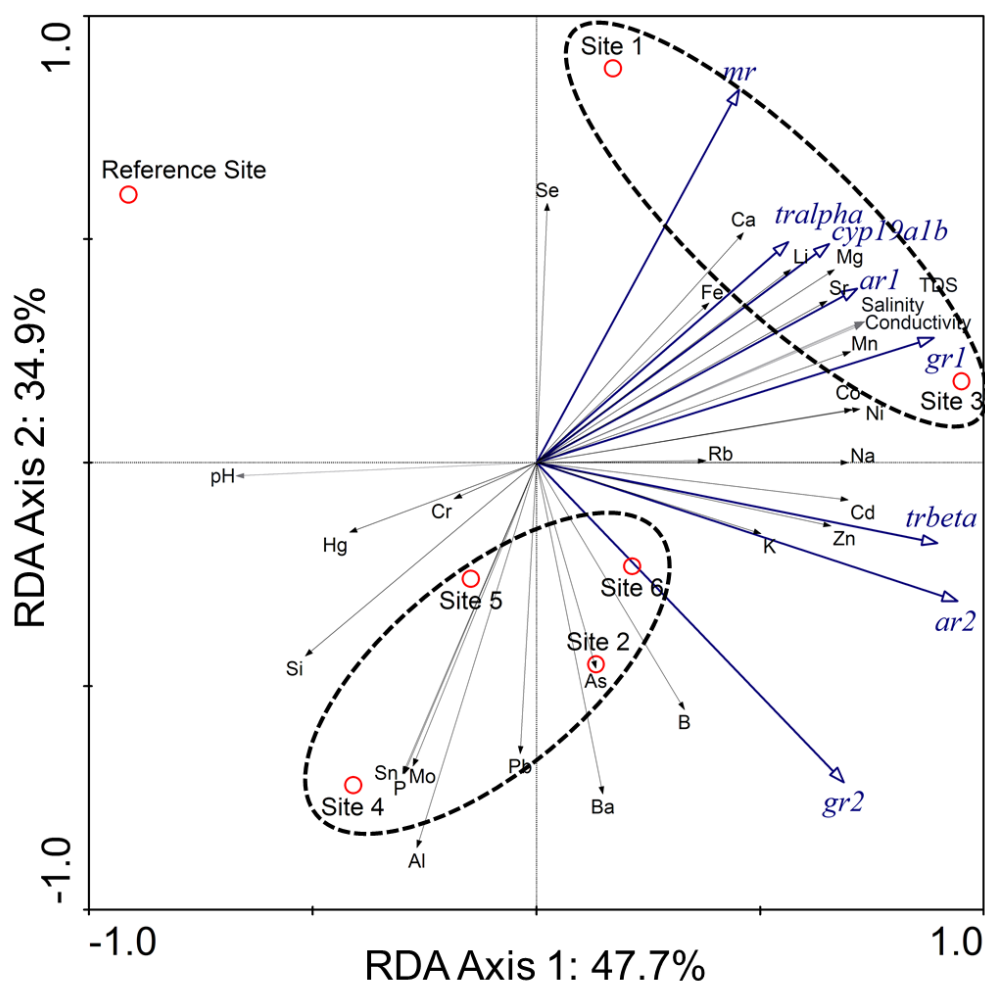


Figure 5.5: A Redundancy Analysis (RDA) triplot indicating the (dis)similarities among the seven sampling locations in regard to the expression of eight genes *thyroid hormone receptor alpha* [*tralpha*], *thyroid hormone receptor beta* [*trbeta*], *androgen receptor-1* [*ar1*], *ar2*, *glucocorticoid receptor-1* [*gr1*], *gr2*, *mineralocorticoid receptor* and *aromatase* [*cyp19a1b*], basic water chemistry parameters (i.e., salinity, electrical conductivity, total dissolved solids and pH) and the concentrations of 23 elements. The mean of gene expression per treatment group was used, and all data applied in the RDA was log transformed.

5.4 Discussion

Acid mine drainage (AMD) water is known to be toxic, being characterized by a low pH accompanied by high metal concentrations and severely impacted watercourses are known to be virtually void of aquatic vertebrates (Parsons, 1977). The treatment/de-toxification of AMD has become more prevalent through processes which in most cases are focused on increasing the pH to near neutral, subsequently causing the precipitation of the majority of metals. Organisms living in streams and rivers receiving treated AMD may, however, still be impacted, seeing that treated AMD is known to still contain inorganic contaminants including certain metals (Cravotta and Trahan, 1999).

The current investigation explored the impacts of a stream catchment receiving a high portion of neutralised AMD (HDS limestone/lime treated) originating from defunct gold mines by assessing the concentrations of selected metals and elements as well as the impact on the aquatic vertebrate endocrine system, using the expression of selected genes in juvenile *O. mossambicus* as biomarkers.

The current sites 1, 2 and 3 within the Tweelapie stream can be generally characterized as mine-impacted, and has very limited other anthropogenic impacts. Sites 4, 5 and 6 are subject to a combination of agricultural impacts (i.e., runoff and/or spray drift) and the effluent of a waste water treatment plant via the respective Riet and Blougat streams. The three distinct groupings of sites observed in the metal-, other element concentrations and water quality data based PCA biplot illustrates the general land cover dependent signature of contamination in the Bloubank stream drainage (i.e., sites 1, 2 and 3: “mine impacted”; sites 4, 5 and 6: “agricultural runoff/WWTP effluent”; reference site: “natural”).

The concentrations of Mn observed at sites 1, 2 and 3 were high ($\sim 10 \text{ mg.L}^{-1}$) relative to natural streams, exceeding the WHO drinking water guidelines (WHO, 2011) and being approximately 200 times higher than the concentration observed at the reference site. The higher concentrations of Mn, Ni, Co, Fe, Ca and Mg, and the associated increased EC and salinity observed at sites 1, 2 and 3 relative to the downstream sites 4, 5 and 6 as well as the reference site corresponds to other reports in the literature regarding contaminant loads in treated AMD (Caraballo *et al.*, 2009; Cravotta and Trahan, 1999). The absence of phosphate in the mine impacted sites 1, 2 and 3, and presence, yet longitudinal decrease from site 4 to site 6 is as expected, seeing that these sites are located downstream of the Riet and Blougat streams hence receiving agricultural runoff and WWTP effluent.

Other elements such as Fe were low relative to other reports of AMD indicating that the HDS plant which discharges into the Tweelopie stream was efficient at the time of sampling. AMD neutralization is known to be effective in the removal of Fe and Al from the water, whereas Mn, Ni, Co in some cases less successfully (Caraballo *et al.*, 2009; Cole *et al.*, 2001; Cravotta and Trahan, 1999; Porter and Nairn, 2010) as is evident in the current results.

Oberholster *et al.* (2013) reported high concentrations of Al ($\sim 312 \text{ mg.L}^{-1}$) and Fe ($79 - 312 \text{ mg.L}^{-1}$) at the exact locations as the current sites 1 and 2. In contrast, Al was not detected in the samples collected from Sites 1 and 2 in the current study and the Fe concentration was at least 500 times lower than those reported by Oberholster *et al.* (2013). The pH of the water from sites 1, 2 and 3 ranged between 3 and 4 in contrast to pH of between 6.58 and 6.98 currently observed (Table 5.1). Oberholster *et al.* (2013) collected water during June 2011 and May 2012, and the HDS plant has been upgraded since then, explaining the improved quality in terms of pH and metal loads currently observed as has been shown in detail by Hobbs (2013).

The expression of eight genes associated with the endocrine system (*thra*, *thrb*, *ar1*, *ar2*, *gr1*, *gr2*, *mr* and *cyp19a1b*) was quantified in 32 dpf *O. mossambicus* juveniles, exposed to water collected from the Bloubank drainage as well as a buffered reverse osmosis water control. In general, the gene expression responses were moderate and very few treatment groups exhibited mRNA transcript abundance which differed significantly from that observed in the reference site or RO water “control” treatments.

The PCA biplot depicting (dis)similarities among the eight treatment groups, based on the expression of *thra*, *thrb*, *ar1*, *ar2*, *gr1*, *gr2*, *mr* and *cyp19a1b* further indicated high intra-treatment and low inter-treatment variation. The high degree of similarity between the responses of fish exposed to water from site 1 (consisting of approximately 95% treated AMD) and the reference site treatment on the PCA biplot is of interest, suggesting limited disruption by neutralized AMD on the expression of a compilation of eight genes selected as representative of the endocrine system, in the study species. Published investigations assessing sub-organismal biological impacts of treated AMD in fish are scant, but generally indicate limited health impairment (Cole *et al.*, 2001; Porter and Nairn, 2010). For example, Cole *et al.* (2001) investigated the impacts of raw and neutralized AMD on the plasma sodium and glucose levels of brook char after short term exposures and observed no significant difference between the neutralized AMD and control treatments. Porter and Nairn (2010) reported no significant differences in hepatosomatic index, condition factor and condition index among bluegill exposed to bed ash or lime treated AMD. The biological impacts of lime treated acidic lakes and rivers (acidified primarily through atmospheric

deposition [Baker *et al.*, 1991]) on fish are more prevalent and furthermore indicates improved fish health and fecundity (Brown *et al.*, 1990; Clair and Hindar, 2005; Sangalang *et al.*, 1990; Vuorinen *et al.*, 2004). The current observation of limited endocrine disruption in the site 1 treatment, based on the expression of a compilation of genes as representative of the endocrine system, therefore, corresponds to previous reports of treated AMD or other acidic waters.

When the expression of the genes is, however, considered independently, some evidence of endocrine disruption associated with treated AMD is evident. In particular, the significant increase in *mr* expression in the site 1 treatment (95% treated AMD) relative to the reference site indicates 'n degree of disruption. Moreover, the higher *gr1* expression in the site 1 and 3 treatments as well as the higher *thra*, *thrb* and *ar1* expression observed in the site 3 treatment relative to the reference site treatment is likely also linked to the contaminants originating from the AMD treatment facility, seeing that site 3 (located marginally downstream of the Krugersdorp Game Reserve) has no other obvious sources of contamination in its catchment. The only other significant difference in gene expression relative to the reference site treatment that was observed was in *gr2*, being higher in the site 2, 3, 4, 5 and 6 treatments.

Corticosteroids (i.e., mineralocorticoids and glucocorticoids) are involved in osmoregulation and stress response and function primarily to regulate homeostatic mechanisms (Hontela, 1998). The expression of *mr*, *gr1* and *gr2* is known to increase in *O. mossambicus* brains in response to salinity and handling stress (Aruna *et al.*, 2012a; Aruna *et al.*, 2012b). The significantly increased expression of *gr1* and *mr* in the site 1 treatment, as well as the higher expression of *gr2* in the site 2, 3, 4, 5 and 6 treatments are, therefore, likely stress related. Aruna *et al.* (2012a) showed co-localization yet differential expression patterns of *gr1*, *gr2* and *mr* among organs associated with osmoregulation in *O. mossambicus*, hence unveiling the complex coordinated response of these corticosteroid receptors to salinity stress. The differential expression currently observed in *gr1*, *gr2* and *mr* transcripts within each treatment group (Figure 5.3), further indicated by the weak correlation observed among these genes in the PCA biplot (Figure 5.4), is not surprising in light of the findings of Aruna *et al.* (2012a), suggesting a complex coordinated (stress) response to the contaminants and other stressors these fish were subject to. The varied expression of *gr1*, *gr2* and *mr* currently observed among treatment is difficult to interpret due to the complex nature of corticosteroid signalling and the fact that the fish were exposed to a diversity of metals, other elements and organic contaminants. Nonetheless, certain anecdotal predictions can be made based on the water chemistry data and the RDA triplot. In particular, *gr2* was weakly correlated with

salinity on the RDA triplot suggesting that salinity stress was not the primary driver of *gr2* expression observed in the site 2, 3, 4, 5 and 6 treatments, and salinity was in fact low at sites 4, 5 and 6. The increased *gr2* expression observed in the fish exposed to water collected from sites 2, 3, 4, 5 and 6 at least suggests that the animals were stressed, although it is difficult to elucidate the stressor. The close correlation between salinity, conductivity and quantitative *gr1* expression on the RDA triplot provides evidence suggesting that the higher *gr1* expression observed at sites 1 and 3, and higher mean expression at site 2 relative to the control and reference site treatments may be linked to increased salinity.

The high concentration of Ca observed at site 1 ($\sim 1 \text{ g.L}^{-1}$) is not surprising, likely originating from the lime (Ca(OH)_2) and limestone (CaCO_3) applied in the HDS plant. The close correlation of *mr* with Ca on the RDA triplot, provides evidence that the increased *mr* expression in the site 1 treatment may be associated with hydromineral balance maintenance, seeing that Ca concentration is known to be a driver of ion exchange in fish (Lin *et al.*, 2011).

A number of metals have been linked to interrenal disruption in teleost fish including Pb (Ramesh *et al.*, 2009), Cu (Craig *et al.*, 2009), Cd, Hg (Hontela, 1998) and Ni (Prophete *et al.*, 2006). We could not locate previous studies indicating altered corticosteroid receptor expression in response to metal exposures in fish. The close correlation of Co, Ni and Mn with *gr1* on the RDA triplot, as well as the close positioning of the site 3 treatment with these metals is of interest. Prophete *et al.* (2006) observed a significant decrease in plasma cortisol levels in Japanese medaka exposed to a low dosage of $125 \mu\text{g.L}^{-1}$ of Ni after 7 days, a concentration which exceeds the current Ni concentrations observed at sites 2 and 3. Nickel may, therefore, have been involved in the altered corticosteroid receptor expression observed at site 1, 2 and 3.

Site three had the highest concentrations of Cd, Ni, Co and Mn among all the sites sampled. The significant increase in *thra*, *thrb* and *ar1* relative to both the control and reference site may be related to the presence of one of these metals or a combined action. The location of site 3 on the RDA triplot provides some anecdotal support for this prediction, and further work such as single and mixtures of metal exposure studies may shed light on this prediction.

Both exposure to Cd and Hg has been shown to alter thyroid hormone concentrations in certain fish (Brown *et al.*, 2004; Carr and Patino, 2011). The altered *thra* and *thrb* expression in the site 3 treatment relative to the other treatments may, therefore, be linked to the higher Cd concentration observed at site 3.

Amutha and Subramanian (2013) observed reproductive disruption associated with a low dosage of 100 ng.L^{-1} of Cd in juvenile, adult male and female *O. mossambicus* after 50 to 150 days exposure. In particular, significant increases in estradiol and testosterone concentrations as well as altered gonadosomatic index were observed. A Cd concentration of 160 ng.L^{-1} was observed at site 3. The significant increase in *ar1* expression observed in the site 3 treatment relative to the reference site and control treatments suggests a degree of reproductive disruption and may be related to Cd exposure.

Interestingly, there was a discrepancy in the gene expression signature of the site 2 treatment relative to the site 1 and 3 treatments, evident in figures 5.4 and 5.5. This segregation is likely associated with indirect or direct targets of *thra* and *mr* expression seeing that both *thra* and *mr* expression was lower in site 2 than both sites 1 and 3, although not significantly in all cases. These differences are, however, difficult to interpret seeing that the said localities are all within the Tweelapie stream and, therefore, subject to no other apparent impacts aside from neutralized AMD. The grouping of sites 1, 2 and 3 on the PCA biplot describing the association between metal and other element concentrations and the different sampling localities suggest that the degree of segregation of site 2 from sites 1 and 3 in terms of gene expression (Figures 5.4 and 5.5) may be the result of other factors such as organic contaminants unaccounted for in the current investigation.

The fact that only site 4 was segregated from both the reference site and the control treatments in the gene expression PCA biplot (Figure 5.4), is as expected because site 4 is the first site downstream of the Percy Steward WWTP as well as the agriculture runoff via the Riet stream and these impacts are known to be prominent sources of EDCs (Diamanti-Kandarakis *et al.*, 2009). A number of contaminants typically found in WWTP effluents including bisphenol A and nonylphenol have been linked to aromatase inhibition in teleost fish (Cheshenko *et al.*, 2008; Wang *et al.*, 2010). The fact that aromatase (*cyp19a1b*) expression was significantly lower in the site 4 than the control and sites 1, 3, 5 and 6 treatments may be associated with the WWTP effluent entering the Bloubank stream via the Blougat stream just prior to site 4. Moreover, certain fungicides are potent aromatase inhibitors in teleost fish (Ankley *et al.*, 2005; Hinfray *et al.*, 2006) and may also be culprits in the altered *cyp19a1b* expression in the site 4 treatment seeing that there are agricultural lands in both the Riet and Blougat stream catchments.

Pb concentrations as low as 1 ng.L^{-1} significantly altered the concentrations of four corticosteroids, estrogen, progesterone and testosterone in catfish ovary tissue exposed *ex vivo* (Chaube *et al.*, 2010). Pb may, therefore, have contributed to the altered aromatase activity observed in the site 4 treatment seeing that the water contained 330 ng.L^{-1} of Pb.

pH was recorded in the different treatments during the commencement of the 48 hour exposure, but unfortunately not during exposure termination. Factors such as the excrements of the fish may have altered pH which could have influenced metal bio availability in the different treatments (Olaniran *et al.*, 2013). pH fluctuation is, therefore, a potential confounding factor in the current investigation in terms of the identification of links between metals and altered gene expression.

5.5 Conclusions

Although little is known regarding the biological impacts of treated/neutralized AMD on aquatic vertebrates, the literature generally suggests that impacts are limited. The current results based on a compilation of eight genes representing the endocrine system also suggest limited disruption in juvenile *O. mossambicus* after short term exposure. When the expression of these genes are, however, considered individually among treatment groups, significant changes in *gr1*, *gr2*, *mr*, *thra*, *thrb* and *ar1* in fish exposed to water containing contaminants originating from an AMD treatment facility were observed. Water chemistry data could be applied to predict links between certain metals and the observed changes in gene expression although these predictions are anecdotal. Gene expression based biomarkers cannot be used as evidence of physiological impairment, but rather functions as a screening tool to assess the need for further investigation (Hutchinson *et al.*, 2006). In this study, we have identified various potential targets of treated AMD on the endocrine system of a teleost fish and further investigations are merited. The static exposure regime and small volume of water applied in the current investigation (i.e., 800 mL) may have reduced the intensity of responses. Chronic exposure studies such as a fish partial life cycle test or the *Xenopus* Metamorphosis Assay will provide further and more concrete insight into the endocrine disruptive potency of neutralized AMD (Opitz *et al.*, 2005; OECD, 2011). The occurrence and nature of AMD in terms of chemical composition is dependent on the mineralogical identities of mines and may be variable among regions (Valente and Gomes, 2009). The nature of treated AMD may, therefore, vary similarly as well as the associated biological impacts, and it is important to assess such impacts at local scale.

