The assessment of Namibian water resources for endocrine disruptors

by

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Declaration

I declare that *The assessment of Namibian water resources for endocrine disruptors* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

André K. Faul

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January 2018

Dedication

I dedicate this work to my parents who provided me with all the opportunities to follow my dreams and reach my goals.

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Keywords

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Cytotoxicity

Neurotoxicity

Immunotoxicity

Cytokines

Chemokines

Proteome Profiling

Potable Water

Abstract

The assessment of Namibian water resources for endocrine disruptors

Namibia is the driest sub-Saharan country in Africa and it is characterised by low and variable rainfall. As a result, potable water in this mostly semi-arid country is considered an extremely valuable resource. Given the variety of anthropogenic and natural chemicals released into the environment by a growing human population, many water resources worldwide present health risks to both man and wildlife. Many of these chemicals are classified as endocrine disruptors which are chemicals with the ability to adversely affect the physiological systems regulated by the endocrine systems of organisms. These include, among others, reproductive, neurological and immunological effects. Endocrine disrupting chemicals include: natural and synthetic hormones such as estrogen, estrone, estriol and testosterone; heavy metals such as tri-butyltin, lead and cadmium; pesticides such as organophosphates and organochlorides; and a number of compounds such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls.

Windhoek, the capital city of Namibia, has for long been at the forefront of water reclamation by being the first town in the world to reclaim sewage for direct potable re-use. Presently, reclaimed sewage contributes approximately a third of the potable water utilised in Windhoek, with the remaining water being sourced mainly from a three-dam system: the S von Bach, Swakoppoort and Omatako dams, as well as from boreholes tapping into the Windhoek aquifer.

Prior to the research conducted for this thesis, no studies have been undertaken to determine the endocrine disrupting potential of the reclaimed sewage in Windhoek. Likewise, no such studies have been performed on any of the surface water sources in this country, including the three-dam system supplying Windhoek.

During 2010 and 2011, raw sewage, treated sewage and reclaimed sewage samples from Windhoek were collected at different stages of the wet and dry

season. These samples were analysed for cytotoxicity using a lactate dehydrogenase (LDH) assay, for neurotoxicity using an acetylcholinesterase (AChE) inhibition assay, for inflammatory activity using enzyme-linked immunosorbent-assays (ELISAs) to determine interleukin-6 (IL-6) and interleukin-10 (IL-10) concentrations, as well as for the presence and quantification of three selected steroid hormones: estradiol, estrone and testosterone using ELISAs. Simultaneously, surface water from nine dams in Namibia were collected and analysed for the same parameters.

High estradiol, estrone and testosterone levels were detected in the raw sewage. The sewage treatment plant process significantly reduced the concentration of these hormones, but levels were still in the range where adverse effects can be expected in organisms exposed to this water. The reclamation process successfully removed these residual hormones. The AChE inhibition and inflammatory activity of the treated sewage was also significantly lower than in the raw sewage and were completely removed in the reclaimed water. Cytotoxicity was only present in the raw sewage.

In all the dam waters, no samples showed cytotoxicity. Estrone was the only hormone detected at low levels, once in the Avis dam water sample and once in the Goreangab dam water sample. The highest acetylcholinesterase inhibition was noted in the Goreangab dam water. Water from all the dams induced high IL-6 production with the highest levels being in the Goreangab and Swakoppoort dam water. IL-10 was lower than IL-6 concentrations in all samples, but was also highest in the Goreangab and Swakoppoort as well as the Avis dam water samples.

During 2017 the efficiency of the reclamation process of treated sewage in Windhoek was assessed using a range of immunotoxicological bioassays on the water samples. This again included LDH and AChE inhibition assays as well as IL-6 and IL-10 production. In addition interferon-γ (IFN-γ) and macrophage inflammatory protein (MIP) -1β production were also determined using ELISAs. As a broad screen for immunotoxicity, proteome profiling was performed to test

for 36 different chemokines and cytokines. This is the first time that proteome profiling is used for determining the immunotoxicity of treated sewage reclaimed for direct potable water use.

For the 2017 assays, no cytotoxicity was detected in treated sewage or reclaimed water. Based on the ELISAs, treated sewage induced IL-6, MIP-1β and IL-10 production, but not IFN-γ. The corresponding test results for the reclaimed water were negative. The proteome profile indicated the presence of interleukin-1ra (IL-1ra), Monocyte Chemoattractant Protein-1 (MCP-1), MIP-1α/MIP-1β, IL-6 and interleukin-1β (IL-1β) in culture supernatants exposed to treated sewage, but not to the reclaimed water.

In conclusion, the results of the studies indicated the usefulness of the *in vitro* bioassays to test the endocrine disrupting potential of water sources. Results indicated that intake water at the reclamation plant in Windhoek contains contaminants that can adversely affect human health. The reclamation process however successfully removed these. However, routine monitoring is required to ensure continued delivery of safe potable water. The study further indicated the usefulness of proteome profiling as a quick, cost-effective screen for the immunotoxicity of water sources. The proteome profile can be followed up with cytokine-specific ELISAs to better quantify the inflammatory potential of water sources.