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Chapter 6: An evaluation of the endocrine disruptive potential of crude oil water accommodated fractions and crude oil contaminated surface water to freshwater organisms using *in vitro* and *in vivo* approaches

Submitted for publication in Environmental Toxicology and Chemistry

Declaration by the candidate

With regard to Chapter 6, the nature and scope of my contribution were as follows:

Nature of contribution Extent of contribution (%)

Nature of contribution	Extent of contribution
Conceptual design, experimental work, manuscript writing.	75%

The following co-authors have contributed to Chapter 6:

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Abstract

Knowledge regarding the potential impacts of crude oil on endocrine signalling in freshwater aquatic vertebrates is limited. In this study, the expression of selected genes as biomarkers for altered endocrine signalling were studied in African clawed frog, *Xenopus laevis* tadpoles, and juvenile Mozambique tilapia, *Oreochromis mossambicus* exposed to weathered bunker and un-weathered refinery crude oil water accommodated fractions (WAFs). In addition, the expression of the aforementioned genes was quantified in *X. laevis* tadpoles exposed to surface water collected from the proximity of an underground oil bunker. The (anti)estrogenicity and (anti)androgenicity of crude oil, crude oil WAFs and surface water were furthermore evaluated using recombinant yeast. *Thyroid hormone receptor beta* expression was significantly downregulated in *X. laevis* in response to both oil WAF types, whereas, a further thyroid linked gene, *type 2 deiodinase* was upregulated in *O. mossambicus* exposed to a high concentration of bunker oil WAF. In addition, both WAFs altered the expression of the adipogenesis-linked *peroxisome proliferator-activated receptor gamma* in *X. laevis*. The crude oil and WAFs exhibited anti-estrogenic and anti-androgenic activity *in vitro*. However, *O. mossambicus androgen receptor 2* was the only gene, representing the reproductive system, significantly affected by WAF exposure. Estrogenicity, anti-estrogenicity and anti-androgenicity were detected in surface water samples; however, no significant changes were observed in the expression of any of the genes evaluated in *X. laevis* exposed to surface water. The responses varied among the two model organisms used, as well as among the two types of crude oil. Nonetheless, my data provide evidence that crude oil pollution may lead to adverse health effects in freshwater fish and amphibians due to altered endocrine signalling.

6.1 Introduction

Crude oil pollution is known to be hazardous to wildlife and a global environmental concern. Terrestrial areas and freshwater systems are frequently contaminated with crude oil due to natural disasters, war and leakages in pipelines (Vandermeulen and Ross, 1995), and some of the largest oil spills in history have been on land (e.g., Kuwait due to the Gulf War, Mingbulak oil spill in the Fergana Valley, Uzbekistan, and the Usink oil spill in Northern Russia). The amount of published research describing effects of crude oil on freshwater organisms is, however, only a fraction of the literature on marine species, and was estimated as 5.5% in 1995 (Vandermeulen and Ross, 1995).

The endocrine system plays a key role in the regulation of various biological processes including growth and development, reproduction, immunity and metabolism. A number of

compounds originating from crude oil or other fossil fuels have been shown to modulate the endocrine systems of vertebrates. Some examples of such endocrine disruptive hydrocarbons include certain polycyclic aromatic hydrocarbons (PAHs) (Hawliczek *et al.*, 2012), alkyl phenols (Lee *et al.*, 2003) and naphthenic acids (Wang *et al.*, 2015). However, although the understanding of biological action associated with a single chemical is helpful to predict hazards to wildlife, it is well known that chemicals may react differently when in mixture (Kortenkamp, 2007), and such interactions need to be accounted for when polluted natural systems such as rivers and lakes are considered. Crude oil consists of a complex mixture of potentially hazardous chemicals, and the “mixture-effect” is, therefore, important to account for in risk assessments.

Surprisingly little is known regarding the impacts of crude oil on endocrine signalling in aquatic vertebrates, and these studies are generally biased towards marine fish (Alkindi *et al.*, 1996; Stephens *et al.*, 1997a; Martin-Skilton *et al.*, 2006; Tollefsen *et al.*, 2011; Rhee *et al.*, 2013), whereas peer reviewed publications on freshwater fish are limited (Arukwe *et al.*, 2008; Salaberria *et al.*, 2014; Kim *et al.*, 2016), and amphibian studies non-existent. Conversely, the endocrine disruptive potential of oil sands process-affected water, and produced water from North Sea offshore oil platforms have received more attention (Hersikorn and Smits, 2011; Kavanagh *et al.*, 2013; Bakke *et al.*, 2013).

In order to mimic the natural dispersal of crude oil in waterbodies, crude oil assessment exposure experiments mostly include a natural (physical) water soluble fraction (WSF), a water accommodated fraction (WAF), or a chemical enhanced water accommodated fraction (CEWAF) of the oil being studied. The WSF and WAF provide a realistic representation of the hydrocarbons aquatic animals will be directly exposed to within the water column during an oil spill. A further advantage of the use of WSFs or WAFs in toxicity studies is more repeatable results that are comparable to other literature reports (Singer *et al.*, 2000; Barron and Ka'aihue, 2003; Aurand and Coelho, 2005).

Certain crude oils have been shown to exhibit estrogenic, anti-estrogenic and anti-androgenic activity *in vitro* (Vrabie *et al.*, 2010; Vrabie *et al.*, 2011). The question, however, remains to what extent the crude oil WAF exhibit similar potential than the oil itself and, therefore, to what extent endocrine disruptive hydrocarbons are taken up in the water column. A further question is whether the response predicted by *in vitro* assays for a particular oil and its WAF will occur in exposed animals. Although the majority of sex and thyroid hormones are conserved among vertebrates, sensitivity towards hormone receptor agonists and endocrine disruptors with other modes of action, may vary markedly among taxa (Schiller *et al.*, 2014), even at species level (Lange *et al.*, 2012). Variable sensitivity

among wildlife species is, therefore, an important factor to account for when the risk/hazard of a particular substance is assessed. Therefore, in terms of assessing anthropogenic effects on freshwater systems, in particular aquatic vertebrates, the use of both fish and amphibian species is, therefore, favourable (Kerby *et al.*, 2015).

Weathering of oil, either as a spill or as bunkered oil, may significantly affect the toxicity associated with a particular crude oil (Neff *et al.*, 2000). Although it is generally thought that degradation will lead to a decrease in toxicity, it may not always be the case (Holth *et al.*, 2014), and certain derivatives of for instance PAHs are in fact more biologically active than the parent compounds (Andersson and Achten, 2015). Studies comparing the biological activity of weathered/aged versus non-weathered oil to vertebrates are, however, limited (Incardona *et al.*, 2013; Incardona *et al.*, 2014; Esbaugh *et al.*, 2016), and to my knowledge the effect of weathering on endocrine disruptive potential is yet to be tested.

In this study, a combination of *in vitro* and *in vivo* bioassays were applied to evaluate the endocrine disruptive potential of aged crude oil collected from an underground oil bunker as well as “fresh” oil obtained from a refinery. The investigation furthermore included the evaluation of the WAFs of the said crude oils, as well as surface water collected in the close proximity of the bunker from which crude oil was collected.

The aims of the present investigation were: (1) to describe the potential effects of crude oil exposure on the expression of selected genes associated with the thyroid hormone system, adipogenesis and reproduction in two freshwater model organisms: African clawed frog *Xenopus laevis* tadpoles and juvenile Mozambique tilapia *Oreochromis mossambicus*; (2) to describe potential estrogen and androgen receptor agonistic and antagonistic activity associated with crude oil and crude oil WAFs, using yeast reporter gene assays; (3) to, by use of the aforementioned *in vivo* and *in vitro* assays, compare the biological activity weathered crude oil collected from an underground crude oil bunker and un-weathered oil obtained from a refinery using the aforementioned bioassays; (4) evaluate the endocrine disruptive potential of surface water collected from two freshwater pans and two streams located in the close proximity of an underground oil bunker.

The expression of *thyroid hormone receptor beta (thrb)* and *deiodinase type 2 (dio2)* were described as representatives of thyroid signalling, because both these genes are integral counterparts within the thyroid cascade (Brown *et al.*, 2004). *Peroxisome proliferator-activated receptor gamma (pparg)* expression was described as representative of adipogenesis, being a well-known molecular target of endocrine disruptors which drive a positive energy balance (i.e., obesogens) (Grün and Blumberg, 2009). As representatives of

the reproductive system, *cytochrome P450, family 19, subfamily a, polypeptide 1 (cyp19a1)* (aromatase), *androgen receptor (ar)* and *steroid 5 alpha-reductase type 1 (srd5a1)* were quantified in *X. laevis*, whereas *cyp19a1b*, *ar1* and *ar2* expression was described in *O. mossambicus*. Aromatase regulates estrogen synthesis (Diotel *et al.*, 2010), whereas *ar* and *srd5a1* are principally involved with the regulation of androgen action (Langlois *et al.*, 2010). Amphibians and most other vertebrates have a single aromatase coding gene (*cyp19a1*), whereas teleosts have two, *cyp19a1a* and *cyp19a1b*, expressed predominantly in the ovary and brain, respectively (Diotel *et al.*, 2010). Similarly, teleosts have two androgen receptor genes, *ar1* and *ar2*, whereas other vertebrates including amphibians have only one (Lorin *et al.*, 2015). The different aromatase and androgen receptor genes evaluated among species in the present study were, therefore, due to genetic differences among the species studied. 11 Ketotestosterone is the principal and most biologically active androgen in teleost fish, whereas, unlike in amphibians, birds and mammals, dihydrotestosterone (DHT), and therefore steroid 5 alpha reductase (Srd5a), plays a less prominent role in the regulation of the male reproductive system (Martyniuk *et al.*, 2014). The expression of *srd5a1* was, therefore, only described in *X. laevis* tadpoles.

6.2 Materials and Methods

6.2.1 Crude oil

Weathered crude oil was obtained from a coal mine, transformed into an underground storage bunker. The crude oil has been in storage for at least 30 years, and portions of the oil mass were in contact with groundwater during this period. The specific bunker sampled is one of three located in the region. The location and further details regarding these bunkers are classified. Fresh crude oil was furthermore provided by Chevron (Cape Town Refinery, South Africa). The oil samples were maintained in the dark at 4 °C in sealed glass containers.

6.2.2 Water accommodated fraction preparation

Water accommodated fractions (WAF) of crude oil were prepared as prescribed by Singer *et al.* (2000), with slight modifications, as described in detail in appendix 1 of the supporting material. The WAF stock solutions were prepared using 25 g of crude oil per litre of buffered reverse osmosis (RO) water (containing 250 mg.L⁻¹ marine salt and 80 mg.L⁻¹ NaHCO₃, pH 7). Different concentrations of WAFs were prepared from the 25 g.L⁻¹ stock, and hence following variable dilution approach suggested by Barron and Ka'aihue (2003).

6.2.3 Sampling locations and surface water collection

Surface water was collected from two pans located in the close proximity of an underground oil bunker (Pan1 and Pan2), as well as from two different streams (Stream1 and Stream2), which has the underground bunker within their catchments (Figure 6.1). Ground water is periodically pumped out of the underground bunker, to prevent the oil mass from rising to the surface. The extracted water, however, contains crude oil clusters, which are removed using an oil-water separation plant. The water fraction is subsequently released through a pipeline into Pan1, located near an active open-cast coal mining site (Figure 6.1). Pan2 is surrounded by dry-land agriculture land use, and located near the closed-up entrance of the historic coal mine (converted to an oil bunker) (Figure 6.1). The land use upstream of the Stream1 site consists of dry-land agriculture and grazing areas, whereas the Stream2 site is located downstream of dry-land agriculture and active coal mines.

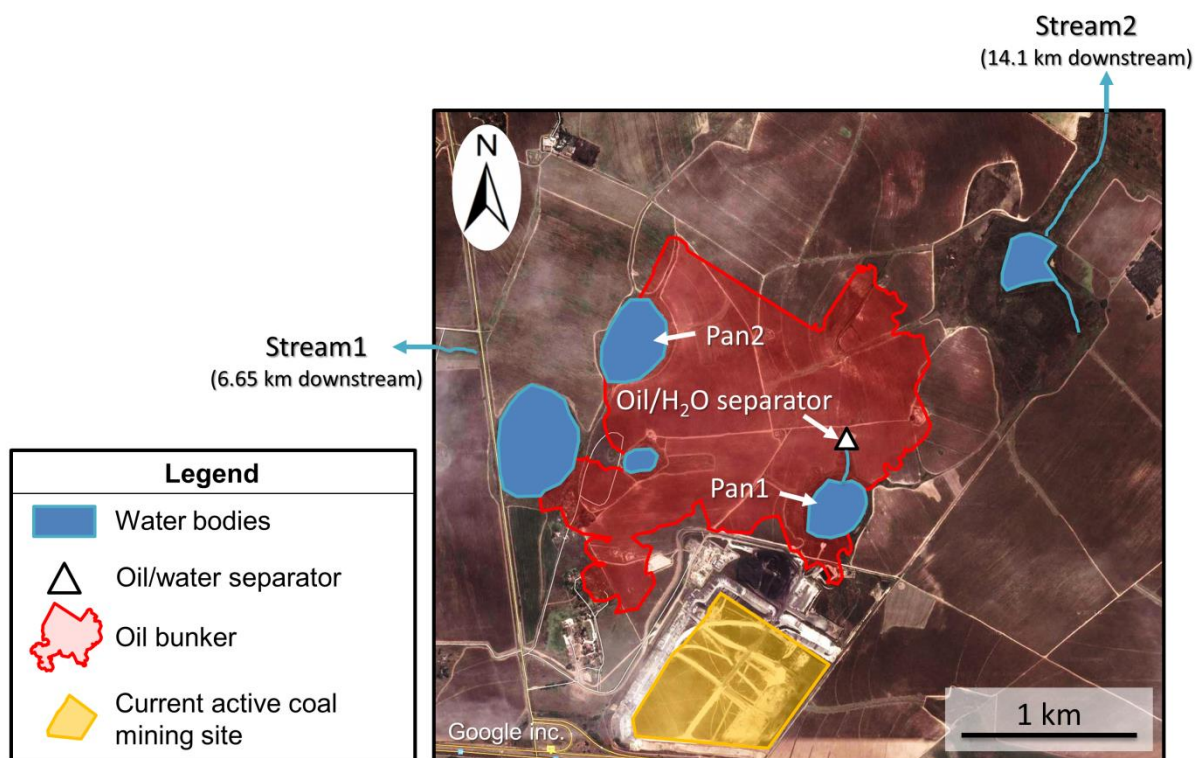


Figure 6.1: Map indicating the study area. The location is confidential and GPS coordinates are, therefore, not provided.

6.2.4 C18 solid phase extraction

Non-polar and slightly polar organic compounds were extracted from surface water samples collected during January, May, August and November 2014, as well as crude oil WAFs, using 6 mL 500 mg Discovery® DSC18 solid phase extraction (SPE) columns (Supelco,

Sigma, ZA) under vacuum (Visiprep manifold, Supelco, Sigma, ZA) at ~5 mL/min. Surface water samples were passed through 1.2 µm glass fibre filters (Munktell, DE) prior to C18 extraction. The SPE columns were conditioned using absolute methanol and ultrapure H₂O. Organic extracts were eluted from dried SPE columns using a solvent mixture (40% hexane, 45% methanol, 15% isopropanol). Eluents were subsequently dried under a gentle stream of N₂ (WAFs), or air (surface water samples), and reconstituted in absolute ethanol (EtOH). The WAF samples assigned to gas chromatography (GC) were eluted from SPE columns using ethyl acetate. A negative reverse osmosis (RO) water control was included during each extraction event.

6.2.5 Yeast bioassays

Estrogenic, anti-estrogenic, androgenic and anti-androgenic activity of crude oil, WAFs and surface water C18 extracts were evaluated using the Yeast Estrogen Screen (YES), Yeast Anti-estrogen Screen (YAES), Yeast Androgen Screen (YAS), and Yeast Anti-androgen Screen (YAAS) recombinant yeast bioassays (See section 3.2.4 of this dissertation).

6.2.5.1 Yeast exposures

Bunker and refinery crude oil were diluted in absolute ethanol (100 mg per 1 mL), sonicated using an Omni Ruptor ultrasound sonicator (Omni-ruptor 400, Omni International Inc., USA) at 20% power for 20 seconds, vortexed for 15 seconds, centrifuged for 5 minutes at 1000 x g, 4 °C and stored at -20 °C. A 12 point two-fold dilution series of bunker crude oil in EtOH ($1.22 \times 10^{-3} \text{ g.L}^{-1}$ to 2.5 g.L^{-1}), as well as concentrated WAF C18 extracts of both oil types (0.31 g.L^{-1} to 625 g.L^{-1}) were tested using YES, YAES, YAS and YAAS. Surface water C18 extracts were assayed at a 10x concentrated state (1x concentrated denotes the state in nature) allowing higher assay sensitivity, and were also screened using all four yeast bioassays. Each assay plate included a 12 point two-fold serial dilution of the appropriate hormone or chemical standard (i.e., E₂: $2.72 \times 10^{-7} \text{ g.L}^{-1}$ – $1.33 \times 10^{-10} \text{ g.L}^{-1}$; TAM: $18.57 \times 10^{-5} \text{ g.L}^{-1}$ – $9.07 \times 10^{-8} \text{ g.L}^{-1}$; DHT: $1.45 \times 10^{-5} \text{ g.L}^{-1}$ – $7.09 \times 10^{-9} \text{ g.L}^{-1}$; FLU: $27.62 \times 10^{-3} \text{ g.L}^{-1}$ – $1.35 \times 10^{-5} \text{ g.L}^{-1}$). The wells assigned to screen anti-estrogenicity and anti-androgenicity were supplemented with 1.43 nM E₂ and 7.14 DHT nM respectively. All standards, crude oil and environmental samples were loaded to assay plates using 10 µL EtOH which was then evaporated to dryness, prior to the introduction of yeast-containing medium (Vrabie *et al.*, 2010). All samples and standards were tested in duplicate on at least two assay plates, supplied with different yeast stocks and loaded independently with oil or C18 extracts. At least six wells loaded with solvent only were included per plate.

6.2.5.2 Calculations

"Absolute" EC50 or IC50 values for E₂, DHT, TAM and FLU as well as crude oil and crude oil WAFs were calculated according to the guideline of Sebaugh (2011) using GraphPad Prism V6 (GraphPad Software, USA). Absorbance values (OD540 nm) were normalised to cell density (OD620 nm) and expressed as agonistic activity (AA) (equation 1) (section 3.2.4.3). AA was further normalised using the solvent control AA and maximal response AA and expressed as a percentage, where 100% denotes the maximal response and 0% that of the solvent blank (negative control), indicative of zero hormone receptor agonism (equation 2). The assay wells representing the maximal response were in all cases from the hormone (E₂, DHT) or chemical (TAM, FLU) standard range.

$$\text{Agonistic Activity (AA)} = \text{Abs 540 nm}_{(\text{sample})} - [\text{Abs 620}_{(\text{sample})} - \text{Mean Abs 620 nm}_{(\text{solvent control})}] \quad (1)$$

$$\text{Normalised AA (NAA)} = \frac{\left| \left(\frac{\text{AA}_{(\text{sample})}}{\text{Mean AA}_{(\text{solvent control})}} \right) - 1 \right|}{\left| \left(\frac{\text{AA}_{(\text{max})}}{\text{Mean AA}_{(\text{solvent control})}} \right) - 1 \right|} \times 100\% \quad (2)$$

Crude oil potency was expressed as E₂, TAM, DHT and FLU induction equivalents (e.g., IEQ_{E2}, µg [E₂ potency]/g [crude oil]), calculated as follows (Ziccardi *et al.*, 2002):

$$\text{IEQ}_{\text{E}_2} = \frac{\text{EC50}_{\text{E}_2}}{\text{EC50}_{\text{crude oil}}} \quad (3)$$

$$\text{IEQ}_{\text{TAM}} = \frac{\text{IC50}_{\text{TAM}}}{\text{IC50}_{\text{crude oil}}} \quad (4)$$

In addition, TAM and FLU equivalents (TEQ and FEQ) were calculated for 100% bunker and refinery oil WAFs (25 g.L⁻¹). In particular, firstly, the expected NAAs for 25 g.L⁻¹ were calculated through interpolation, using regressions function obtained from the WAF NAA data. TEQs and FEQs were subsequently calculated through interpolation, using the expected WAF NAAs interpolated, using the TAM and FLU regression functions derived from the standard curves generated during the present experiment. Estradiol, dihydrotestosterone, tamoxifen and flutamide equivalents for surface water samples were calculated through interpolation, using plate-specific regression functions (of the appropriate

standard) (Section 3.2.4.3; Grover *et al.*, 2011). Cell densities (OD 620 nm) less than three standard deviation values lower than the mean of the solvent blank wells were considered cytotoxic, and samples below this limit were excluded from the analysis.

6.2.6 Tadpole and fish exposures to crude oil WAF

6.2.6.1 Breeding

Frog spawning and fertilization was induced with human chorionic gonadotropin (HCG), administered subcutaneously into the dorsal lymph sacs of adult *X. laevis*. Male and female frogs were primed with 100 IU and 50 IU HCG respectively, followed by a final 100 IU for males and 400 IU for females. *Oreochromis mossambicus* is a (maternal) mouth brooder. Adult *O. mossambicus* were allowed to breed naturally and larvae removed from females' mouths once yolk sacs were resorbed (12 dpf) (Reddy and Lam, 1992).

Frog tadpoles and juvenile fish were maintained in aerated buffered RO water (250 mg iodated marine salt, 80 mg NaHCO₃ per litre) until the appropriate developmental stage was reached. Tadpoles were fed Sera Micron (Sera, USA) and fish tilapia pellets (AquaNutro, ZA), at least four times per week during the rearing period.

6.2.6.2 Exposures

Each treatment (surface water, crude oil WAF or negative control) was represented by two replicates containing individuals sourced from separate females. Each replicate tank contained 15 tadpoles during the WAF exposure. The fish WAF exposure was performed using 10 individuals per replicate, as was the case for the tadpole surface water exposure.

Tadpole exposure commenced at Nieuwkoop and Faber (Nieuwkoop and Faber, 1994) NF stage 48, whereas 29 – 31 dpf fish were used. The WAF treatment groups included 0.025, 0.25 and 2.5 g.L⁻¹ refinery oil WAF and 0.025, 0.25, 2.5 and 25 g.L⁻¹ bunker oil WAF, and a negative control (buffered RO water) (0.8 L liquid in 1 L glass vessels). A 25 g.L⁻¹ treatment could not be included for the refinery oil WAF because 100% mortality occurred at this concentration. The surface water treatment groups included: Pan1, Pan2 (75% diluted based on preliminary toxicity data), Stream1 and Stream2, and a negative control (2 L liquid in 3 L glass vessels). The aforementioned environmental samples were collected during January 2014 and stored at 4 °C in the dark for 14 to 15 days before the exposures commenced.

The tadpoles and fish were transferred to glass exposure vessels containing buffered RO water, at least 20 h prior to exposure, in order to acclimate to physical conditions (i.e., vessel size, liquid volume and temperature). The tadpoles and fish were then exposed for 24 h at

23 ± 1 °C, and 24 ± 1 °C respectively, and subject to a 14h:10h light:dark cycle. Surface water treatments were constantly aerated, but WAF treatments not. Tadpoles assigned to surface water were fed (~70 mg of Sera Micron (Sera, USA) per tank) throughout acclimation and exposures, seeing that fasting may alter the expression of thyroid linked genes, and the surface water samples likely contained algae and other micro-organisms to forage on (Truter *et al.*, 2014). The WAF exposure tadpoles and fish were only fed during the acclimation period. All tadpoles and fish were euthanized in 0.1% Benzocaine (Heynes Mathew, Ltd., South Africa), snap frozen in liquid nitrogen or placed directly into 900 µL of TRIreagent (Sigma, ZA) and stored at -80 °C.

Animal husbandry, treatment and handling were performed according to the South African Standard: the care and use of animals for scientific purposes (SANS 10386:200X) and under the approval of the Stellenbosch University Research Ethics Committee: Animal Care and Use (Protocol: SU-ACUM14-00002).

6.2.7 RNA isolation and cDNA synthesis

Homogenates of tadpoles and fish were prepared in TRIreagent using an ultrasound sonicator (Omni-ruptor 400, Omni International Inc., USA). Total RNA was isolated from tadpole and fish homogenates according to the TRIreagent technical bulletin. The RNA was subsequently DNase I treated (Zymo Research, USA), and complementary DNA (cDNA) was prepared from 2 µg of total RNA in 20 µL reaction volumes using Revert-Aid (tadpoles) and Maxima H-minus (fish) reverse transcriptase kits (Thermo Scientific, USA) as prescribed by the manufacturer. The reverse transcription reactions were performed using a combination of oligo(dT)₂₃ primers and random nonamers.

6.2.8 RT-qPCR

Gene expression was evaluated in tadpole and fish whole body homogenates through real-time RT-qPCR with *actin beta* (*actb*) as reference gene using a CFX-96 light cycler (Bio-Rad, DE). The PCRs were performed as 15 µl reactions containing 2 µl cDNA as template (5 ng/reaction for *actb* and 10 ng/reaction for the other genes), 7.5 µl Jumpstart® SYBRgreen mix (Sigma, ZA), 0.33 µM of each primer and nuclease free water. The PCR programs for all primer pairs included an enzyme activation step at 95 °C (9 minutes), followed by 40 cycles of denaturing at 95 °C (15 seconds), annealing at 55 – 63 °C (30 seconds) (Supporting Tables S6.1 and S6.2) and elongation at 72 °C (45 seconds). Each PCR plate contained an internal non-template control (no cDNA). In order to determine plate-specific PCR efficiency, a six point two-fold serial dilution of cDNA transcribed from the RNA of a negative control

exposed individual was included per plate. All samples and controls were run in triplicate and amplicon quality was assessed through melting curve analyses.

Gene expression was quantified using the Pfaffl method (Pfaffl, 2001), relative to the control treatment group per experiment. Amplification efficiencies were determined for each primer pair per PCR programme. Outliers were identified per treatment group using Grubbs' test (Burns *et al.*, 2005) and removed. Primer sequences were either obtained from the literature or designed against the appropriate Genbank sequences using Primer Premier 6 (Premier Biosoft, USA). The sequences and sources of the primers are described in tables S6.1 and S6.2. The PCR products of *X. laevis thrb*, *dio2*, *pparg*, *cyp19a1*, *ar*, *srd5a1* and *actb* were sequenced by the Stellenbosch Central Analytical Facility to confirm the validity of the primers. The *X. laevis thrb* primers amplifies the *thrb-a*, *thrb-b1* and *thrb-b2* transcripts, whereas all the other primers are specific, amplifying a single transcript.

6.2.9 PAH analysis

The concentration of the 16 EPA priority PAHs were determined in two independent crude oil WAF preparations per crude oil type. Chromatographic analysis of the PAHs was performed using a Thermo Scientific TRACETM 1310 gas chromatograph coupled to a TSQ 8000 triple quadrupole MS, (Thermo Scientific, Italy). Separation was performed on a non-polar (95% dimethylpolysiloxane) Rxi ®-5Sil MS w/Integra-Guard® (15 m, 0.25 mm ID, 0.25 µm film thickness) capillary column (Part number: 13620-127, Restek, USA). The oven temperature was programmed initially at 75 °C, held for 5 min, then raised at a rate of 10 °C/ min up to 280 °C and held for 3 minutes and finally up to 300 °C and a final hold time of 5 minutes. The samples were injected on a GC injector temperature maintained at 250 °C operated in a splitless mode. Helium at a flow rate of 1 mL/min was used as carrier. The transfer line temperature was held at 300 °C. The ionization source temperature was set at 250 °C. Emission current was 75 µA with argon as collision gas. The WAF samples were tested in conjunction with an eight point dilution series of a PAH reference standard mixture containing the 16 compound tested for (EPA 8270 LCS Mix, Supelco, Sigma). An ultrapure water blank was also included in the analyses. Each sample, standard and blank was supplemented with deuterated internal standards (naphthalene-d₈; acenaphthene-d₁₀; chrysene-d₁₂; perylene-d₁₂, 25 µg.L⁻¹) prior to the C18 solid phase extraction. The samples were eluted using 4 mL ethyl acetate (EA) which was subsequently dried to an approximate volume of 100 µL under a gentle stream of N₂, and then supplemented with EA to a final volume of 250 µL. The LOD was deemed as the lowest point on the calibration curve of each compound.

6.2.10 Statistical analyses

Normality and homogeneity of variance of the data were assessed using the respective Shapiro-Wilk's and Levene's tests. Boxcox transformations were applied to datasets in which the homogeneity of variance assumption was not met. Non-normal data was analysed using the Kruskal-Wallis ANOVA followed by multiple comparisons of mean ranks to assess pairwise differences. Parametric data was analysed using one-way ANOVA followed by Tukey HSD Spjotvol/Stoline unequal N post hoc test. All linear analyses were performed using Statistica version 12 (StatSoft, Inc., 2015). Probability values (P) lower than 0.05 were deemed significant.

6.3 Results

6.3.1 Gene expression biomarkers

The expression of the thyroid-linked *thrb* was significantly downregulated in tadpoles exposed to 0.25 g.L⁻¹ of bunker and refinery oil WAF, but did not vary significantly from the control in any of the other concentrations tested (Figure 6.2a). In *O. mossambicus*, WAF exposure had no significant effect on *thrb* expression. In contrast, *dio2* expression was significantly upregulated in fish exposed to a high dosage of 25 g.L⁻¹ bunker crude oil WAF (Figure 6.3a). The adipogenesis-linked *pparg* exhibited a U-shaped dose response in tadpoles exposed to bunker oil WAF, and was significantly downregulated at 0.25 and 2.5 g.L⁻¹ WAF, and significantly upregulated at 25 g.L⁻¹ (Figure 6.2b). Conversely, *pparg* expression was not significantly downregulated in any of the refinery oil WAF treatments, yet significantly upregulated at 2.5 g.L⁻¹, whereas a response to 25 g.L⁻¹ could not be evaluated due to 100% mortality. Unlike in *X. laevis*, *pparg* expression did not vary significantly among WAF exposed *O. mossambicus* and the controls. Moreover, the expression of *ar*, *srd5a1* and *cyp19*, representing the reproductive system, did not vary significantly among bunker or refinery crude oil WAF exposed *X. laevis* tadpoles and controls. Similarly, the expression of both *ar1* and *cyp19a1b* was unchanged in *O. mossambicus* exposed to crude oil WAFs. The expression of *ar2* was, however, significantly higher in fish exposed to 25 g.L⁻¹ bunker oil WAF than the control treatment (Figure 6.3b). There were no significant differences in the expression of *thrb*, *dio2*, *ar*, *srd5a1*, *cyp19* and *pparg* in *X. laevis* tadpoles exposed to surface water relative to the control treatment.

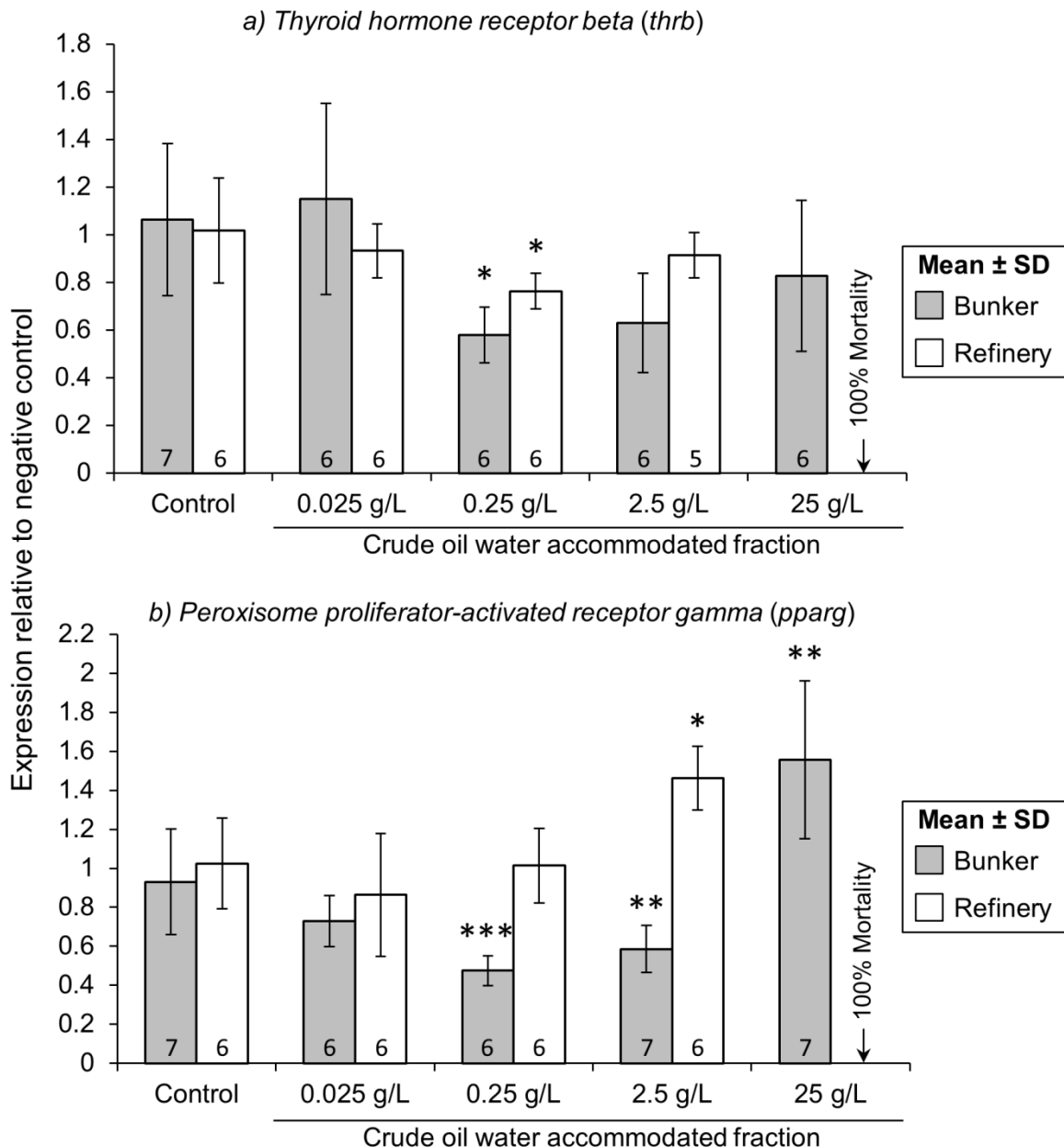


Figure 6.2: Expression of (a) *thyroid hormone receptor beta (thrb)* and (b) *peroxisome proliferator-activated receptor gamma (pparg)* in *Xenopus laevis* tadpoles (Nieuwkoop and Faber (Nieuwkoop and Faber, 1994) stage 48 whole body homogenates) exposed for 24 h to the water accommodated fractions (WAF) of crude oil obtained from and underground bunker as well as a refinery. The concentrations given portray the mass represented relative to the original crude oil mass used to prepare the WAF and 25 g.L⁻¹, therefore, equates 100% WAF. Asterisks indicate significant differences (Tukey HSD *post hoc* test with Spjotvolle/Stoline correction, **P* < 0.05; ***P* < 0.01, ****P* < 0.001). The numbers within the horizontal bars indicate the number of tadpoles representing each treatment.

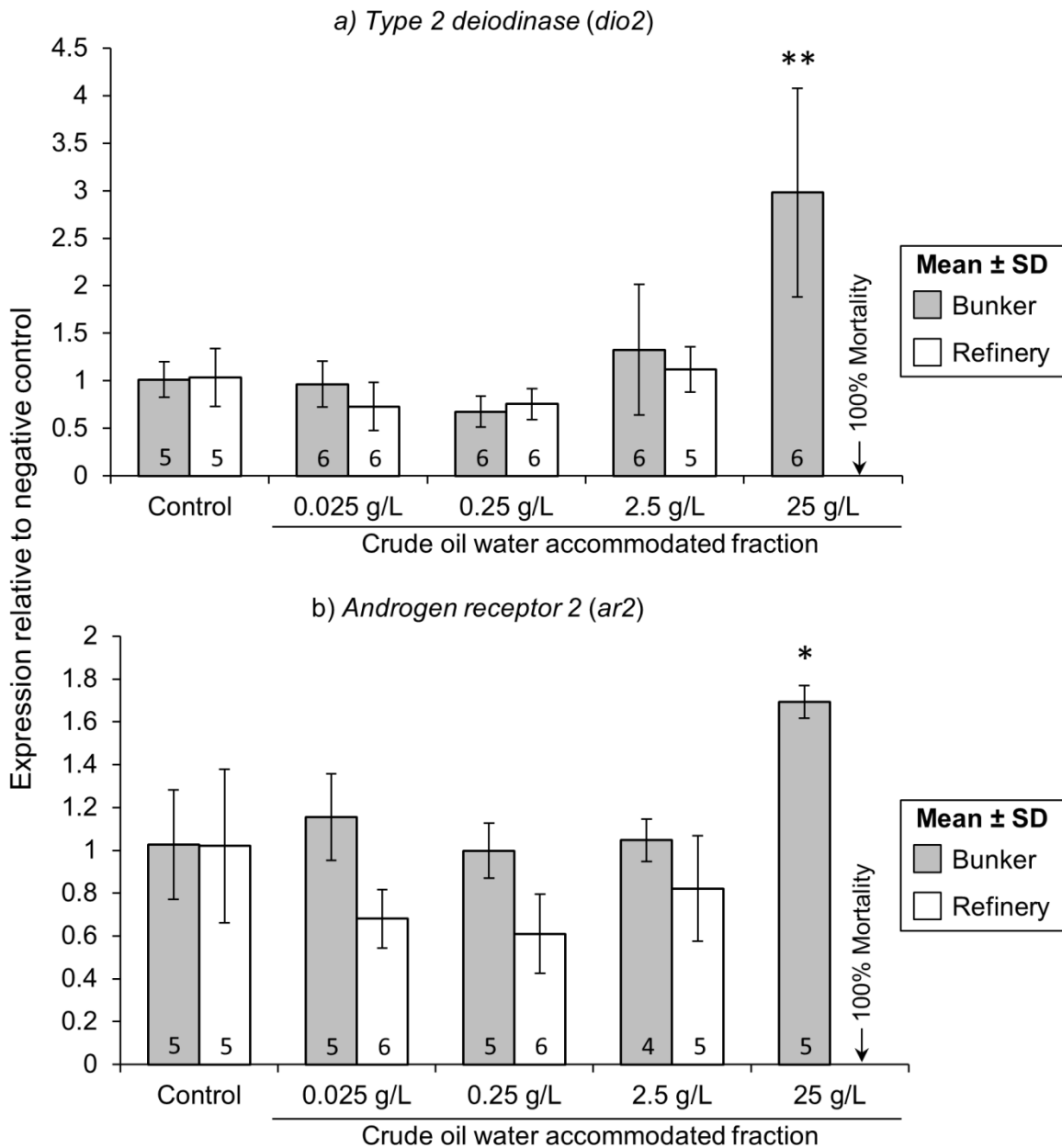


Figure 6.3: Expression of (a) *deiodinase type 2 (dio2)* and (b) *androgen receptor 2 (ar2)* in 29 – 31 dpf *Oreochromis mossambicus* (whole body homogenates) exposed for 24 h to the water accommodated fractions of crude oil obtained from and underground bunker as well as a refinery. The concentrations given portray the mass represented relative to the original crude oil mass used to prepare the WAF and 25 g.L⁻¹, therefore, equates 100% WAF. Asterisks indicate significant differences (Tukey HSD *post hoc* test with Spjotvolle/Stoline correction, **P* < 0.05; ***P* < 0.01, ****P* < 0.001). The numbers within the horizontal bars indicate the number of fish representing each treatment.