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List of Abbreviations

µl	Microlitre
µm	Micrometre
Abs/cm	Absorbance per centimetre
AChE	Acetylcholinesterase
ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
APC	Antigen-Presenting Cell
ATCI	Acetylthiocholine iodide
BC	Before Christ
BCR	B cell Receptor
C	Complement Component (e.g., C3, C3a, C5a)
CCL	Chemokine (C-C motif) ligand (e.g., CCL2, CCL5)
CD	Cluster of differentiation (e.g., CD4, CD8)
cDC	Conventional Dendritic Cell
cfu	Colony-forming Unit
CR	Complement Receptor
CXCL	Chemokine (C-X-C motif) ligand (e.g., CXCL1
DDT	2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DTNB	5,5-dithiobis (2-nitro-benzoic acid)
E1	Estrone
E2	Estradiol
ed.	Editor
EDC	Endocrine Disrupting Chemical / Endocrine Disrupting Compound
ELISA	Enzyme-Linked Immunosorbent-Assay
FAS	First Apoptosis Signal
GSI	Gonadosomatic Index
GSTP	Gammams Sewage Treatment Plant

GWRP	Goreangab Water Reclamation Plant
h	Hour
HRP	Horseradish Peroxidase
HSI	Hepatosomatic index
ICAM	Intercellular Adhesion Molecule
IFN	Interferon (e.g., IFN-α, IFN-β)
Ig	Immunoglobulin (e.g., IgG, IgM)
IL	Interleukin (e.g., IL-6, IL-10, IL-1β)
ILC	Innate Lymphoid Cell
l	Litre
LDH	Lactate Dehydrogenase
LOQ	Limit of quantification
LPS	Lipopolysaccharide
LT	Lymphotoxin (e.g., LT-α)
M	Molar
M^3	Cubic Metre
MAC	Membrane Attack Complex
MASP	MBL-Associated Serine Protease
MBL	Mannose-Binding Lectin
MCP-1	Monocyte Chemoattractant Protein-1
MDP	Muramyl Dipeptide
mg	Milligram
MHC	Major Histocompatibility Complex
MIF	Macrophage Migration Inhibitory Factor
min	Minute
MIP	Macrophage Inflammatory Protein (e.g., MIP-1α, MIP-1β)
ml	Millilitre
mm	Millimetre
mS/m	Millisiemens/meter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFAT	Nuclear Factor of Activated T-Cells
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cell

ng	Nanogram
NK Cell	Natural Killer Cell
NLR	NOD-like receptor (e.g. NOD1, NOD2)
nm	Nanometre
nM	Nanomolar
NOD	Nucleotide-Binding Oligomerization Domain
NR	Neutral Red
NRF	National Research Foundation
NTU	Nephelometric Turbidity Unit
PAC	Powder Activated Carbon
PAF	Platelet-Activating Factor
PAH	Polycyclic Aromatic Hydrocarbon
PAI-1	Human Plasminogen Activator Inhibitor-1
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCB	Polychlorinated Biphenyl
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cell
PFU	Plaque-forming Unit
pg	Picogram
PHA	Phytohaemagglutinin
PNEC	Predicted-No-Effect-Concentration
POP	Persistent Organic Pollutant
PRR	Pattern Recognition Receptor
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
ROS	Reactive Oxygen Species
RPMI	Roswell Parks Memorial Institute
SD	Standard Deviation
Serpin	Serine Protease Inhibitor
SPE	Solid-Phase Extraction
STW	Sewage Treatment Works
T	Testosterone

T_{FH}	T Follicular Helper
TGF	Transforming Growth Factor (e.g., TGF-β
T_H	T Helper (e.g., T_H1, T_H2)
TKN-N	Total Kjeldahl Nitrogen
TLR	Toll-Like Receptors
TNF	Tumor Necrosis Factor (e.g., TNF-α)
T_{reg}	Regulatory T Cells
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
v/v	Volume/Volume %
VTG	Vitellogenin
WHO	World Health Organization
WINGOC	Windhoek Goreangab Operating Company

Research Output List

Conference Presentations: Posters

FAUL, A.K., JULIES, E. & POOL, E.J. 2011. Endocrine disrupting potential of water from the Gammams Sewage Treatment Plant and Goreangab Water Reclamation Plant in Windhoek, Namibia. International Symposium on Toxicity Assessment, Hong Kong.

Publications

FAUL, A.K., JULIES, E. & POOL, E.J. 2013. Oestrogen, testosterone, cytotoxin and cholinesterase inhibitor removal during reclamation of sewage to drinking water. *Water SA*. **39**: 499–506.

FAUL, A.K., JULIES, E. & POOL, E.J. 2014. Steroid hormone concentrations and physiological toxicity of water from selected dams in Namibia. *African J. Aquat. Sci.* **39**: 37–41.

Chapter 1

Introduction

1.1 Introduction

"*The next war in the Middle East will be fought over water, not politics.*" This famous quote from 1985 by former United Nations Secretary General, Boutros Boutros Ghali, sums up the importance of fresh water and the pressure that the human population is placing on this renewable, but limited source. In fact, wars have possibly already involved securing of water supplies as is the case in the Battle of Beersheba of 1917 and the Six-Day war of 1967 (http://en.wikipedia.org/wiki/Battle_of_Beersheba_(1917); Seliktar 2005). While water resources remained relatively unthreatened historically, during the last 10 000 years have seen the human civilization starting to take shape and this has culminated in a population explosion during the last two centuries (Smol 2002). The beginning of the industrial revolution some 250 years ago permanently changed the future of water sources worldwide. Increased manufacturing, intensive agriculture and large scale mining became a way of life and together with that, improved health care lead to an explosion in the human population. The downside of this improved lifestyle was, however, numerous adverse impacts on the ecology of the environment in which we live. This was not limited to the degradation of the immediate environment through various forms of pollution and destruction, but also ecosystems in remote locations with limited or no human inhabitants at all. In fact, today it is believed that there is not a single location left on earth where anthropogenic disturbances have not yet occurred (Western 2001).