6.3.2 Yeast bioassays

Both the bunker and refinery oils, as well as WAFs, exhibited anti-estrogenic and anti-androgenic action *in vitro* (Figure 6.4, Table 6.1). Both oil types (diluted in ethanol), however, did not inhibit up to 50% of ESR1 mediated transactivation within the range of concentrations tested (Figure 6.4a). Conversely, the bunker and refinery WAFs inhibited ESR1 transactivation more extensively, although, at high concentrations, with IC50s of 44.57 and 306 g.L⁻¹, respectively, being observed (Figure 6.4a, Table 6.1a). The crude oils and WAFs were more potent in regard to inhibition of AR transactivation in comparison to ESR1 (Figure 6.4b, Table 6.1b). The crude oil itself inhibited AR transactivation at concentrations nearly three orders of a magnitude lower than the WAFs (Figure 6.4b, Table 6.1b). Moreover, the refinery oil and refinery oil WAF were more anti-androgenic than the bunker oil and bunker oil WAF (Figure 6.4b, Table 6.1b). The pure bunker and refinery oil WAFs (100%, 25 g.L⁻¹) had TAM equivalents (TEQs) of 26.34 and 81.61 µg.L⁻¹ respectively, and FLU equivalents (FEQs) of 0.56 and 3.34 mg.L⁻¹ (Table 6.1). The bunker and refinery crude oil and the associated WAFs did not exhibit estrogenic or androgenic activity (Table 6.1).

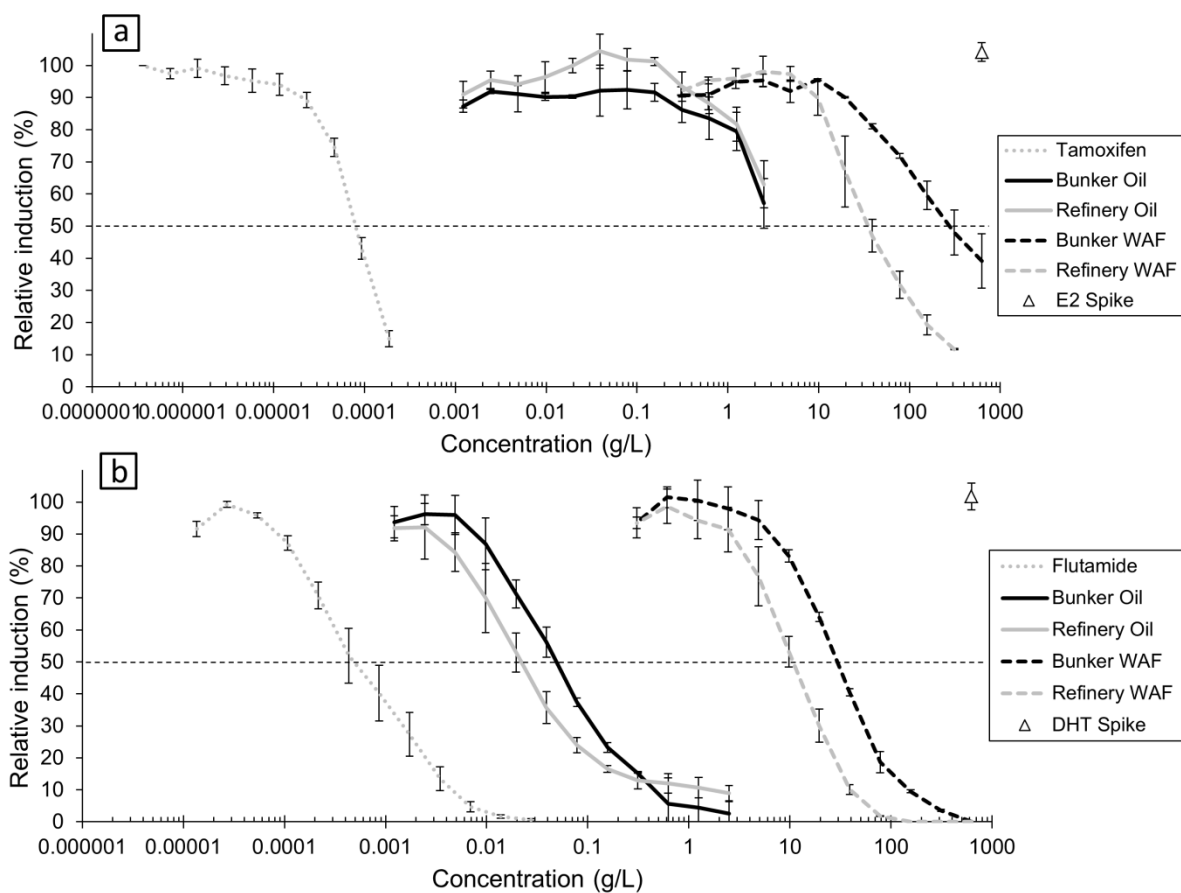


Figure 6.4. Anti-estrogenic (a) and anti-androgenic (b) activity of bunker and refinery crude oil as well as the water accommodated fractions of these oils, as determined using recombinant yeast hormone receptor transactivation assays (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998). The error bars indicate the standard deviation among assay plates.

Table 6.1: (a) The half maximal effective concentration (EC50) for estrogen receptor 1 (ESR1) agonism, estradiol induction equivalents (IEQ_{E2}), half maximal inhibitory concentration (IC50) for ESR1 antagonism, and tamoxifen (TAM) induction equivalents (IEQ_{TAM}), and TAM equivalents (TEQ) of bunker and refinery crude oil. (b) The half maximal effective concentration (EC50) for androgen receptor (AR) agonism, dihydrotestosterone (DHT) induction equivalents (IEQ_{DHT}), half maximal inhibitory concentration (IC50) for AR antagonism, flutamide (FLU) induction equivalents (IEQ_{FLU}), and FLU equivalents (FEQ) of bunker and refinery crude oil. NC: could not be calculated.

a)

Test substance	ESR1 agonism		ESR1 antagonism		
	EC50 (mg.L ⁻¹)	IEQ _{E2} (mg/g)	IC50 (mg.L ⁻¹)	IEQ _{TAM} (mg/g)	TEQ (µg.L ⁻¹)
17β-estradiol	3.35 x 10 ⁻⁵	-	-	-	-
Tamoxifen	-	NC	0.08	-	-
Bunker Oil	NC	NC	NC	NC	-
Refinery Oil	NC	NC	NC	NC	-
Bunker Oil WAF	NC	NC	306.00 x 10 ³	2.58 x 10 ⁻⁴	26.34
Refinery Oil WAF	NC	NC	44.57 x 10 ³	1.77 x 10 ⁻³	81.61

b)

Test substance	AR agonism		AR antagonism		
	EC50 (mg.L ⁻¹)	IEQ _{DHT} (mg/g)	IC50 (mg.L ⁻¹)	IEQ _{FLU} (mg/g)	FEQ (µg.L ⁻¹)
Dihydrotestosterone	1.90 x 10 ⁻³	-	-	-	-
Flutamide	-	NC	0.55	-	-
Bunker Oil	NC	NC	50.31	10.93	-
Refinery Oil	NC	NC	24.61	22.35	-
Bunker Oil WAF	NC	NC	29.58 x 10 ³	18.59 x 10 ⁻³	0.56 x 10 ³
Refinery Oil WAF	NC	NC	10.63 x 10 ³	51.74 x 10 ⁻³	3.34 x 10 ³

Estrogenicity was detected in only three of the 16 surface water samples tested, with activities ranging between 3.01 and 3.98 ng.L⁻¹ E₂ equivalents (EEQs), but limited to Pan1 and Pan2 (Figure 6.5a). Anti-androgenicity was more widespread than estrogenicity and FEQs ranging between 23.38 and 592.32 µg.L⁻¹ were observed (Figure 6.5b). In particular, anti-androgenicity was detected at all four sampling locations during January (Summer), as well as during May (Autumn) at Pan1 and Pan2, and during August (Spring) and November (Early summer) in Pan2 (Figure 6.5b). Anti-estrogenic activity was only detected during January (Summer) and at concentrations ranging between 3.06 and 10.89 µg.L⁻¹ TEQs, with the highest potency being in Pan2 water (Figure 6.5c). No androgenic activity was detected in any of the samples tested.

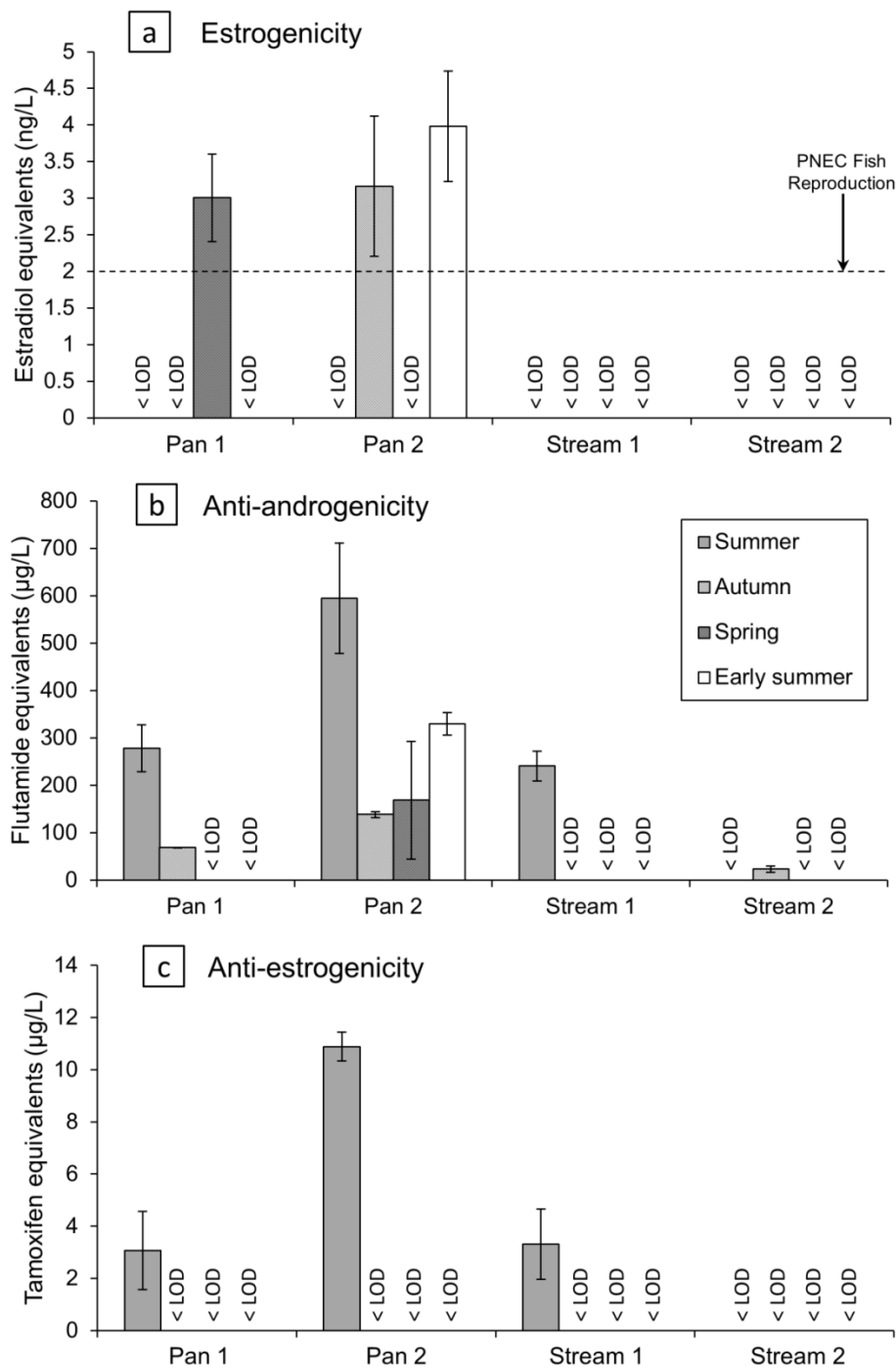


Figure 6.5: (A) Estrogenic, (B) anti-androgenic and (C) anti-estrogenic activity of surface water collected from two pans and two streams located in the close proximity of a historical coal mine converted into an underground crude oil bunker. Hormone receptor transactivation activity was determined using the YES, YAES and YAAS yeast bioassays. Samples that were below the limit of detection (LOD) are indicated. The horizontal dashed line in figure A indicates the predicted no effect concentration (PNEC) for reproduction in fish (Caldwell *et al.*, 2012).

6.3.3 PAH analysis

The only PAHs that were detected in the WAFs tested included anthracene, benzo(g,h,i)perylene and naphthalene (Table 6.2). Anthracene and benzo(g,h,i)perylene were detected only in the bunker oil WAF, whereas naphthalene was detected in both the WAF types tested (Table 6.2).

Table 6.2: Concentrations of polycyclic aromatic hydrocarbons (PAHs) measured in bunker and refinery crude oil water accommodated fractions, as well as the limits of detection (LOD) and recoveries of the individual compounds tested for.

Compound	Concentration ($\mu\text{g.L}^{-1}$)			Matrix recovery (%) (100 ppb)
	LOD	Bunker	Refinery	
Acenaphthene	1	<LOD	<LOD	98.26
Acenaphthylene	1	<LOD	<LOD	91.00
Anthracene	1	1.01 \pm 0.08	<LOD	80.71
Benzo(a)anthracene	10	<LOD	<LOD	52.56
Benzo(a)pyrene	1	<LOD	<LOD	143.588
Benzo(b)fluoranthene	5	<LOD	<LOD	132.20
Benzo(g,h,i)perylene	2.5	3.45 \pm 3.98	<LOD	53.97
Benzo(k)fluoranthene	5	<LOD	<LOD	65.35
Chrysene	10	<LOD	<LOD	82.30
Dibenzo(a,h)anthracene	10	<LOD	<LOD	70.19
Fluoranthene	5	<LOD	<LOD	86.69
Fluorene	10	<LOD	<LOD	87.52
Indeno(1,2,3-cd)pyrene	5	<LOD	<LOD	62.10
Naphthalene	0.5	45.66 \pm 7.25	89.43 \pm 11.15	101.91
Phenanthrene	10	<LOD	<LOD	61.65
Pyrene	2.5	<LOD	<LOD	92.43

6.4 Discussion

Crude oils are responsible for the release of complex mixtures of biologically active compounds, including PAHs, naphthenic acids, heterocyclic aromatic compounds and alkyl phenols, into the environment. These hydrocarbon-based compounds are also associated with other fossil fuel related industries, including hydraulic fracturing, coal mining and oil sands. The chemical signature of different crude oils as well as other fossil fuels may, however, vary considerably depending on the source and history, and the potential impact to wildlife may, therefore, also vary. Nonetheless, due to the limited data available describing the endocrine disruptive potential of hydrocarbons, the findings of crude oil can be used to identify priority questions to address for other fossil fuels or related industries as well. Although a number of oil spills have devastated freshwater ecosystems in the past, and smaller scale pollution frequently occurs (Vandermeulen and Ross, 1995), surprisingly little is known regarding the effects of crude oil on freshwater aquatic vertebrates. In the present study, *in vitro* and *in vivo* biological assays were applied to evaluate the potential influence of crude oil on selected molecular targets related to endocrine signalling in freshwater aquatic vertebrates.

The present data indicate disrupted thyroid signalling associated with crude oil WAF exposure in both an amphibian and teleost species. In particular, *thrb* expression was significantly downregulated in response to 0.25 g.L⁻¹ (1%) of both bunker and refinery crude oil WAF, in *X. laevis* pre-metamorphic tadpoles. Moreover, the expression of *dio2* was significantly upregulated in juvenile *O. mossambicus* exposed to a high concentration of bunker oil WAF (25 g.L⁻¹; 100%), whereas the expression of this gene was unchanged in *X. laevis* tadpoles. No significant changes in *thrb* or *dio2* expression were, however, observed in *X. laevis* tadpoles exposed to surface water collected from water bodies located in the close proximity of an underground oil bunker.

In a recent study, Kim *et al.* (2016) indicated the potential of Iranian crude oil WAF to disrupt thyroid signalling by describing the expression of a number of genes representing the HPT axis in zebrafish, *Danio rerio*, exposed for 120h, as well as in GH3 rat pituitary cells. A significant increase in the expression of selected thyroid linked genes were observed in *D. rerio*, and in particular, *type 1 deiodinase (dio1)*, *hematopoietically expressed homeobox (hhex)*, *paired box protein 8 (pax8)* and *transthyretin (ttr)* in response to a high dosage of 12.5 g.L⁻¹ (50%) WAF, whereas *uridine diphosphate glucuronosyltransferase (ugt)* was significantly upregulated in both the 10 g.L⁻¹ and 12.5 g.L⁻¹ WAF treatments. As was the case for *O. mossambicus* in the present study, no significant differences were observed in the expression of *thrb* (Kim *et al.*, 2016). In addition, *dio2*, *corticotrophin releasing hormone*

(*crh*), *thyroid stimulating hormone β* (*tshb*), *thyroid stimulating hormone receptor* (*tshr*), *thyroglobulin* (*tg*) and *NK2 homeobox1* (*nkx2.1*) was not significantly affected by WAF exposure in *D. rerio* and *dio1*, *dio2*, *thra*, and *thrb* expression in GH3 cells (Kim *et al.*, 2016). The expression of *tshb* was, however, significantly downregulated in GH3 cells in response to 1.25 g.L⁻¹ WAF (0.05%), corresponding to the effect observed in response to a T₃ treatment (Kim *et al.*, 2016). Kim *et al.* (2016) furthermore reported significant decreased levels of plasma triiodothyronine (T₃) and thyroxine (T₄) in *D. rerio* exposed to a high dosage of WAF.

In a further study, the expression of *thyroid hormone receptor* (*thr*) was shown to be significantly down-regulated in sea otters, *Enhydra lutris* exposed to type-c heavy fuel oil (Bowen *et al.*, 2007), as was observed in *X. laevis* in the present study. No other published records of the effects of crude oil or other fossil fuels on *thr* (*a* or *b*) or *dio2* could be located. In fact, there is a limited amount of literature on the effects of crude oil on the thyroid systems of fish (Alkindi *et al.*, 1996; Stephens *et al.*, 1997a; Stephens *et al.*, 1997b), and to my knowledge, no published reports describing effects in amphibians. Alkindi *et al.* (1996) observed significant changes in plasma T₃ and T₄ levels in flounders, *Pleuronectes flesus*, exposed to crude oil WSF. Stephens *et al.* (1997a) observed a dose-dependent increase in whole-body T₄ concentrations in juvenile turbot *Scophthalmus maximus* exposed to crude oil WSF, whereas T₃ was unchanged, and the T₃:T₄ ratio, therefore, decreased as WSF dose increased. A similar response was observed in *S. maximus* larvae at a high concentration of crude oil WSF, T₃ being increased, and T₄ unchanged (Stephens *et al.*, 1997b). None of these aforementioned reports, however, include data on *thrb* or *dio2* expression. The decreased T₃:T₄ ratio reported by Stephens *et al.* (1997a; 1997b) suggests lowered deiodinase mediated T₃ synthesis.

Exposure to a mixture of naphthenic acids did not affect T₃ or T₄ levels in northern leopard frogs, *Lithobates pipiens* (Smits *et al.*, 2012), whereas water from oil sands reclamation wetlands caused significant increase in the time to metamorphosis, and a decreased T₃:T₄ ratio in the same species (Hersikorn and Smits, 2011), as was observed in *S. maximus* exposed to crude oil WSF (Stephens *et al.*, 1997b; Stephens *et al.*, 1997c). Moreover, a significant delay in time to metamorphosis was observed in western clawed frogs, *Sirulina tropicalis* exposed to a mixture of naphthenic acids (Melvin and Trudeau, 2012), which may have been associated with thyroid disruption. In a further study, a mixture of chemicals associated with hydraulic fracturing waste water including phenols and other hydrocarbons were shown to antagonise the human thyroid hormone receptor (THR) *in vitro* (Kassotis *et al.*, 2015). When tested individually, ethoxylated nonylphenol, ethoxylated octylphenol,

ethylene glycol, and the PAH, naphthalene were the most potent THR antagonists (Kassotis *et al.*, 2015).

PAHs are probably the best studied constituents of crude oil in terms of toxicity and other biological effects in humans and wildlife. However, surprisingly little is generally known regarding the potential targets of PAHs, or their derivatives, within the thyroid cascade. The only report on either *thrb* or *dio2* expression in fish or amphibians exposed to PAHs is a study on marbled rockfish, *Sebastes marmoratus* embryos, where *thrb* expression was shown to be significantly downregulated in response to pyrene exposure (He *et al.*, 2012a). Certain thyroid disruptors have their action by interfering with thyroid hormone transport. Transthyretin (TTR) is an important T₄ transport protein in vertebrates (Schussler, 2000). Bekki *et al.* (2009), through the use of the TTR-binding assay, tested the potential of 25 PAH derivatives (hydroxides, ketones and quinones) to interfere with T₄ transport. In addition, interaction with the human thyroid hormone receptor beta (THRB) (i.e., agonism and antagonism) of the 25 compounds was evaluated, using the TR β -CALUX reporter gene system. Fifteen of the PAH derivatives tested, exhibited (human) TTR binding affinity, of which benzo[c]phenanthrene-[1,4]-quinone was the most potent, displacing 91% of the T₄ in solution, providing evidence that PAH derivatives, and potentially (parent) PAHs, can disrupt T₄ transport in exposed animals. In addition, no THRB agonism or antagonism was observed; however, five of the PAH ketones, and two of the PAH quinones potentiated THRB agonism when tested in combination with T₃. The results of Bekki *et al.* (2009), therefore, suggested that the compounds tested for did not interact directly with THR, but affected THR transactivation indirectly (by for example targeting co-factors), hence the potentiation. There is, however, evidence that certain PAHs or PAH derivatives interact directly with THRs. In particular, pyrene was shown to bind weakly, and one of its derivatives, 1-aminopyrene, to bind more strongly, to THRA and THRB, using competitive binding immunoassays (Koike *et al.*, 2014). Moreover, as mentioned earlier, naphthalene was shown to antagonise human THRB in a mammalian cell reporter gene assay (Kassotis *et al.*, 2015), as was the case for two metabolites of naphthalene, 1-naphthol and 2-naphthol (Sun *et al.*, 2008).

There is clearly a gap in the literature regarding the potential effects of crude oil, other fossil fuels, and their constituents (e.g., PAHs and naphthenic acids) on the thyroid systems of fish and amphibians, and more research is required. Future studies performing longer term exposures, utilizing for instance the *Xenopus* Metamorphosis Assay (XEMA) (Opitz *et al.*, 2005), are needed to test for potential adverse effects of crude oil or associated hydrocarbons on the thyroid system.

Research on PPAR gamma as molecular target of endocrine disruptive contaminants has increased considerably in recent years, due to a growing body of evidence suggesting causative links between chemical exposure and obesity (Grün and Blumberg, 2009). In the present study, significant changes in *pparg* expression were observed in *X. laevis* tadpoles exposed to both crude bunker and refinery crude oil WAFs. This effect was most pronounced in the bunker oil WAF treatments, with a significant downregulation occurring at a low concentration of 0.25 g.L⁻¹ (1% WAF). The expression of the said gene was, however, unchanged in crude oil WAF exposed juvenile *O. mossambicus*, and in *X. laevis* exposed to surface water samples. Little is known regarding the influence of crude oil on PPAR gamma signalling. As presently observed in *X. laevis* exposed to 2.5 g.L⁻¹ refinery oil WAF and 25 g.L⁻¹ bunker oil WAF, a significant increase in *pparg* expression was shown in marine medaka, *Oryzias melastigma* exposed to crude oil WAF (20%, 5 g.L⁻¹), but not in response to a lower concentration of 2.5 g.L⁻¹ WAF (Rhee *et al.*, 2013). Conversely, Mississippi Canyon block 252 crude oil WAF did not induce the expression of (human) PPAR gamma (in a mammalian cell reporter gene assay), whereas a COREXIT 9500 WAF, and a COREXIT:252 oil WAF resulted in a dose dependent increase in PPARG transactivation (Temkin *et al.*, 2015).

Certain PAHs have been shown to affect the expression of the (human and mouse) *PPARG* gene (Jin *et al.*, 2014; Yan *et al.*, 2014; Yan *et al.*, 2014), and the changes in *X. laevis pparg* expression in response to crude oil WAF exposure may, therefore, have been associated with PAHs. The potential of naphthenic acids and other constituents of fossil fuels to alter PPAR gamma signalling are yet to be described. A recent epidemiological study reported a significant association between urinary PAH levels and childhood obesity in the USA (Scinicariello and Buser, 2014), suggesting a causative link between PAH exposure and obesity. Research on the effects of crude oil, PAHs as well as other hydrocarbons on lipid metabolism, are, therefore, important from a human health perspective as well as for wildlife.

The epigenetic modification of *PPARG* is a further important parameter, apart from direct interaction (e.g., transactivation), to consider when the potential of chemicals to disrupt PPAR gamma signalling is assessed (Janesick and Blumberg, 2012). The F1 and F2 offspring of mice treated prenatally with a mixture of eight PAHs exhibited significantly higher *Pparg* expression, as well as physical weight, than control individuals (Yan *et al.*, 2014). Significant changes in the methylation of the *Pparg* promotor of both the F1 and F2 generations were furthermore shown (Yan *et al.*, 2014). Further research on the epigenetic effects of crude oil on lipid homeostasis is needed and will be of value for risk assessment purposes.

Crude oil has been shown to exhibit estrogenicity, but the potency seems to vary considerably among different kinds of crude oil (from different locations), and occur predominantly via ESR2, and not ESR1 (Vrabie *et al.*, 2010; Vrabie *et al.*, 2011). The fact that the yeast screen applied in the present study only represented ESR1, therefore, possibly explains the absence of estrogenicity observed for the crude oils tested.

Both the bunker and refinery crude oils and their WAFs were anti-estrogenic; however, the activity of the WAFs occurred at a considerably higher concentration than the crude oil, dissolved in ethanol. Moreover, the crude oil did not totally inhibit ESR1 transactivation, within the range tested, whereas the WAFs did so to a greater extent, allowing IC50s to be calculated. Using recombinant yeast, Vrabie *et al.* (Vrabie *et al.*, 2010) evaluated four crude oil types for potential ESR1 and ESR2 antagonistic activity, and only Romanian crude oil was found to be anti-estrogenic, and antagonised only ESR1. In addition, Arcaro *et al.* (2001) reported anti-estrogenicity for three crude oil types using estrogen-sensitive cell growth and tissue restructuring in MCF-7 human breast-cancer cells as biomarkers, together with competitive ESR binding assays. The findings of Arcaro *et al.* (2001) suggest that the anti-estrogenicity was due to both increased catabolism of E₂ and antagonistic binding to ESRs, although it was not determined whether the interaction was specific to ESR1 or ESR2. Similarly, oil sands process-affected water was shown to be anti-estrogenic *in vitro* (Leclair *et al.*, 2015), as was the case for water impacted by hydraulic fracturing (Kassotis *et al.*, 2014). Although the exact compounds responsible for the anti estrogenicity observed in the present study remains unclear, it may have been due to naphthenic acids, as well as certain alkyl phenols, PAHs and PAH derivatives, which are known to be anti-estrogenic (Santodonato, 1997; Hayakawa *et al.*, 2007; Kassotis *et al.*, 2014; Leclair *et al.*, 2015; Kassotis *et al.*, 2015).

Even though anti-estrogenic compounds can potentially alter *cyp19a1b* expression (Kuhl and Brouwer, 2006), the bunker and refinery oil WAFs had no significant effect on the expression of aromatase in both *X. laevis* (*cyp19*) and *O. mossambicus* (*cyp19a1b*), despite the anti-estrogenic potential. In a further study where fish were exposed to crude oil, the expression of *cyp19a1b* was unchanged in *D. rerio* exposed to weathered North Sea crude oil WSF (Salaberria *et al.*, 2014). Moreover, Cyp19 (enzyme) activity did not vary significantly in turbot *S. maximus* exposed to North Sea crude oil (Martin-Skilton *et al.*, 2006). There are, however, some reports in the literature suggesting that crude oil exposure affect aromatase. In particular, a significant downregulation of *cyp19a1b* was reported for *D. rerio* exposed to weathered crude oil WSF (Arukwe *et al.*, 2008), as was the case for both *cyp19a1a* and *cyp19a1b* in *O. melastigma* exposed to crude oil WAF (Rhee *et al.*, 2013).

The bunker and refinery crude oil tested in the present study exhibited anti-androgenic action, as have been shown for Romanian crude oil, distillate marine grade oil, bilge oil, heavy nautical fuel oil (Vrabie *et al.*, 2010), Type-C heavy fuel oil (Kizu *et al.*, 2000), North Sea offshore oil platforms produced water (Tollefsen *et al.*, 2007; Thomas *et al.*, 2009), oil sands process-affected water (Leclair *et al.*, 2015), and hydraulic fracturing impacted water (Kassotis *et al.*, 2014). In an effect-directed investigation, the constituents of North Sea produced water were fractionated using HPLC, allowing individual fractions to be screened for estrogenicity and androgenicity, together with chemical analysis (Thomas *et al.*, 2009). Naphthenic acids were found to be responsible for approximately 65% of the total estrogenicity, whereas the remainder of the activity was attributed to alkyl phenols (Thomas *et al.*, 2009). Similarly, naphthenic acids were found to be responsible for the most of anti-androgenic activity, but, unlike with estrogenicity, PAHs contributed nearly equally (than naphthenic acids), and alkyl phenols to a lesser extent (Thomas *et al.*, 2009). A number of other studies have also suggested the anti-androgenic potential of selected PAHs (Vinggaard *et al.*, 2000; Kizu *et al.*, 2000; Hawliczek *et al.*, 2012), naphthenic acids (Leclair *et al.*, 2015; Kassotis *et al.*, 2015) and alkyl phenols (Kassotis *et al.*, 2015). The anti-androgenic action observed in the present study was, therefore, likely due to PAHs, naphthenic acids and alkyl phenols, as was the case with anti-estrogenicity. Although Vrabie *et al.* (Vrabie *et al.*, 2010) did not report AR antagonism IC₅₀s for the oil tested, the IC₅₀s presently observed for refinery and bunker crude oil (50.31 and 24.61 mg.L⁻¹ respectively) seems to correspond generally to the results for Romanian crude oil. Interestingly, the pure bunker and refinery oil WAFs (25 g.L⁻¹ crude oil) had lower FEQs (i.e., 0.56 mg.L⁻¹ and 3.33 mg.L⁻¹) than previously reported for North Sea produced water (i.e., 5.90 mg.L⁻¹ to 6.89 mg.L⁻¹) (Thomas *et al.*, 2009).

As suggested by *in vitro* androgen receptor antagonism data, the anti-androgenic action of crude oil, other fossil fuels or constituents thereof, has been shown in fish. In particular, plasma testosterone (T) concentrations were significantly decreased in *D. rerio* exposed to North Sea crude oil WSF (Arukwe *et al.*, 2008). Similarly, plasma T was significantly lowered in turbot *S. maximus* exposed to North Sea crude oil (Martin-Skilton *et al.*, 2006). Moreover, male fathead minnows, *Pimephales promelas* exposed to oil sands process-affected water had lowered testosterone and 11-ketotestosterone levels, as well as a reduced number of reproductive tubercles (Kavanagh *et al.*, 2012). In the present study, *ar1* expression was unchanged in juvenile *O. mossambicus*, as well as *ar* and *srd5a1* in *X. laevis* tadpoles exposed to crude oil WAF, and the increased expression of *ar2* in *O. mossambicus* exposed to a high concentration of bunker oil WAF (25 g.L⁻¹; 100%) was the only significant change observed in a gene representing the male hormone system. There are a number of potential

confounding factors that may have contributed to the lack of significant changes in gene expression in the present study, including the fact that juvenile animals/tadpoles were used (i.e., life stage), the testing of whole body-homogenates and not gonads, the short exposure duration, and variable species sensitivity. Other studies, in which fish were exposed to crude oil or associated compounds, have similarly observed no changes in biomarkers representing the male hormone system, as was the case for *X. laevis* in the present study. In particular, North Sea crude oil had no significant effect on plasma T levels in Atlantic cod *Gadus morhua*, although, as mentioned earlier, plasma T was significantly decreased in *S. maximus* in the same investigation (Martin-Skilton *et al.*, 2006). In addition, the expression of *ar* was unchanged in male fathead minnows exposed to oil sands process-affected water, but significantly downregulated in female livers (He *et al.*, 2012b). Moreover, in a study applying the sensitive three-spined stickleback *Gasterosteus aculeatus* androgenised female spiggen assay, naphthenic acids did not have anti-androgenic action, and in fact, was shown to be androgenic (Knag *et al.*, 2013).

The effluent of a water/oil separation plant is periodically released into Pan1, and the said pan is, therefore, the most impacted by chemicals originating from crude oil among the waterbodies evaluated. However, based on the present results, Pan2 was more estrogenic, anti-estrogenic and anti-androgenic than Pan1, as well as Stream1 and Stream2, during all four seasons sampled. Unlike Pan1, Pan2 has no direct (obvious) pathway of crude oil contamination, but is largely surrounded by maize fields, and pesticide contamination is, therefore, a real risk. Certain pesticides are known to be anti-androgenic, anti-estrogenic (Birkhoj *et al.*, 2004; Orton *et al.*, 2009; Orton *et al.*, 2011) or estrogenic (Grunfeld and Bonefeld-Jorgensen, 2004; Kojima *et al.*, 2004). Pesticide analyses were, however, not performed on the surface water tested in the present study and further investigation is, however, required determine the contribution of pesticides on endocrine disruptive potential in Pan2.

The *X. laevis* tadpole exposures were performed using surface water collected during summer, the period when estrogenicity, anti-estrogenicity and anti-androgenicity was at a peak, with EEQs, TEQs, and FEQs of up to 3.98 ng.L⁻¹, 10.89 µg.L⁻¹ and 595.32 µg.L⁻¹ respectively. No significant changes in the expression of any of the genes linked to reproduction, evaluated in *X. laevis* tadpoles exposed to surface water samples, were, however, observed.

6.5 Conclusions

In the present study, the endocrine disruptive potential of weathered crude oil extracted from an underground oil bunker, un-weathered refinery crude oil, as well as surface water collected from the proximity of an underground crude oil bunker towards freshwater vertebrates was evaluated using a combination of *in vitro* and *in vivo* bioassays.

Gene expression biomarkers for disruption of thyroid signalling indicated that both freshwater fish and amphibians may be affected by crude oil exposure. In particular, the expression of *thrb* was significantly downregulated in response to the WAFs of both oil types in *X. laevis* tadpoles, whereas *dio2* expression was significantly upregulated in *O. mossambicus* exposed to a high concentration of bunker oil WAF. Literature reports on the impacts of crude oil on the thyroid systems of aquatic vertebrates are limited, and further studies are needed.

The expression of *pparg* was altered in *X. laevis* tadpoles exposed to the WAFs of both bunker and refinery oil, which was not the case for *O. mossambicus*. My data suggest that oil pollution may disrupt lipidogenesis in amphibians like the African Clawed frog, *X. laevis* and possibly other aquatic vertebrates. Further studies describing the effects of crude oil on *pparg* signalling in other species as well as different life stages are required. Some evidence of epigenetic changes affecting *pparg* expression associated with hydrocarbon exposure exists, and future studies evaluating such effects in wildlife exposed to crude oil will be of value.

The *in vitro* screens indicated that both types of crude oil exhibited anti-estrogenic and anti-androgenic activity, as has been previously shown for other crude oil types (Kizu *et al.*, 2000; Vrabie *et al.*, 2010), as well as produced water from North Sea offshore oil platforms (Tollefsen *et al.*, 2007). The WAFs were less anti-estrogenic and anti-androgenic than the crude oil itself, yet were able to antagonise ESR1 to a greater extent (than the oil) and fully antagonise AR transactivation. Anti-estrogenicity or anti-androgenicity does not necessarily indicate antagonism through direct binding with hormone receptors, but may also be due to catabolism of hormones or interference with transcriptional activation at another level. Nonetheless, the response will result in the inhibition of hormone receptor mediated gene expression. The weathered bunker crude oil and the WAF of this oil was consistently less biologically active than the un-weathered refinery oil. Although the *in vitro* data suggest that the reproductive systems of animals exposed to bunker and refinery oil WAFs will be disrupted, the expression of the majority of genes studied as biomarkers for disruption of the hypothalamus-pituitary-gonadal axis was unaffected. In particular, only *ar2* expression, and

only in *O. mossambicus*, was significantly upregulated in response to a high concentration of bunker oil WAF. The *in vivo* data, therefore, do not confirm the risk towards reproductive impairment predicted by the *in vitro* data. However, the life stage of the model organisms utilized as well as the fact that gene expression was quantified in whole body homogenates are some potential confounding factors explaining the *in vitro/in vivo* discrepancy.

Estradiol equivalents exceeding the predicted no-effect concentration for reproductive impairment in fish (Caldwell *et al.*, 2012) were detected in two of the water bodies investigated, whereas anti-estrogenicity was detected in three waterbodies. In addition, anti-androgenic activity was detected throughout the study area, and at the highest potency in surface water collected from the Pan2 site during all four seasons investigated. Pan1 receives the effluent of an oil-water separation plant and, therefore, constituents of crude oil, but was less anti-estrogenic and anti-androgenic than Pan2, with no apparent contamination vector for crude oil or its constituents. The receptor antagonism activity may have been associated with bio-active pesticides from agricultural fields in the close proximity of the water investigated. There were no significant changes in the expression of any of the genes described in *X. laevis* tadpoles exposed to surface water samples, despite the estrogenic, anti-estrogenic and anti-androgenic potential of these samples, suggesting that the sole use of *in vitro* data for risk assessments may be misleading.

6.6 References

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6.7 Supplementary material

6.7.1 Water accommodated fraction preparation

Water accommodated fractions (WAF) of oil were prepared as described by Singer *et al.* (2000), with the variable dilution approach suggested by Barron and Ka'aihue (2003). In particular, 25 g.L⁻¹ WAF was prepared in air-tight glass containers with a fixed liquid-free head space of ~20% (with the water level being below the vessels' sloping shoulders). The bunker crude oil was weighed on a stainless steel disc and frozen at 80 °C. The frozen oil mass was then dispensed into the appropriate volume of buffered RO water (containing 250 mg.L⁻¹ marine salt and 80 mg.L⁻¹ NaHCO₃, pH 7) using a chilled spatula. The refinery oil was poured directly into the buffered RO water placed on top of a scale ensuring the addition of the correct mass of oil. Containers were subsequently sealed from air and light using a screw cap and aluminium foil respectively. The solution was then incubated at room temperature for 24 to 26 h on a magnetic stirrer at a low speed (~240 rpm) (Figure S6.1).

The WAFs were extracted from glass containers using Pasteur pipettes fitted with Tygon® tubing. The pipettes were enclosed in a paper sheath that was gently broken once the tip of the pipette have passed through the oil slick and pure WAF without any crude oil micro-droplets could, therefore, be extracted (Figure S6.1).