Water covers about three quarters of our planet's surface. It has long been realised that our impact on freshwater ecosystems through pollution by products such as pesticides, organic waste, agricultural run-off and sewage discharge has made many water sources unsuitable for human or animal consumption (Cabra 2010; Andersson et al. 2012). In marine environments, the adverse effects of these pollutants have always been believed to be insignificant, since oceans were seen as being resistant to the effects of pollutants mainly as a result of the ocean's volume and thus a dilution effect (Cohen 2001). However, the amount of pollutants reaching the oceans and specifically coastal areas are raising serious concern. With more than seven billion people living on earth, and exploiting its

resources to make their lives more comfortable, man must take a closer look at the pollutants reaching our aquatic resources and the way they impact on biota and man.

Laws to prevent pollution of the environment and water resources are not an entirely new thing. Laws dating back to 500 B.C. were in place for proper refuse disposal in Greece, and in ancient Rome, pollution of the Tiber River were prohibited. The practice of drinking water purification and treatment in ancient Egypt dates back even further to about 2000 B.C. (Kartaginer 2004). This is seen in inscriptions on Egyptian walls and medical texts (1440 - 2000 B.C.) that shows primitive purification devices and talks about keeping and treating water in copper vessels. Initial concerns regarding water quality therefore were mostly focussed on the elimination of waterborne disease in humans.

More recently, during the middle of the 19th century, discoveries by scientists like Filippo Pacini, Robert Koch, Joseph Lister and John Snow, showed that diseases like Cholera spread through bacterial contamination of drinking water (Page 2004; Department of Environmental Engineering 2005). Advances in water treatment over the next 200 years have led to the eradication of most water borne diseases in the developed world. These have been so successful that even the reclamation of sewage for human consumption has become a reality. With the elimination of waterborne disease in drinking water the focus of many researchers has moved to the chemical contamination and pollution of aquatic ecosystems and the effect that these may have on both humans and wildlife. Already, towards the end of the 19th century, American naturalist George Perkins Marsh warned about the way man has changed the woods and streams of Western Europe (Pullin 2002), but it was not until the 1960s that the threat of contamination of water resources became a major concern. Often this change in attitude towards pollution, especially of water resources, is accredited to the publication of the books like Silent Spring by Rachel Carson. Today thousands of publications exist on all forms of aquatic pollution, the threats that they pose, and its mitigation or remediation. Among all these pollutants is a group called the endocrine disrupting chemicals (EDCs) that pose health threats to both man and wildlife.

Water scarce countries, like Namibia, has to take special precautions when it comes to the protection of its potable water sources. With the only perennial rivers being on the northern and southern borders, some 1 250 km apart and shared with the neighbouring countries, Namibia relies mainly on underground aquifers and the storing of rain water in man-made and natural reservoirs. Two main alternative water sources are utilised and these are a sewage reclamation plant in Windhoek, the first of its kind in the world, and a desalination plant on the central coast.

Despite the vulnerability of Namibia in terms of its water resources, no research has been conducted with regard to the endocrine disrupting potential of the various surface and sub-surface water sources. For completion of this thesis multiple biomarkers have been employed for the first time ever, to determine the endocrine disrupting potential of various surface water sources (water from storage dams) and of reclaimed sewage in Windhoek. The literature review (Chapter 2) provides a brief discussion on the nature and history of EDCs before reviewing the most frequently used monitoring and screening methods for EDCs in the environment. It further provides a short summary of cytotoxicity, neurotoxicity and immunotoxicity as physiological biomarkers for EDCs in aquatic habitats and presents an overview of the immune system to create a better understanding of immunotoxicity and the use of immunological biomarkers for toxicity testing.

Chapter 3 and Chapter 4 assesses the successful removal of some selected potential EDCs in the sewage treatment and reclamation process in Windhoek as well as in nine surface water sources throughout Namibia. This is achieved by determining oestrogen and testosterone levels as well as cytotoxic, neurotoxic and immunotoxin potential of the water sources. Chapter 5 focuses only on the reclamation process of treated sewage and whether it successfully removes pollutants with immunomodulatory potential. It further investigates the potential of using proteome profiling for quick and cost effective screening of reclaimed water for direct potable reuse.

1.2 Problem Statement

Potable water is becoming an increasingly scarce resource and anthropogenic impacts on remaining water sources pose a number of health problems. In Windhoek, Namibia, reclamation of treated sewage has been an important source of potable water for almost five decades. While microbial contamination and aesthetic characteristics of reclaimed water are strictly monitored, the endocrine disrupting potential of the reclaimed water, as well as other surface water sources, has never been investigated. The potential impact of potable water on physiological systems of the Namibian, and specifically Windhoek, population are therefore not known.