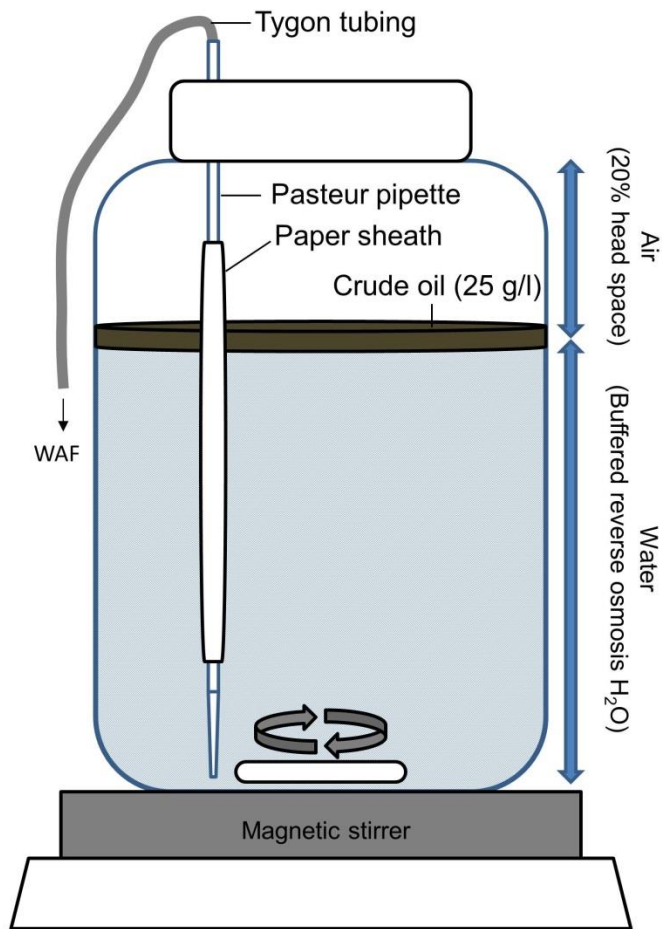


Figure S6.1: Water accommodated fraction (WAF) preparation (Singer *et al.*, 2000). Glass containers were covered with aluminium foil during the 24 h WAF preparation period. The Pasteur pipette with paper sheath was only inserted after the WAF preparation.

6.7.2 Sequences and sources of primers applied in real-time RT-qPCR analyses.Table S6.1: Primer sequences for *Xenopus laevis* in the 5' to 3' direction applied for RT-qPCR.

Target	Primer sequence	Ta (°C)	Source ¹
<i>thrb</i>	F: AAAGTGCCAGGAAGGTTTCCTC	55	NM001087781.1
	R: GGTCGGTGACTTTCATCAGCA		
<i>dio2</i>	F: ACATTGACGAGGCACATC	63	Searcy <i>et al.</i> , 2012
	R: GAGACACCATAGGCAACAT		
<i>pparg</i>	F: TTCGCCATTCGCTTCAACTCC	63	NM001087843
	R: CCGCAGGTCCGTCATCTTCT		
<i>cyp19a1</i>	F: CGGTTCCATATCGTTACTTC	63	AB031278.1
	R: ATATTCTCCAGGCATCTTCC		
<i>ar</i>	F: GAGGAAATGTTATGAAGCTGGG	63	Bogi <i>et al.</i> , 2002
	R: ACGGTCATTTGGTCGCTTAC		
<i>srd5a1</i>	F: ACAATCCGAGTTAGGCATC	63	NM001098696
	R: GAATCCAGTCTGAGGCATAG		
<i>actb</i>	F: ATGCCAATACTGTTCTGTCT	63	NM001088953.1
	R: CATACTCCTGCTTGCTGAT		

¹Genbank accession number

Table S6.2: Primer sequences for *Oreochromis mossambicus* in the 5' to 3' direction applied for RT-qPCR.

Target	Primer Sequence	Ta (°C)	Source
<i>thrb</i>	F: AATGTGTTATTGACAAAGTG	63	Shiao <i>et al.</i> , 2007
	R: GATCGGATGAAAGCAGGATA		
<i>dio2</i>	F: TACAACAGAGAAAGATTGCCTACC	57	Chapter 3
	R: TTCAAGACTCCTACCGTTTACCA		
<i>pparg</i>	F: TGCGAGGGCTGTAAGGGTTT	59	Chapter 4
	R: ACTTGTTGCGGGACTTCTTG TG		
<i>cyp19a1b</i>	F: GAGCGTCAGAAGTCACTGC	60	Esterhuysen <i>et al.</i> , 2008
	R: GCTCAA AATCAGGGTCTCCC		
<i>ar1</i>	F: CTATCAAGAGTGGGCCTTCGG	65	Ijiri <i>et al.</i> , 2008
	R: GCGCCTTAAACTGCGATCTG		
<i>ar2</i>	F: AGGGTGAGGTCGGCGAAT	58	Ijiri <i>et al.</i> , 2008
	R: TGGACTCAAACCTGGTGTCGT		
<i>actb</i>	F: TGTGATGGTGGGTATGGG	63	Esterhuysen <i>et al.</i> , 2008
	R: CTGTGGTGGTGAAGGAGTAG		

Table S6.3: Expression of a selection of genes in (a) NF48 *Xenopus laevis* and (b) exposed for 24 h to the water accommodated fractions of crude oil obtained from and underground bunker as well as a refinery. The concentrations given portray the mass represented relative to the original crude oil mass used to prepare the WAF and 25 g.L⁻¹, therefore, equates 100% WAF. No significant differences were observed among the treatments.

a) *Xenopus laevis*

Treatment	<i>dio2</i>		<i>cy19a1</i>		<i>ar</i>		<i>srd5a1</i>	
	Bunker	Refinery	Bunker	Refinery	Bunker	Refinery	Bunker	Refinery
Control	1.08±0.26	1.01±0.15	1.09±0.61	1.24±1.07	1.06±0.26	1.01±0.17	0.98±0.28	1.02±0.19
25 mg/L	1.00±0.26	1.02±0.18	0.99±0.43	0.79±0.13	1.45±0.38	0.86±0.24	0.78±0.15	0.89±0.16
250 mg/L	0.97±0.15	0.83±0.14	0.64±0.24	0.60±0.20	0.71±0.18	0.75±0.20	1.19±0.18	0.84±0.10
2500 mg/L	1.24±0.89	0.81±0.08	1.02±0.44	0.75±0.13	0.91±0.83	1.01±0.34	1.18±0.17	1.23±0.38
25000 mg/L	1.30±0.43	-	1.27±0.82	-	1.52±1.01	-	1.20±0.37	-

b) *Oreochromis mossambicus*

Treatment	<i>thrb</i>		<i>pparg</i>		<i>cy19a1b</i>		<i>ar1</i>	
	Bunker	Refinery	Bunker	Refinery	Bunker	Refinery	Bunker	Refinery
Control	1.02±0.23	1.01±0.20	1.01±0.14	1.04±0.32	1.01±0.13	0.95±0.22	1.04±0.31	1.05±0.36
25 mg/L	1.12±0.20	0.82±0.24	1.18±0.32	0.85±0.19	1.08±0.17	0.88±0.33	1.18±0.21	0.66±0.14
250 mg/L	0.95±0.70	0.69±0.17	0.81±0.11	0.86±0.22	0.82±0.20	0.69±0.22	0.91±0.13	0.70±0.19
2500 mg/L	1.17±0.29	1.03±0.63	1.09±0.61	1.34±0.93	0.85±0.19	1.07±0.69	1.20±0.33	0.87±0.25
25000 mg/L	1.30±0.24	-	1.10±0.19	-	0.92±0.12	-	1.54±0.43	-

i.e.,

6.7.3 References

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Chapter 7: *In vitro* screening for endocrine disruptive activity in selected South African harbours and river mouths

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Declaration by the candidate

With regard to Chapter 7, the nature and scope of my contribution were as follows:

Nature of contribution Extent of contribution (%)

Nature of contribution	Extent of contribution
Conceptual design, experimental work, manuscript writing.	80%

The following co-authors have contributed to Chapter 7:

Name	Email address and institutional affiliation	Nature of contribution	Extent of contribution
Prof JH van Wyk	jhvw@sun.ac.za Department of Botany and Zoology, Stellenbosch University	Conceptual design, manuscript editing.	20%
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Abstract

Various waterborne anthropogenic contaminants disrupt the endocrine systems of wildlife and humans, targeting reproductive pathways, among others. Very little is known, however, regarding the occurrence of endocrine disruptive activity in South African freshwater ecosystems, and coastal ecosystems have not been studied in this regard. In a first attempt to investigate coastal endocrine distributive activity in South African coastal waters, surface water samples collected from harbours, river mouths and estuaries in three metropolitan municipalities, eThekweni (which includes Durban), Nelson Mandela (specifically Port Elizabeth Harbour) and City of Cape Town, were screened for (anti)estrogenicity and (anti)androgenicity using recombinant yeast bioassays. Moreover, levels of the female hormone 17β -estradiol (E_2) were determined in all samples, as well as a selection of hydrocarbons in the eThekweni samples. A high proportion of samples collected from eThekweni were estrogenic, whereas none from Port Elizabeth Harbour and only a single river mouth in City of Cape Town were estrogenic. E_2 was detected in all the samples tested, but at higher concentrations at the eThekweni and City of Cape Town localities than Port Elizabeth Harbour. In addition, the recombinant yeast assays revealed that anti-androgenicity was widespread, being detected in the majority of samples screened apart from those representing Port Elizabeth Harbour. Conversely, no anti-estrogenic or androgenic activity was detected. Anti-androgenicity did not associate with hydrocarbon loads, providing evidence that other anti-androgens were responsible for the observed activity. The present data suggests potential reproductive disruption in marine and estuarine fauna inhabiting the eThekweni and City of Cape Town regions.

7.1 Introduction

Estuaries are the final phase of surface-water runoff (from drainage basins) into oceans and, therefore, act as repositories for a range of contaminants of agricultural, residential and industrial origin (Kennish, 1994). Certain marine fish species use estuaries as nurseries; in particular, these fish spawn at sea and enter estuaries as juveniles, where they remain until just prior to sexual maturity. Other marine or freshwater fish occasionally enter estuaries, but this is not a feature of their life cycle (Whitfield, 1999; Elliot *et al.*, 2007). The estuarine ichthyofaunal assemblage, therefore, consists of marine and freshwater species and estuaries can be a contact point for pollutants to enter both marine and freshwater food webs. Various anthropogenic and natural chemicals have been identified as endocrine disruptors (i.e., endocrine disrupting chemicals [EDCs]) (Colborn *et al.*, 1993). Reproductive impairment associated with EDC exposure has been observed in freshwater and marine

organisms (Jobling *et al.*, 1998; Allen *et al.*, 1999; Ford *et al.*, 2007; Kidd *et al.*, 2007). The presence of EDCs that modulate the reproductive system may affect fecundity in marine fish species that use estuaries as nurseries, because these fish are exposed during the maturation of their reproductive organs. Crustaceans and molluscs that inhabit estuaries represent a further route of entry of EDCs, such as certain persistent organic pollutants (POPs), into food webs (Geyer *et al.*, 2000; Vos *et al.*, 2000). In fact, it has been shown that invertebrate hormone systems and other key physiological processes such as cellular metabolism and immunity, as well as embryonic development, can be affected by certain EDCs (Depledge and Billingham, 1999; Zhou *et al.*, 2010, 2011). The evaluation of surface waters in estuarine habitats for endocrine disruptive activity is, therefore, important for risk assessment and holistic conservation efforts.

Harbours are important entrance points to the marine environment for pollutants (Young *et al.*, 1979; Soclo *et al.*, 2000), especially petrochemicals from vessel exhaust emissions and spillage (Soclo *et al.*, 2000; Mestres *et al.*, 2010). Certain petrochemicals are known endocrine disruptors (Tollefsen *et al.*, 2007; Vrabie *et al.*, 2010; Wang *et al.*, 2010) and organisms occurring in the proximity of harbours may, therefore, be affected.

In a recent review on the status of marine pollution research in South Africa, Wepener and Degger (2012) highlighted the need for more studies on organic pollutants in South African coastal waters. Studies on EDCs in the South African coastal environment are extremely limited, and apart from Marshall and Rajkumar (2003), who observed imposex in the mollusc *Nassarius kraussianus*, report on contaminant loads only (Fatoki and Awofolu 2004; Bollmohr *et al.*, 2007; Ogata *et al.*, 2009; Ryan *et al.*, 2012; Wepener and Degger, 2012; La Guardia *et al.*, 2013). It is difficult, however, to accurately predict endocrine disruptive (biological) activity based on chemical data due to mixture interactions (Kortenkamp, 2007); bioassays, *in vivo* exposures or animal tissue are more reliable predictors.

The aim of this study was to screen surface water from selected South African rivers, harbours and estuarine environments in three metropolitan municipalities on the South African coastline, namely eThekweni (which includes Durban), Nelson Mandela (specifically Port Elizabeth Harbour) and the City of Cape Town, for endocrine modulation effects (hormone receptor interaction) and female reproductive hormone contamination. The specific objectives were to measure (anti)estrogenic and (anti)androgenic activity in surface water samples using recombinant yeast bioassays (interaction with human steroid receptors), and environmental concentrations of the female hormone 17 β -estradiol (E₂).

A further aim was to explore the association between hydrocarbon loads and endocrine disruptive activity in surface water samples. For this purpose the concentrations of a selection of hydrocarbons were determined in Durban Bay and selected rivers and estuaries in the greater eThekweni metropolitan area.

7.2 Materials and methods

7.2.1 Sample collection and extraction

Surface water was collected at 10 localities in the eThekweni region in August 2012, and three and six locations were sampled in the Nelson Mandela (Port Elizabeth Harbour) and City of Cape Town regions, respectively, during October 2012 (Figure 7.1, Supplementary Table S7.1). The samples were collected in 500 mL acid-cleaned, amber glass bottles with PTFE-lined caps, kept on ice and shipped to the laboratory within 24 h. The samples were subsequently filtered through 0.5 µm glassfibre filters (MN 85/90 Machery-Nagel, DE), and the pH adjusted to 3 using 37% HCl. Non-polar and slightly polar compounds were extracted from 250 mL of water within 96 h of collection using 500 mg DSC-18 columns and a Visiprep® manifold system (~10 mL.min⁻¹) (Sigma, ZA). The columns were flushed with 50 ml of Milli-Q water directly after environmental samples were passed through, in order to remove salts. A Milli-Q negative control was included during each extraction event. The SPE columns were subsequently air-dried overnight, after which compounds were eluted from the column using a solvent mixture (40% hexane, 45% methanol and 15% 2-propanol), air-dried, reconstituted in absolute ethanol and stored at -20 °C.

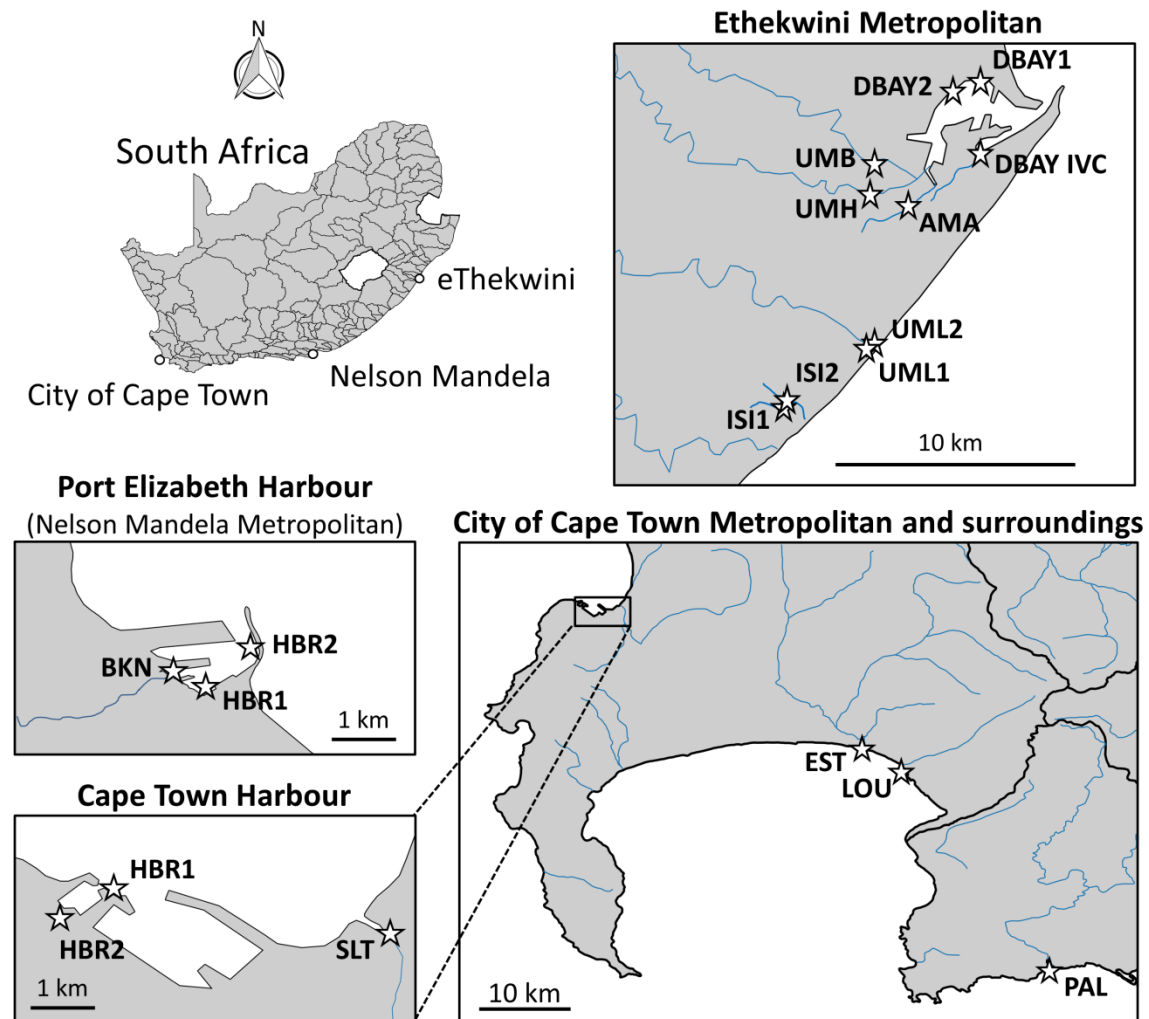


Figure 7.1: Maps indicating the sampling locations in the metropolitan municipalities of eThekweni (which includes Durban), Nelson Mandela (specifically Port Elizabeth Harbour) and City of Cape Town and surroundings. Secondary catchments and rivers are also indicated. Details of locations are provided in Supplementary Table S7.1. Note that the Palmiet Estuary falls outside the Cape Town metropolitan region.

7.2.2 *In vitro* recombinant yeast assay

Estrogenic, anti-estrogenic, androgenic and anti-androgenic activity of surface water C18 extracts (representing non-polar and slightly polar compounds) were evaluated using the respective Yeast Estrogen Screen (YES), Yeast Anti-estrogen Screen (YAES), Yeast Androgen Screen (YAS) and Yeast Anti-androgen Screen (YAAS) recombinant yeast bioassays (Section 3.2.4; Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998). The water extracts and C18 (Milli-Q) negative controls were assayed in sterile 96-well flat-bottom plates (Costar, Corning, USA) at a 10× and 5× concentrated state ('1× concentrated' denotes the state in nature) as described in section 3.2.4.2 of this dissertation.

Estradiol, dihydrotestosterone (DHT), tamoxifen (TAM) and flutamide (FLU) equivalent values (expressing the relative potency of surface water samples) were calculated per assay plate using the plate-specific regression functions (Grover *et al.*, 2011). These equivalents express biological activity (i.e., hormone receptor agonism [E_2 and DHT] or hormone receptor antagonism [FLU and TAM]) equivalent to the activity associated with the particular hormone or pharmaceutical standard represented. The calculations were performed as described in section 3.2.4.3 of this dissertation.

7.2.3 Enzyme-linked immunosorbent assays (ELISAs)

17 β -estradiol levels were measured in the C18 SPE extracts of water collected from the 19 localities using a commercially available ELISA kit (DRG International Inc., USA) according to the manufacturer's instructions. The extracted samples in ethanol (2 000 \times concentrated) were diluted 1/20 in a 0.1% w/v human serum albumin and 0.9% NaCl solution, and assayed (Swart and Pool, 2007).