1.3 Focus of the Study

This study will focus on determining the endocrine disrupting potential and presence of EDCs in treated and reclaimed sewage and selected surface water sources in Namibia. It will further investigate the use of proteome profiling as a rapid and cost-effective screen for immunotoxicity in reclaimed water.

1.4 Aims and Objectives

The aim of the study is to assess the baseline endocrine disrupting potential of treated and reclaimed sewage as well as surface water sources in Namibia. This will be achieved through the following objectives.

Objective 1: To determine the baseline steroid hormone concentrations, specifically estradiol, estrone and testoasterone, in raw, treated and reclaimed sewage at the Gammams Sewage Treatment Plant (GSTP) and Goreangab Water Reclamation Plant (GWRP) in Windhoek.

Objective 2: To determine cytotoxicity, neurotoxicity and immunotoxicity of raw, treated and reclaimed sewage at the Gammams Sewage Treatment Plant (GSTP) and Goreangab Water Reclamation Plant (GWRP) in Windhoek, using selected biomarkers,.

Objective 3: To determine the baseline steroid hormone concentrations, specifically estradiol, estrone and testoasterone, in nine dams in Namibia.

Objective 4: To determine cytotoxicity, neurotoxicity and immunotoxicity, using selected biomarkers, in nine dams in Namibia.

Objective 5: To, for the first time ever, do proteome profiling of treated and reclaimed sewage at the GSTP and the GWRP, to determine the immunotoxicity of reclaimed sewage for direct potable reuse.

1.5 References

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Chapter 2

Endocrine Disrupting Chemicals, their Screening Methods, and the Use of Physiological Endpoints as Biomarkers for EDCs in Aquatic Environments

2.1 What are Endocrine Disrupting Chemicals?

In the 1980's and 1990's several scientists in the field of toxicology shifted their attention to a collection of chemicals that would be termed endocrine disrupting chemicals or endocrine disrupting compounds (EDCs). EDCs are chemicals, both natural and synthetic, with the ability to mimic or antagonize the effects of hormones, alter the pattern of synthesis and metabolism of hormones, and modify hormone receptor levels (Colborn et al. 1993; Burkhardt-Holm 2010). Today, various definitions for EDCs are in use. The World Health Organization (WHO) defines it as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (WHO 2002), while the United States Environmental Protection Agency (US EPA) defines EDCs 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" (Markey et al. 2003). Furthermore, the WHO defines a potential endocrine disruptor as "an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations" (WHO 2002).

When the first evidence of chemicals altering hormonal systems of wildlife surfaced, the number of chemicals suspected of having this ability were largely limited to pesticides and some industrial chemicals. Today, hundreds of chemicals are either suspected to, or known to have endocrine disrupting properties. Although many of these EDCs are of anthropogenic origin, there are also those that occur naturally in the environment. As highlighted by Markey et al. (2003) and Burkhardt-Holm (2010), the major recognised groups of EDCs today are:

- Natural EDCs
    - Natural hormones like estradiol, progesterone and testosterone
    - The metabolites of natural hormones like estrone and estriol
    - Phyto- and myco-estrogens

- Heavy metals, like cadmium and lead

- Synthetic EDCs
  - Synthetic hormones like those used in hormonal treatments and contraception, e.g., 17-α-ethinylestradiol
  - Pesticides and their metabolites, like organochlorine and organophosphate
  - Chemicals, like polychlorinated biphenyls (PCBs), plasticizers, surfactants and polycyclic aromatic hydrocarbons (PAHs)

2.2 A Brief History of Endocrine Disrupting Chemical Research

The majority of research on EDCs has mainly been conducted in the last two to three decades. However, the potential effects of EDCs were already predicted in the middle of the 20th century although it was not termed "endocrine disruptor" at that stage (Matthiessen 2003; Markey et al. 2003). In the 1940's to 1960's it was noted that various bird species and animals like minks showed reduced reproductive success and declining population numbers (Niering 1968; Markey et al. 2003). These effects were linked to the presence of sewage, agricultural run-off as well as chemicals like PCBs and dioxin in aquatic habitats. During the same time, livestock showed endocrine disrupting effects believed to be as a result of natural and synthetic substances (Bennetts 1946; Burlington 1950). However, despite evidence pointing towards the harmful effects of some chemicals on wildlife, society at large remained either ignorant towards it or ignored the warnings expressed. The publication *Silent Spring* by Rachel Carson (1962) was met with some scepticism by industrialists and agriculturalists (Cottam and Scott 1963; Niering 1968) and this further illustrates the disregard shown towards environmental threats due to pollutants during the middle to latter part of the 20th century.

During the 1970's and 1980's increasing evidence of endocrine disruption in wildlife surfaced. For example, studies on tadpoles exposed to 2,2-bis (p-chlorophenyl)-1,1,1-trichloroethane (DDT) showed retardation in tail

generation (Weis 1975) while gull eggs injected with DDT showed feminization of male embryos (Fry and Toone 1981). In the United States of America scientists found that alligators exposed to industrial and agricultural chemicals like DDT showed reproductive disorders (Markey et al. 2003), while sex changes were observed in female dog-whelks as a result of exposure to tri-butyltin and anti-fouling paints (Gibbs et al. 1988). Abnormal thyroid pathology in herring gulls and salmon were also attributed to exposure to environmental contaminants (Moccia et al. 1981; Moccia et al. 1986). In the early 1990's a strong focus and awareness in research into the endocrine disruptive behaviour of chemicals ensued. One of the key moments in EDC research was the Wingspread Conference held in Raccine, Wisconsin in 1991 (Markey et al. 2003). Here, focus was on the large number of man-made chemicals that had the potential to disrupt the endocrine systems of animals and possibly humans. Following the Wingspread conference, it seems, the term endocrine disruption was first published in 1992 (Matthiessen 2003; Markey et al. 2003). During the rest of the 1990's the presence and dangers of EDCs in the environment became well established and it became a major research field. A large number of research publications provided evidence for the presence and effects of EDCs in the environment and these typically had a strong focus on aquatic ecosystems and often on fish as affected species. Publications ranged from the occurrence of intersex and vitellogenin (VTG) production in wild and caged fish exposed to estrogenic river water (Harries et al. 1997; Tyler and Routledge 1998) to the possible link between environmental estrogens and male infertility (Colborn 1993; Sharpe 1998). By 1998 various testing methods for reproductive and developmental effects of EDCs on wildlife existed, but these were largely insufficiently validated for regulatory testing (Jobling 1998). However, the existing research field laid the foundation for regulatory action, including controls on certain EDCs, as well as the phasing out of certain chemicals, like phthalates (Jobling 1998). The last 15 years have seen an enormous global interest in the study of EDCs and their effects and a multitude of tests have been developed for the detection and monitoring of EDCs in the environment.

Today a quick search on endocrine disruption using science-based databases can deliver thousands of results. These include literature on the testing of chemicals for endocrine disrupting effects, determination of the presence of, and quantification of, such chemicals in ecosystems, the adverse health effects of EDCs on organisms and the development of biomarkers and bioassays to monitor environmental health. Research has become more and more specific with regard to the chemicals that have an endocrine disrupting potential as well as the potential use of biomarkers or bioassays for the early, cost-effective and easy detection of EDCs. The number of potential EDCs and the continued production of new chemicals, the number of different ecosystems on which they can impact, as well as the different effects they can cause in organisms, together with their degradation pathways and removal from systems, warrants continued research.

2.3 Screening and Monitoring for Endocrine Disrupting Chemicals

The presence of EDCs in the environment is a cause for concern as a result of its effects on wildlife as well as its potential effects on humans. Due to ethical considerations, the effects of EDCs have been much better studied in animals than in humans, but one can expect that many of the effects on animals will also be present in humans. As a result, it is important to be able to monitor environments, water sources and food sources of both wildlife and humans for the presence of EDCs as well as the consequences of its presence on biological systems.

2.3.1 Quantification with Analytical Methods

Analytical methods are often employed to accurately measure the quantity of a specific EDC in a sample (Snyder et al. 2008). The development of advanced instrumentation has significantly increased the ability to detect and analyse for EDCs (Sosa-Ferrera et al. 2013). Analytical methods however do not give an indication of the overall effect of EDCs on organisms as well as the combined effects of multiple EDCs in a system.

2.3.1.1 High-Performance Liquid Chromatography

High-performance liquid chromatography is one of the most frequently used analytical techniques for determination of EDCs. Various modern techniques such as hyphenated chromatography-mass spectrometry, the use of monolith columns, liquid chromatography conducted at high temperatures, and liquid chromatography at ultra-high pressures using columns packed with sub-2-micron particles provided increased sensitivity and/or reduced time required for the analyses (Sodré et al. 2010; Liu et al. 2011; Sosa-Ferrera et al. 2013).

2.3.1.2 Mass Spectrometry

Mass spectrometry is a very sensitive and selective method that also provides information on the molecular structure of the compounds analysed (Snyder et al. 2008; Sosa-Ferrera et al. 2013). Mass spectrometry can be combined with other analytical techniques to reach different objectives and to improve results (Chang et al. 2009). For example, when chromatography is combined with mass spectrometry, mixtures can be separated into individual compounds or elements which can then be analysed qualitatively and quantitatively (Sosa-Ferrera et al. 2013).

2.3.2 *In Vitro* Bioassays

The *in vitro* and cell-based assays target specific EDCs and allow for relatively high throughput at costs lower than those of the *in vivo* animal studies while allowing high reproducibility (Gross et al. 2003; Shanle and Xu 2011). Additional advantages include their use for studying modes of action, screening the effects of mixtures, and detecting interaction effects. However, in the *in vivo* systems, EDCs may behave differently if they undergo enzymatic conversions, this being the major limitation of the *in vitro* assays (Gross et al. 2003).

2.3.2.1 Receptor-Binding Assays

Receptor-binding assays are useful to monitor for and identify specific EDCs. Certain EDCs will bind to specific receptors, for example estrogenic EDCs will

interfere with estrogenic signalling by binding to estrogen receptors (Holmes et al. 1998; Shanle and Xu 2011). Receptor-binding assays to identify chemicals interacting with estrogen receptors include transcriptional reporter assays, bioluminescence or fluorescence resonance energy transfer and fluorescence polarization assays (Shanle and Xu 2011).