7.2.4 Hydrocarbon analysis

The concentrations of an array of hydrocarbons (the polycyclic aromatic hydrocarbon (PAH) isomers benzene, ethylbenzene, toluene, o-xylene, m'p'-xylene (collectively BTEX), naphthalene, acenaphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene, indeno(1,2,3-c,d)pyrene, benzo(a)pyrene and dibenzo(a,h)anthracene), and total petroleum hydrocarbon (TPH) carbon equivalent ranges C10–C12, C12–C16, C16–C21, C21–C30, C30–C35 and C35–C40, were analysed in surface water samples collected in the eThekweni region. The analyses were performed by the commercial analytical laboratory Eurofins Analytico (Barneveld, Netherlands), using accredited methods that are proprietary. Briefly, internal standards were added to aliquots of water samples. BTEX was analysed using a headspace gas chromatograph mass spectrometer. TPH's were analysed using large volume injection-gas chromatograph-flame ionisation detection with a heating profile between 175 and 575 °C. PAHs were extracted using dichloromethane, exchanged into acetonitrile, injected into a high pressure liquid chromatograph column, and eluted using a water/acetonitrile gradient. The PAHs were detected using ultraviolet absorbance. Quantification was performed against internal standards, but certified reference material was not analysed for purposes of quality assurance and control.

7.2.5 Statistical analyses

The correlation between E_2 concentration and estrogenicity was evaluated using the Spearman Rank test (Statistica 12, Statsoft, USA). Potential associations between hydrocarbons and anti-androgenic activity among sampling locations were evaluated using a principal component analysis biplot (Canoco 5, Microcomputer Power, USA). A p -value <0.05 was considered significant.

7.3 Results

Estradiol was detected in all samples analysed, although at concentrations <1 ng.L⁻¹ in Port Elizabeth and Cape Town harbours and in samples from the Lourens and Palmiet estuaries (Figure 7.2). The highest E_2 concentration was detected in the mouth of the Salt River in Cape Town (20.96 ng.L⁻¹), followed by the Isipingo Estuary in the eThekweni region (17.41 ng.L⁻¹).

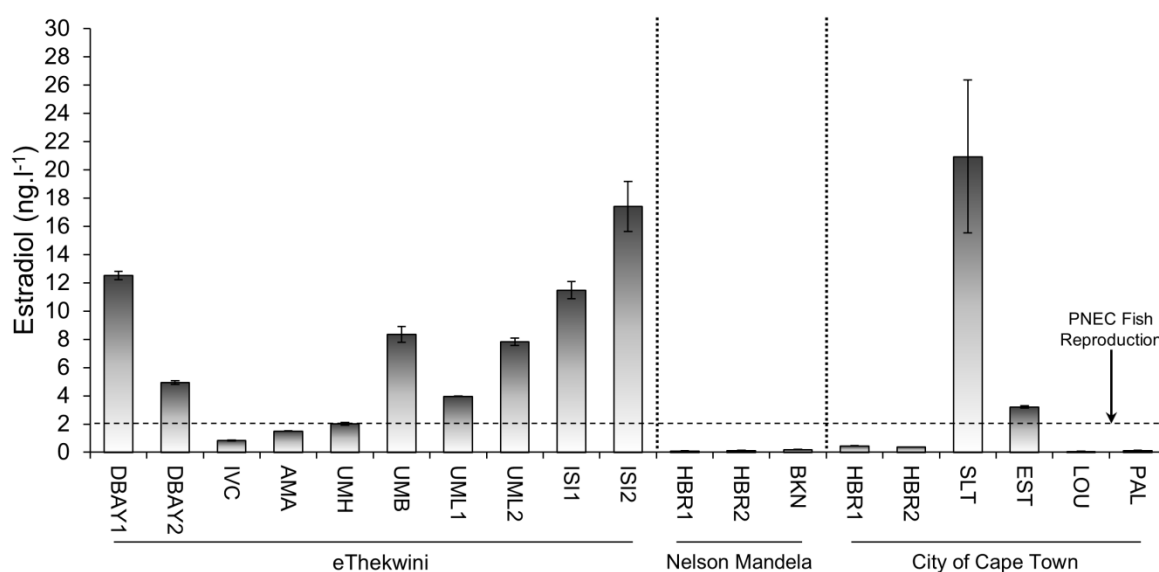


Figure 7.2: Estradiol concentrations for surface water samples collected in rivers, estuaries and harbours in the eThekweni, Nelson Mandela and City of Cape Town metropolitans, South Africa (see Figure 7.1 for sampling locations). Error bars indicate the standard deviation among replicates within an immuno assay plate. The horizontal dashed line indicates the predicted no-effect concentration (PNEC) for reproductive impairment in fish (Caldwell *et al.*, 2012).

Estrogenicity (binding to the human estrogen receptor [ER]) was detected in seven of the 19 samples analysed (Figure 7.3). The eThekweni region had the highest proportion of estrogenic samples, with activities ranging between 1.33 and 8.01 ng.L⁻¹ E_2 equivalents

(EEQs) (Figure 7.3). Conversely, no activity was detected in the Port Elizabeth Harbour samples. The sample collected at the mouth of Salt River in Cape Town was the most estrogenically active, with an EEQ of 12.82 ng.L⁻¹. Although the EEQs (Figure 7.3) followed a similar trend as E₂ concentrations (Figure 7.2), for example, being highest in the mouth of the Salt River, EEQs were generally lower than E₂ concentrations measured using ELISA. There was a significant correlation between E₂ concentrations and estrogenic activity when the three study regions were evaluated collectively (Spearman Rank $r = 0.82$, $p < 0.05$).

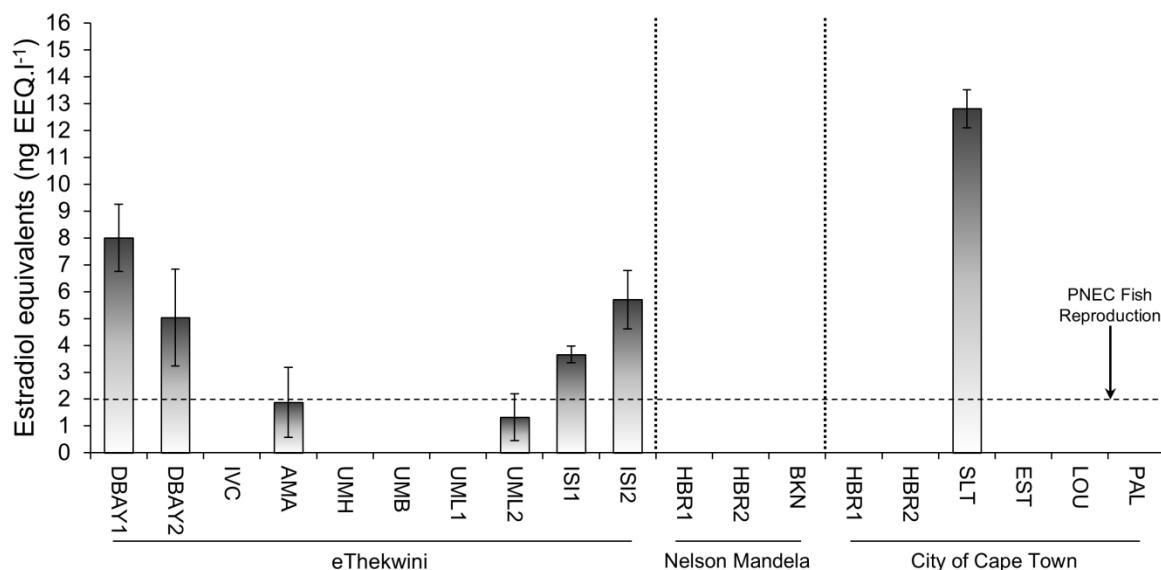


Figure 7.3: Estrogenicity expressed as estradiol equivalents for surface water samples collected in rivers, estuaries and harbours in the eThekweni, Nelson Mandela and City of Cape Town metropolitans, South Africa (see Figure 7.1 for sampling locations). Error bars indicate the standard deviation among assay plates. The horizontal dashed line indicates the predicted no-effect concentration (PNEC) for reproductive impairment in fish (Caldwell *et al.*, 2012).

Anti-androgenicity (inhibition of androgen binding to the human androgen receptor [AR]) was more widespread than estrogenicity and detected in 14 of the 19 samples analysed (Figure 7.4). All samples collected in the eThekweni region were anti-androgenic, with flutamide equivalents (FEQ) ranging between 89.10 and 604.44 $\mu\text{g.L}^{-1}$. None of the Port Elizabeth Harbour samples were anti-androgenic. Similarly, no inhibition of androgen binding to AR was observed in the Cape Town Harbour samples, whereas all three of the estuarine and river mouth samples collected in the City of Cape Town region were anti-androgenic, ranging between 59.60 and 196.86 $\mu\text{g FEQ.L}^{-1}$ (Figure 7.4).

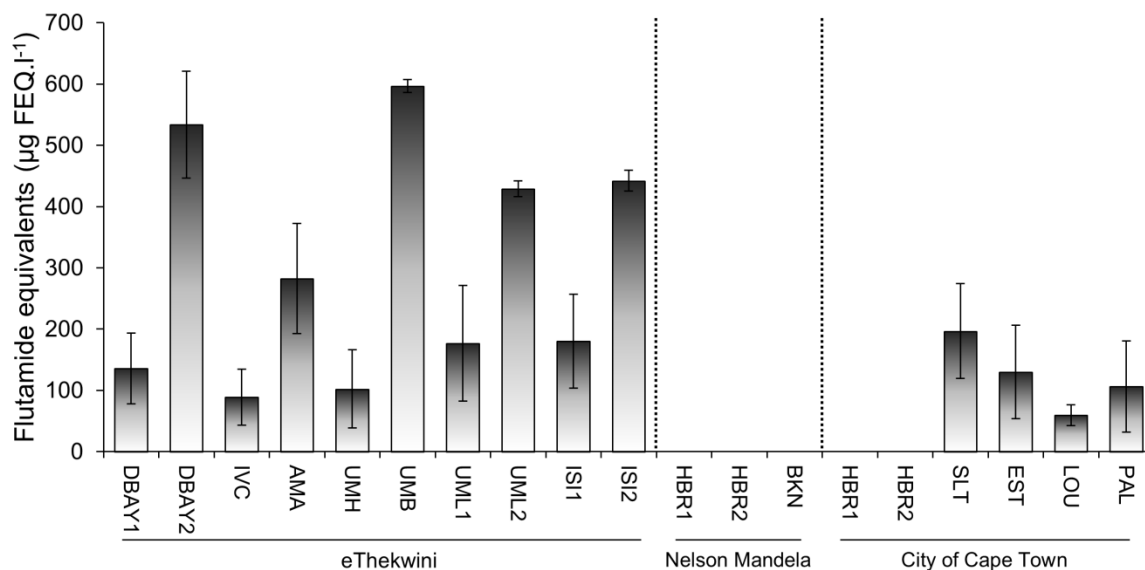


Figure 7.4: Anti-androgenicity expressed as flutamide equivalents for surface water samples collected in rivers, estuaries and harbours in the eThekweni, Nelson Mandela and City of Cape Town metropolitans, South Africa (see Figure 7.1 for sampling locations). Error bars indicate the standard deviation among assay plates.

No anti-estrogenic or androgenic activity was detected in any of the samples analysed.

A biplot of a principal component analysis of hydrocarbons and anti-androgenicity indicates three major groupings for sampling locations in the eThekweni region (Figure 7.5). In particular, locations AMA and ISI1 (see Figure 7.1) grouped together and were associated with the majority of TPHs analysed, PAH isomers pyrene, fluoranthene, anthracene and phenanthrene, and the sum of all PAH isomers. In addition, locations DBAY1, DBAY2, IVC, UML2, UMB and ISI2 grouped together and were more correlated with anti-androgenicity than other samples analysed. Anti-androgenicity was not closely correlated to any of the hydrocarbons analysed (Figure 7.5). Hydrocarbon concentrations are presented in the supplementary material (Tables S7.2 and S7.3). All BTEX components in the eThekweni study area were below the method detection limit. (Table S7.2). Conversely, the total petroleum hydrocarbon (TPH) analyses show the presence of TPHs at all the locations sampled, apart from UMH and UML1 (Table S7.2). The highest diversity of PAHs was detected at the IVC locality in Durban Bay (sum PAH: $0.22 \mu\text{g.L}^{-1}$), followed by AMA, which had the highest abundance of PAHs in the study area (sum PAH: $0.28 \mu\text{g.L}^{-1}$) (Table S7.3). However, virtually no PAHs were detected at the other locations sampled (Table S7.3).

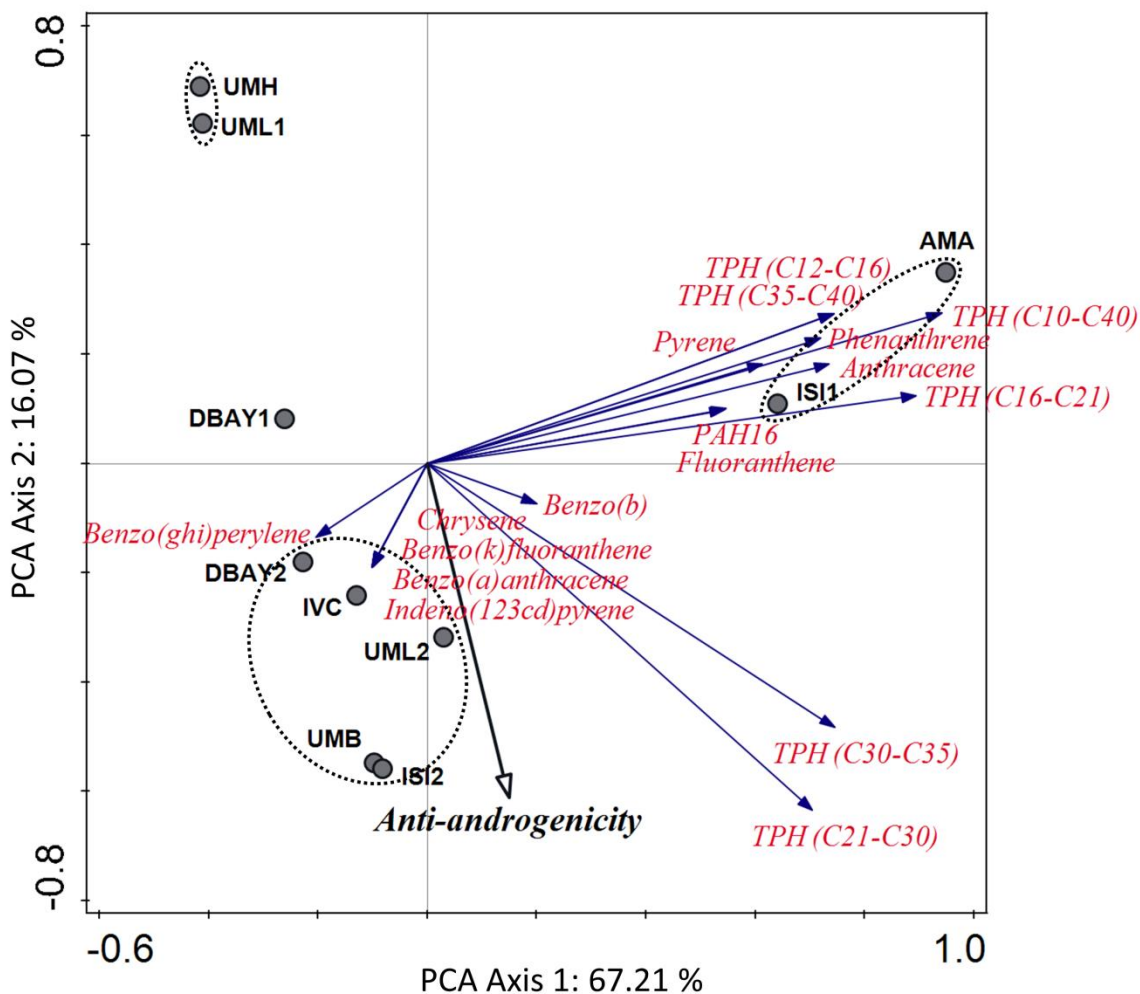


Figure 7.5: Principal component analysis biplot of anti-androgenicity in reference to selected hydrocarbons detected in surface water collected in the eThekweni Metropolitan.

7.4 Discussion

Published records providing concentrations of organic pollutants such as pesticides and industrial chemicals in the South African coastal environment are extremely limited (Wepener and Degger, 2012). Moreover, to my knowledge, no study of the South African coastal environment has described biological endpoints of endocrine disruptive activity, apart from Marshall and Rajkumar (2003), who observed imposex in a mollusc species.

The present study confirms estrogenic and anti-androgenic activity in surface waters collected in Durban Bay and a number of rivers, river mouths and estuaries in the eThekweni and Cape Town regions. Conversely, no such activity was detected in the Cape Town and

Port Elizabeth harbours, indicating good quality in terms of EDCs targeting estrogen or androgen receptors. Durban Bay is a highly transformed estuarine embayment that receives inflows from three rivers and surface runoff from a multitude of stormwater outfalls and canals. Conversely, Port Elizabeth Harbour receives inflow from a single small river, historically an estuary, transformed to built-up land (Veldkornet *et al.*, 2015), and subject to urban surface runoff. There is no riverine inflow to Cape Town Harbour. The higher estrogenic and anti-androgenic activity observed in Durban Bay may, therefore, be explained by the sheer number of anthropogenic sources of contaminants entering the bay via its large catchment, compared to the Cape Town and Port Elizabeth harbours.

The estrogenic activity detected in the eThekweni and City of Cape Town regions (1.33–12.82 ng EEQ.L⁻¹) is within the range reported for Chinese rivers and estuaries (Zhao *et al.*, 2011; Rao *et al.*, 2013). Studies in other countries have typically reported lower activity, such as in the Rhine River, Germany (1.1–1.3 ng EEQ.L⁻¹) (Pawlowski *et al.*, 2003), the Shannon River basin, Ireland (0.53–2.67 ng EEQ.L⁻¹) (Kelly *et al.*, 2010), the Seine and Oise rivers, France (0.30–4.52 ng EEQ.L⁻¹) (Cargouet *et al.*, 2004), and in a nationwide study in the Netherlands (0–0.17 ng EEQ.L⁻¹, $n = 90$ samples which included estuaries) (Vethaak *et al.*, 2005). The E₂ levels detected throughout the three study areas correlated well with estrogenic activity, suggesting that the observed estrogenicity was largely due to female hormone contamination and, therefore, likely linked to waste water treatment plant (WWTP) discharges.

The anti-androgenic activity detected in the eThekweni and City of Cape Town regions exceeded 100 µg FEQ.L⁻¹ at 12 of the locations sampled, and although relatively high, they were similar to activities reported for surface waters elsewhere. Some examples include the Zhujiang River estuary, China (135 µg FEQ.L⁻¹; Zhao *et al.*, 2011), the Lambro River, Italy (438.15 µg FEQ.L⁻¹; Urbatzka *et al.*, 2007), and the Ray River, England (5–250 µg FEQ.L⁻¹; Grover *et al.*, 2011). In a modelling study, FEQs of 0–100.12 µg.L⁻¹ were predicted for 30 rivers in the United Kingdom (Jobling *et al.*, 2009).