2.3.2.2 Target-Cell Proliferation and Differentiation Assays

The growth and multiplication of cells, or the differentiation of cells, can be used to screen for EDCs that have the potential to stimulate cell proliferation or differentiation in specific cells. One such assay is the E-screen assay that works on the principle of estrogenic chemicals stimulating for example MCF-7 cells derived from human breast cancer cells (Soto et al. 1995). Chemicals leading to the proliferation of MCF-7 cells are identified as E2 agonists by the assay, but one potential limitation of the study is the fact that it may result in false positives due to the presence of other chemicals that also stimulate cell proliferation such as nutrients, cytokines and growth factors (Gross et al. 2003). Methods for increasing accuracy have however been investigated with some success (Tian et al. 2002).

2.3.2.3 Cell-Based Expression Assays

Biological responses to EDCs may involve the induction or suppression of proteins by specific genes (Gross et al. 2003). One of the most often used endpoints for expression assays to determine estrogenicity of chemicals is the measurement of VTG (Anderson et al. 1996, Tremblay and Van der Kraak 1998). Cells that are used for expression assays include fish hepatocytes, MCF-7 and yeast (Anderson et al. 1996; Tremblay and Van der Kraak 1998; Gross et al. 2003). It should however be noted that the type of cell used will determine the response and cell sensitivities also show great variation. The major advantage of expression assays is that they detect both agonists and antagonists (Gross et al. 2003).

2.3.2.4 Reporter Gene Assays

Reporter gene assays are based on the ability to manipulate eukaryotic cells at a genetic and biochemical level (Liu 2009). Cells can be genetically modified to express specific gene products when they are stimulated. These gene products may be measurable, as is the case in fluorescence assays, or displayed enzymatic activity can be monitored. The luciferase assays for example typically use yeast or mammalian cells which are transfected with an expression vector like one that encodes the human estrogen receptor and estrogen response elements (Baker 2001; Berckmans et al. 2007; Liu 2009).

2.3.2.5 Non-Cellular Bioassays

A number of assays exist that do not rely on cellular material for the *in vitro* quantification of EDCs. A popular example is the Enzyme-Linked Immuno-Sorbent Assays (ELISA) that can, for example, be used to detect presence, or quantify concentrations of, steroid hormones. These typically can be performed using ready-to-use kits that can be bought from a variety of suppliers. A major advantage of these assays is that one does not have to deal with the difficulties often experienced when working with living cells and organisms such as mortalities, varying life stages and individual toxicity responses (Swart 2008). One major disadvantage is that the assays do not provide an indication of the consequences of EDC presence on biological systems.

2.3.3 *In Vivo* Assays

The i*n vivo* assays are not mechanism-dependent and as a result they can identify potential EDCs as well as provide a description of their potential effects. It thus provides more environmentally relevant results than the *in vitro* studies (Gross et al. 2003). The i*n vivo* assays either look at naturally exposed organisms or controlled exposures. Popular endpoints in *in vivo* assays are molecular, biochemical and physiological changes. Biochemical and molecular level changes are typically early indicators of exposures and effects.

The i*n vivo* assays making use of molecular endpoints include receptor analysis, transcriptional-based analysis and differential displays (Gross et al. 2003; Larkin et al. 2003; Lee et al. 2007). An example includes differential display reverse transcription polymerase chain reaction (PCR) that can be used to detect induction and repression of gene expression. The major advantage of molecular endpoints in EDC assays is their sensitivity, but they can be difficult to validate and it may lack relevance to ecological systems (Gross et al. 2003).

Biochemical endpoints include the up or down regulation of a variety of enzymes and proteins in response to EDC exposure. A popular biochemical endpoint is the production of VTG in response to estrogenic EDCs in oviparous species like fish (Harries at al. 1997; Lye et al. 2005), amphibians (Palmer and Palmer 1995; Van Wyk et al. 2003; Selcer and Verbanic 2014) and invertebrates like mussels and crabs (Ricciardi et al. 2008; Ricciardi et al. 2010). However, many different biochemical constituents and enzymes have been studied as potential biomarkers with the advantage of them being more sensitive, less variable and easy to measure (Vijayavel and Balasubramanian 2006). A number of *in vivo* assays measure cell viability as endpoint after exposure to EDCs. Examples include lysozyme and lysosomal investigations in mussels (Farcy et al. 2011; Dailianis et al. 2003).

Some biomarkers do not assess the biochemical or molecular level of EDC effects, but rather at whole organism responses or morphological characteristics. These endpoints include aspects like mortality (Ackermann et al. 2002; Akaishi et al. 2007), growth rate (Jobling et al. 2003; Robinson et al. 2007), reproductive traits (Ackermann et al. 2002; Lye et al. 2005; Dammann et al. 2011), hepatosomatic index (HSI), gonadosomatic index (GSI) and organ histology (Bogers et al. 2007; Dammann et al. 2011). The major advantage of these biomarkers is that they have excellent eco-relevancy as part of *in vivo* research.

2.4 Physiological Endpoints as Biomarkers for Contaminants in Aquatic Environments

Over the last three to four decades a great deal of research was conducted and methods were developed that relate to the detection of environmental pollution. Methods either identify specific chemicals through quantification with analytical methods (Snyder et al. 2008) or *in vivo* and *in vitro* measurement of the presence and/or effects of toxins (Gross et al. 2003) often using physiological endpoints. The following is a brief overview of three physiological systems that are frequently employed for EDC and general toxicity testing of aquatic environments, utilizing *in vivo* and *in vitro* assays. They are cytotoxicity, neurotoxicity and immunotoxicity.

2.4.1 Cytotoxicity

Cytotoxicity (cellular toxicity) is the ability of a substance to adversely affect and potentially kill living cells. When cells are exposed to a cytotoxic chemical it can lead to reduced cell viability, (i.e. reduced/no cell growth and division), cell death as a result of necrosis, and programmed cell death (apoptosis) (Orrenius et al. 2011). Cell viability tests are popular measures for the determination of the cytotoxic potential of chemicals or contaminated water sources (Zhang et al. 1990). Cell viability is often evaluated by quantifying the amount of plasma membrane damage induced by exposing a cell culture to a toxin.

A widely used method for the determination of cell viability is measurement of the amount of lactate dehydrogenase (LDH) that leak through damaged plasma membranes of cells (Zhang et al. 1990). Lactate dehydrogenase is an intracellular enzyme found in most viable cells. The assay is based on the principle that increased cytotoxicity results in increased cell membrane damage, causing the release of intracellular components, and thus a proportional increase in LDH leakage. The amount of LDH present in the extracellular medium is thus representative of the amount of cell damage. A cell culture is for example exposed *in vitro* to a toxin and after exposure LDH can be measured in the supernatant by allowing it, if present, to oxidize lactate to pyruvate (Mosmann 1983).

Chromogenic-based water-soluble tetrazolium salts are then added which reacts with pyruvate to form formazan dye. The amount of dye correlates directly with the LDH and can be determined spectrophotometrically (Mosmann 1983).

Lactate dehydrogenase assays can be used on a wide range of cells, both animal and human. In previous studies mouse macrophage RAW264.7 cell lines were for example exposed to treated sewage (Makene and Pool 2015) and raw and treated textile factory wastewater (Makene et al. 2016). The cytotoxicity of raw sewage, treated sewage and reclaimed sewage (Faul et al. 2013) and various surface water sources (Faul et al. 2014) were determined using human whole blood cultures. The absence of cytotoxicity results in such studies does not necessarily mean that the samples tested had no toxic effects on cells. It is possible that cells are effected intracellularly without any plasma membrane damage.

Another popular method to determine cell viability is the use of neutral red retention assays (Lowe and Pipe 1994; Dailianis et al. 2003; Martínez-Gómez et al. 2015). It is often used to determine lysosomal membrane stability in invertebrates and typically in *in vivo* applications. Typically, haemolymph of test organisms are collected and neutral red dye is added. The dye will freely permeate the cell membranes of granulocytes where it will be taken up by the lysosomes. Dye leakage from the lysosomes back into the cytoplasm of the granulocytes are then monitored against time. Granulocytes with intact lysosomes will retain the neutral red dye for longer than cells that experienced damage as a result of, for example, exposure to toxins. Although neutral red retention assays are very popular for use with invertebrates like mussels (Lowe and Pipe 1994; Dailianis et al. 2003; Rickwood and Galloway 2004) and earthworms (Maboeta et al. 2004; Reinecke and Reinecke 2007) it has also been used on cultured mammalian cells like rat and human hepatocytes (Zhang et al. 1990).

Cell metabolic activity can also be used as a measure of cytotoxicity. Metabolic activity can be determined using the tetrazolium (MTT)-based colorimetric assay (Mosman 1983; Twentyman and Luscombe 1987). The soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is converted into insoluble formazan crystals in a redox reaction that is catalyzed by

oxidoreductases (Twentyman and Luscombe 1987; Parolini et al. 2011). The purple formazan crystals are then dissolved in an organic solvent and the optical density determined in a spectrophotometer. Viable cells with normal metabolic rates will reduce more MTT and hence produce more formazan resulting in increased absorbances.

Bacterial toxicity assays can be used as a sensitive measure of cytotoxicity as they are reproducible and cost-effective (Parvez et al. 2006; Leusch 2015). One example is the use of the gram-negative bacterium *Vibrio fischeri* which produces bioluminescence as a by-product of metabolism. Exposure of the bacteria to toxins can reduce its metabolism and thus the amount of bioluminescence. The reduction in bioluminescence is therefore an indicator of cytotoxicity. Bioluminescence assays have successfully been employed for many years to test the cytotoxic potential of waste water and other pollutants (Fernández et al. 1995; Dizer et al. 2002; Kelly et al. 2004; Ye et al. 2011).

2.4.2 Neurotoxicity

Neurotoxicity refers to the deleterious effects that exposure to toxins may have on nervous system structure and function. This can include cell death, structural damage or disruption of signaling processes (Blake 2004). Neurotoxicity of waste water is typically associated with the presence of pesticides like organophosphates and carbamate (Ghedira et al. 2009), but can include many other toxins like heavy metals (Järup 2003), brominated flame retardants (Hendriks and Westerink 2015), polychlorinated biphenyls (PCBs) (Tilson et al. 1990; Ndountse and Chan 2009) and polycylic hydrocarbons (Maisano et al. 2015).