Both estrogenic and anti-androgenic chemicals may affect reproduction by altering sex organ development and function (Arukwe, 2001), which may have severe consequences for fish populations (Kidd *et al.*, 2007; Jobling *et al.*, 2009). Estradiol concentrations as low as 4 ng.L⁻¹ induce the development of ovarian tissue in testes in Japanese medaka *Oryzias latipes* (Metcalf *et al.*, 2001), and the predicted no-effect concentration (PNEC) for this compound in terms of reproductive impairment in fish has been estimated as 2 ng.L⁻¹ (Caldwell *et al.*, 2012). Estradiol levels exceeding the PNEC for fish were detected in 10 of the 19 samples analysed in the present study. The pharmaceutical anti-androgen, flutamide,

has been shown to disrupt the reproductive system of Asian catfish *Clarias batrachus* at 33 $\mu\text{g.L}^{-1}$ (Chakrabarty *et al.*, 2012; Rajakumar *et al.*, 2012), Murray rainbowfish *Melanitaenia fluviatilis* at 125 $\mu\text{g.L}^{-1}$ (Bhatia *et al.*, 2014), and fathead minnow *Pimephales promelas* at 320 $\mu\text{g.L}^{-1}$ (Filby *et al.*, 2007). The fish populations inhabiting a large proportion of the systems evaluated in the present study, including Durban Bay, are therefore at risk of endocrine disruption, potentially resulting in reproductive disorders such as intersex and impaired reproduction, due to the combined action of estrogens and anti-androgens. Future studies evaluating phenotypic endpoints such as male plasma vitellogenin levels (Vethaak *et al.*, 2005), the expression of marker genes integral to endocrine signalling (Truter *et al.*, 2014), and histopathology of the gonads (Allen *et al.*, 1999) are necessary, however, to confirm the presence of endocrine disruption. Moreover, seasonal investigations on potential endocrine disruptive activity will be of great value as baseline data and to aid conservation efforts.

Petrochemical pollution is an important component of contamination in harbours (Mestres *et al.*, 2010). PAHs have been detected in sediment and mussels in the ports of Cape Town, Port Elizabeth and Durban, and in sediment in rivers and estuaries in the greater eThekweni and City of Cape Town areas (Brent K. Newman, unpublished data; see also Degger *et al.*, 2011; Kampire *et al.*, 2015). The anti-androgenic activity observed in the present study may have been caused by petrochemicals, which are known to be potent androgen receptor antagonists (Kizu *et al.*, 2003; Vrabie *et al.*, 2010). The weak correlation observed between anti-androgenicity and hydrocarbon concentrations in the samples from the eThekweni region (Figure 7.5), however, provides some evidence that androgen receptor antagonists other than hydrocarbons were responsible. Environmental anti-androgens include pesticides, pharmaceuticals and industrial chemicals (Korner *et al.*, 2004). Detailed chemical analyses are required to identify more potential anti-androgens in Durban Bay and the surrounding rivers that were sampled.

The present study is the first on endocrine disruptive activity in the South African coastal environment. It indicated that waters in Durban Harbour, at the Umlazi Canal mouth and in the Isipingo River estuary, and at the mouth of the Salt River in Cape Town, were estrogenic. Moreover, widespread anti-androgenicity was observed in Durban Bay and selected rivers in the eThekweni region, and in three estuaries and a river mouth in the City of Cape Town region, although at a lower potency than in the eThekweni region. Conversely, no endocrine disruptive activity was detected in Port Elizabeth Harbour. The levels of estrogenic and anti-androgenic activity observed are high enough to suggest an effect on the reproduction of certain fish species, and further investigation in this context is required.

7.5 References

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7.6 Supplementary material

Table S7.1: Sampling locations with descriptions and GPS coordinates. ETH = eThekweni, NM = Nelson Mandela, CPT = Cape Town metropolitan municipalities. The Palmiet Estuary falls outside the City of Cape Town municipality, yet within the metropole's surroundings.

Sample ID	Description	GPS Coordinates	
ETH DBAY1	Durban Bay 1	29°51'43.64" S	31°01'31.86" E
ETH DBAY2	Durban Bay 2	29°52'2.06" S	31°00'46.03" E
ETH DBAY IVC	Island View Channel (Feeding DBN Bay)	29°53'37.91" S	31°01'38.92" E
ETH AMA	Amanzimnyana River (Feeding DBN Bay)	29°54'51.94" S	30°59'47.36" E
ETH UMH	Umhlatuzana River (Feeding DBN Bay)	29°54'36.69" S	30°58'46.31" E
ETH UMB	Umbilo River (Feeding DBN Bay)	29°53'47.28" S	30°58'51.02" E
ETH UML1	Umlazi River mouth 1	29°58'9.20" S	30°58'42.14" E
ETH UML2	Umlazi River mouth 2 (Concrete side-canal that receives the discharge of a WWTP)	29°58'7.56" S	30°58'43.45" E
ETH ISI1	Isipingo Estuary 1	29°59'25.03" S	30°56'31.32" E
ETH ISI2	Isipingo Estuary 2	29°59'20.56" S	30°56'28.77" E
NM HBR1	Port Elizabeth Harbour 1	33°58'3.76" S	25°38'14.06" E
NM HBR2	Port Elizabeth Harbour 2	33°57'46.06" S	25°38'34.01" E
NM BKN	Baakens River, at the inlet to Port Elizabeth Harbour	33°57'52.04" S	25°37'51.57" E
CPT HBR1	Cape Town Harbour	33°54'11.68" S	18°25'43.18" E
CPT HBR2	Royal Cape Yacht Club within Cape Town Harbour complex	33°55'13.84" S	18°26'32.43" E
CPT SLT	Salt River mouth	33°54'29.88" S	18°28'21.37" E
CPT EST	Eerste Estuary	34° 04'48.95" S	18°46'2.22" E
CPT LOU	Lourens Estuary	34° 06'3.55" S	18°48'55.20" E
CPT PAL	Palmiet Estuary	34°20'36.77" S	18°59'39.32" E

Table S7.2: Concentrations of monoaromatic hydrocarbons and total petroleum hydrocarbons (TPH) detected in water samples collected in Durban Bay, the Amanzimnyama, Umhlatuzana, Umbilo and Umlazi Rivers, and the Isipingo Estuary in the eThekweni metropolitan municipality.

Chemical	Conc	Location									
		DBAY1	DBAY2	AMA	DBAY IVC	UMB	UMH	UML1	UML2	ISI1	ISI2
Benzene	$\mu\text{g.L}^{-1}$	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
Toluene	$\mu\text{g.L}^{-1}$	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
Ethylbenzene	$\mu\text{g.L}^{-1}$	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
o-Xylene	$\mu\text{g.L}^{-1}$	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
m'p'-Xylene	$\mu\text{g.L}^{-1}$	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
Xylenes (sum)	$\mu\text{g.L}^{-1}$	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40
BTEX (sum)	$\mu\text{g.L}^{-1}$	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
TPH (C10–C12)	$\mu\text{g.L}^{-1}$	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0
TPH (C12–C16)	$\mu\text{g.L}^{-1}$	<5.0	<5.0	12	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
TPH (C16–C21)	$\mu\text{g.L}^{-1}$	<6.0	<6.0	17	<6.0	<6.0	<6.0	<6.0	7.1	9.9	<6.0
TPH (C21–C30)	$\mu\text{g.L}^{-1}$	10	15	35	12	16	<10	<10	12	31	16
TPH (C30–C35)	$\mu\text{g.L}^{-1}$	<5.0	<5.0	14	6.5	6.6	<5.0	<5.0	5.3	10	9.2
TPH (C35–C40)	$\mu\text{g.L}^{-1}$	<8.0	<8.0	8.9	<8.0	<8.0	<8.0	<8.0	<8.0	<8.0	<8.0

Table S7.3: Concentrations of polycyclic aromatic hydrocarbons (PAH) detected in water samples collected in Durban Bay, the Amanzimnyama, Umhlatuzana, Umbilo and Umlazi rivers, and the Isipingo Estuary in the eThekweni metropolitan municipality.

Chemical	Conc	Location									
		DBAY1	DBAY2	AMA	DBAY IVC	UMB	UMH	UML1	UML2	ISI1	ISI2
Naphthalene	$\mu\text{g.L}^{-1}$	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020
Acenaphthylene	$\mu\text{g.L}^{-1}$	<0.050	<0.050	<0.050	<0.050	<0.050	<0.050	<0.050	<0.050	<0.050	<0.050
Acenaphthene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Fluorene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Phenanthrene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	0.11	0.025	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Anthracene	$\mu\text{g.L}^{-1}$	<0.0050	<0.0050	0.017	<0.0050	<0.0050	<0.0050	<0.0050	0.006	<0.0050	<0.0050
Fluoranthene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	0.042	0.034	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Pyrene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	0.095	0.037	<0.010	<0.010	0.019	<0.010	<0.010	0.024
Benzo(a)anthracene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	<0.010	0.019	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Chrysene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	<0.010	0.017	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Benzo(b)fluoranthene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	0.016	0.039	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Benzo(k)fluoranthene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	<0.010	0.011	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Benzo(ghi)perylene	$\mu\text{g.L}^{-1}$	0.011	<0.010	<0.010	0.018	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Indeno(123cd)pyrene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	<0.010	0.016	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Benzo(a)pyrene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Dibenzo(ah)anthracene	$\mu\text{g.L}^{-1}$	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
PAH (Sum)	$\mu\text{g.L}^{-1}$	<0.20	<0.20	0.28	0.22	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20

Chapter 8: General conclusions

The reality of the risk of endocrine disruption in wildlife is realised throughout the developed world. Little is, however, known regarding the status of South African surface waters in terms of potential endocrine disruption, especially for endpoints such as altered thyroid and corticosteroid signalling. In this dissertation, using *in vitro* and *in vivo* techniques, the risk of endocrine disruption was evaluated in a selection of South African surface water bodies subject to a diversity of contamination sources. In addition, associations between different land use areas and specific types of endocrine disruptive potential were identified based on the findings in the different water systems investigated. A further aim of the study was to compare the results of recombinant yeast *in vitro* reporter gene assays with gene expression based biomarkers in juvenile fish and tadpoles exposed *in vivo*, hence determining whether the predicted risks correspond among the *in vitro* and *in vivo* assays. A final aim was to evaluate the potential of *O. mossambicus* as environmental sentinel and source of biomarkers for endocrine disruptive activity, including molecular targets representing the thyroid and interrenal systems.

In vitro reporter gene assays indicate a risk of endocrine disruption in wildlife inhabiting the Upper Olifants River, and foremost so at Waste Water Treatment Plant (WWTP) impacted locations. Estrogenic activity and exceptionally high levels of steroid hormones were detected downstream of a WWTP responsible for the treatment of waste water from Emalaheni, Mpumalanga Province. Alarmingly, this particular plant is one of 212 in South Africa that have been rated as in a “critical state” by the South African Green Drop Certification initiative (DWAS, 2014), and similar impacts can, therefore, be expected in various receiving waters. There is an urgent need for more in depth evaluations on wildlife inhabiting waters impacted by the aforementioned WWTPs. Additionally, the integration of endocrine disruptive activity as parameter tested for in the Green Drop Certification will be valuable. The present study furthermore indicate a risk of altered steroidogenesis in fish and potentially other organisms inhabiting sections of the Upper Olifants River impacted by agriculture, based on changes observed in the expression of the aromatase coding *cyp19a1b* gene in juvenile *O. mossambicus* exposed *in vivo*.

The Loskop Dam *O. mossambicus* population is characterized by a high incidence of obesity and the adipose tissue inflammatory disease, pansteatitis. Potential links between the impaired health of Loskop Dam fish and endocrine signalling was investigated. No association could be shown between the prevalence of obesity and pansteatitis and the

expression of a selection of genes linked to thyroid and corticosteroid signalling as well as peroxisome proliferator-activated receptor gamma in adult *O. mossambicus* inhabiting Loskop Dam. Further investigation on fauna inhabiting Loskop Dam is required and in particular other endocrine pathways such as leptin signalling and epigenetic endpoints need to be studied.

Surprisingly little is known regarding the effects of crude oil on the endocrine systems of freshwater vertebrates. The present study provides data indicating the potential risk of crude oil contamination in fresh water to the reproductive and thyroid systems as well as lipid metabolism in wildlife. No significant changes in the expression of a selection of endocrine linked genes was, however, observed in *X. laevis* tadpoles exposed for a short period to surface water collected from a freshwater pan into which crude oil contaminated water is periodically discharged. Further investigation, and in particular longer term exposures, as well as the evaluation of aquatic fauna collected from fresh water bodies contaminated with crude oil are required.

In addition, the present study indicates a low risk of endocrine disruption associated with exposure to neutralized acid mine drainage (AMD) (containing high levels of metals including Al, Mn, Ni, Co and Cu) based on gene expression based biomarkers in juvenile *O. mossambicus* exposed *in vivo* for a short period. In particular, mineralocorticoid receptor was the only gene evaluated in which the expression varied significantly among the fish exposed to surface water collected directly downstream of an AMD neutralization plant relative to fish exposed to water from a reference stream. More research on the responses of other species and life stages to neutralized AMD exposure will be of value.

Data is furthermore presented indicating a risk of endocrine disruption to wildlife inhabiting selected river mouths and estuaries in the eThekweni and City of Cape Town metropolitans. In particular, *in vitro* estrogenicity and anti-androgenicity were observed in the coastal waters evaluated, suggesting that the fecundity of local fish and potentially other animals may be affected. No published study to date has assessed gonads or testes in marine or estuarine fish captured from the South African coastal environment, and such research will be of value. The screening of potential thyroid disruption in coastal waters is also needed, considering the fact that various thyroid disruptors have been shown to be present (Fatoki and Awofolu, 2004; Ogata *et al.*, 2009; La Guardia *et al.*, 2013).

Gene expression based biomarkers were functional, and allowed the evaluation of multiple pathways. However, it is realised that such an approach can only be used as a screen to indicate the need for further investigation (Hutchinson *et al.*, 2006). Some discrepancies in

the risk predicted by the *in vitro* reporter gene assays and the gene expression biomarkers in fish exposed *in vivo* were evident, as has been previously shown (Hermelink *et al.*, 2010). In particular, although high steroid estrogen loads, estrogenic activity and anti-androgenic activity was detected at a WWTP impacted location in the Upper Olifants River, the expression of a selection of genes representing the reproductive system (i.e., *vitellogenin 1* [*vtg1*], *estrogen receptor 1* [*esr1*] and *aromatase* [*cyp19a1b*]) did not vary significantly among fish exposed to samples collected from the WWTP impacted locality and the negative control fish. Crude oil was shown to exhibit potent anti-androgenic activity *in vitro*, but no significant differences were observed in the expression of selected genes linked to the male reproductive system (i.e., *steroid 5 alpha reductase 1* [*srd5a1*] and *androgen receptor* [*ar*]) in *X. laevis* exposed *in vivo* relative to the control treatment. However, *ar2* was significantly upregulated in *O. mossambicus*, at least indicating a degree of disruption of the male hormone system, as predicted by the *in vitro* assays, although only at a high crude oil concentration.

The discrepancy between *in vitro* results and the gene expression in juvenile *O. mossambicus* exposed for a short period may be due to low sensitivity of the species and the life stage applied. In general, the juvenile *O. mossambicus* proved to be robust, and a very limited number of mortalities were observed in animals exposed to environmental water samples, hormone standards and crude oil water accommodated fractions. However, the juvenile *O. mossambicus* were generally insensitive to estrogenic contaminants, and no significant changes in gene expression was observed in response to 45 ng.L⁻¹ 17 β -estradiol (E₂), or surface water extracts from the Olifants River containing 17 β -estradiol and ethinyl estradiol (EE₂) concentrations of up to 30.80 ng.L⁻¹ and 10.83 ng.L⁻¹ respectively. Conversely, in other fish species significant changes in biomarkers representing the female reproductive system has been observed at concentrations below 10 ng.L⁻¹ E₂ and 1 ng.L⁻¹ EE₂ (Caldwell *et al.*, 2012). Moreover, in the present study, exposure to a triiodothyronine (T₃) concentration of 5 μ g.L⁻¹ induced a significant increase in *thrb* expression in *O. mossambicus* exposed for 48 h, whereas *dio2* expression was unchanged at the same dosage. A further study indicated a significant increase in *thrb* expression in the livers of male Striped parrotfish, *Scarus iseri*, exposed to 13.02 μ g.L⁻¹ T₃, but not in females, and in the brains of both males and females (Johnson and Lema, 2011). In a further study, no significant change in *thrb* and *dio2* expression was observed in goldfish, *Carassius auratus*, exposed to 13.02 μ g.L⁻¹, whereas only *dio2* expression was significantly downregulated in individuals exposed to a higher dosage of 65.10 μ g.L⁻¹ T₃ (Marlatt *et al.*, 2012). Significant increased *thrb* and *dio2* expression have been shown in response to markedly lower T₃ concentrations in amphibians. In particular, 0.33 μ g.L⁻¹ T₃ induced a significant increase in

thrb and *dio2* expression in the brains and gonad–mesonephros complex of *Silurana tropicalis* tadpoles (Duarte-Guterman and Trudeau, 2010; Duarte-Guterman and Trudeau, 2011). Similarly, a significant increase in *thrb* expression in the tail tissue of *X. laevis* tadpoles exposed to $0.31 \mu\text{g.L}^{-1} \text{T}_3$ have been shown (Zhang *et al.*, 2006). Nonetheless, a significant induction of thyroid linked genes (*thra*, *thrb* or *dio2*) in juvenile *O. mossambicus* were observed in response to surface water exposure in three of the chapters of the present dissertation, as well as in response to crude oil WAF. These aforementioned results suggest that thyroid endpoints/biomarkers in juvenile *O. mossambicus* exposed *in vivo* may be functional to screen for thyroid disruption by exogenous substances. Both *X. laevis* tadpoles and juvenile *O. mossambicus* were exposed to the exact same crude oil water accommodated fraction (WAF) samples, in part, to evaluate interspecific variation. Significant changes in the expression of *ar2* and *type 2 deiodinase (dio2)* was observed in the fish, but at a high concentration of 25g.L^{-1} , whereas *thyroid hormone receptor beta (thrb)* and *peroxisome proliferator-activated receptor gamma (pparg)* was significantly altered in *X. laevis*, and at WAF concentrations as low as 0.25g.L^{-1} in both cases. These differences indicate the advantage of using multiple species to evaluate the risk of endocrine disruption.

In summary, the present study indicate the reality of endocrine disruption in selected South African water bodies, and advocate the need for further evaluation. Collection of fish and amphibians from the wild with more endocrine-linked endpoints evaluated including thyroid and metabolism is required. Ill maintained WWTPs are clearly important point sources and the impacts on freshwater bodies needs to be further investigated, especially in the South African context, seeing the at the EDCs released are not only a risk to wildlife, but humans as well. Various South African aquatic systems remain unexplored in regard to endocrine disruption in wildlife and biological activity of surface water. Some examples include the the Berg, Breede, Limpopo, mid and lower Olifants (Mpumalanga), Olifants (Western Cape), Orange, Tugela, and Vaal Rivers. Moreover, most of the South African coastal environment has not been evaluated for EDC risk and effects in wildlife. In addition, the present study suggests that juvenile *O. mossambicus* are not sensitive to estrogenic compounds and other endemic fish sentinals needs to be assessed, or the application of international models such as zebrafish, *D. rerio*, Japanese medaka, *Oryzias latipes*, or three-spined sticklebacks, *Gasterosteus aculeatus*, needs to be considered for testing purposes in South African. The use as *O. mossambicus* as sentinel for thyroid disruption, however, seems more promising. In particular, the present study advocates the use of candidate genes as representatives of the thyroid cascade as biomarkers. Further endpoints such as histopathology of thyroid follicles and plasma hormone levels are yet to be explored as potentially sensitive biomarkers for thyroid disruption associated with exposure to exogenous substances.

There are still many promising research questions relating to endocrine disruptors in the environment that remain unanswered in the South African context. To my knowledge, no study to date has investigated potential epigenetic modification in wildlife exposed to surface water contaminated with EDCs in South Africa, as is the case for the use of transcriptomics, lipodomics, and proteomics approaches in ecotoxicological studies featuring aquatic organisms. The technology to perform the aforementioned investigations is currently available locally, but not applied in the ecotoxicology research sphere, and future collaboration between geneticists and biochemists researching other fields and ecotoxicologists are needed.

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