One of the most frequently employed neurotoxicity assay is the determination of acetylcholinesterase (AChE) inhibition in exposed organisms or cell cultures (Rickwood and Galloway 2004; Ghedira et al. 2009; Solé et al. 2009). Acetylcholinesterase is the enzyme responsible for the hydrolysis of acetylcholine, a neurotransmitter, into choline and acetic acid (Ghedira et al. 2009). Pesticides like organophosphates inhibit AChE irreversibly, thus disrupting the functioning of the nervous system. By measuring the inhibition of AChE in

exposed versus control organisms or cell cultures, one can establish the neurotoxic potential of a chemical or mixture of chemicals. Neurotoxic effects are often associated with raw and semi-purified waste water streams. Acetylcholinesterase assays have for example been performed on human whole blood cultures (Faul et al. 2013; Faul et al. 2014), whole homogenized earthworm extracts (Rault et al. 2008), mussel haemolymph (Dailianis et al. 2003; Rickwood and Galloway 2004), crab gill and hepatopancreas (Ghedira et al. 2009), homogenized whole body or heads only, of small aquatic invertebrates including water fleas, stoneflies and mayflies (Day and Scott 1990), rat blood plasma and homogenized rat brain tissue (Singh and Rishi 2005).

2.4.3 Immunotoxicity

The presence of a multitude of anthropogenic and natural chemicals in the environment have the ability to influence both innate and acquired immunity. These chemicals are often referred to as immunotoxins and their immunotoxic effects can result in immunosuppression, immunostimulation, hypersensitivity and autoimmunity (Duramad and Holland 2011; Luster 2014). The next section first provides a brief overview of immunity before some biomarkers of immunotoxicity are discussed.

2.5 Immunity

Objects or microorganisms foreign to living organisms that, when interacted with, can result in disease or damage, are generally referred to as pathogens. This disease or damage is caused when introduction of a pathogen into a living organism results in an imbalance in the normal homeostasis of an organism, or it causes physical damage to cells, tissues and organs. Immunity is the physiological system responsible for the protection of organisms against pathogens like bacteria, viruses, fungi and parasites as well as protection in cases of injury (Cota and Midwinter 2009; Lilic 2009). In immunity, the specific proteins of pathogens that are recognized by the immune system are referred to as antigens. It is however not only living organisms like bacteria that are potential pathogens that can illicit an

immune response, but also many of the chemicals and pollutants like EDCs we encounter on a daily basis.

In higher vertebrates, the functioning of the immune system, or the immune response, can be classified into two main categories, namely innate immunity (first and second line of defence) and acquired immunity (third line of defence) (Cota and Midwinter 2009). Innate immunity are those elements of the immune system one is born with, while acquired immunity is the immunity one develops as a result of exposure to antigens throughout life (Murphy and Weaver 2016). Innate and acquired immunity are not two entirely distinct processes, but rather one complex, interdependent physiological process, i.e., some steps of the innate immune system are required for processes of acquired immunity to be activated and *vice versa* (Lilic 2009). Typically, once infection by a pathogen occurs, both the innate and acquired immune system are required to rid the organism of the infection. In general, the innate immune system, which is fast acting, keeps the infection suppressed, while the acquired immune system activates through a much longer process and eliminates the infection completely.

The following review of the immune system aims to provide a general overview of the immune system components and functioning. The objective is to provide background to immunity in order to create a better understanding of the field of immunotoxicity addressed earlier, which is an important focal point in the remaining research chapters of this thesis.

2.5.1 Immune Cells

Central to most immune responses are a group of cells collectively referred to as leukocytes or white blood cells. Leukocytes drive or influence both innate and acquired immune systems and develop and mature, like most immune system cells, within the bone marrow (Luster et al. 2005; Murphy and Weaver 2016). Leukocytes originate from two lineages of stem cells, the lymphoid and myeloid lines (Figure 2.1) (Murphy and Weaver 2016). The lymphoid cells are B Cells, T Cells, Natural Killer (NK) Cells and innate lymphoid cells (ILC), while myeloid cells are neutrophils, eosinophils, basophils, monocytes and mast cells. Dendritic

cells can originate from both lineages although it originates mainly from the myeloid lineage. Furthermore, monocytes from the myeloid lineage mature into macrophages within tissues while erythrocytes (red blood cells) and thrombocytes (platelets) also originate from the myeloid lineage of stem cells, but does not form part of this review.

2.5.2 Innate Immune System

The innate immune system is the first line of defence against immunity and it acts by preventing pathogens from entering the body, or when they do enter, to destroy them within minutes. The innate immune system however lacks the specificity and memory which is part of acquired immunity (Lilic 2009). Innate immunity utilises secreted proteins and cell-associated receptors (pattern recognition receptors (PRRs)) to detect pathogens and to prevent self-recognition. It is the part of the immune system that one is born with and the receptors are coded by genes inherited from parents (Murphy and Weaver 2016). Innate immunity comprises of barriers, the complement pathway and phagocytosis.

2.5.2.1 Barriers

Barriers are the first line of defence against infections and include chemical and anatomical barriers. Anatomical (or mechanical) barriers are the epithelial cells (including hairs and cilia) of the skin, respiratory system, gastrointestinal tract, eyes and oral cavity (Cota and Midwinter 2009; Murphy and Weaver 2016). The cells of epithelial linings function by forming tight mats that physically prevent pathogens from penetrating the deeper lying cells and tissues. In turn, the movement of air and fluids over epithelial surfaces aid in removing pathogens that may accrue on their surfaces.

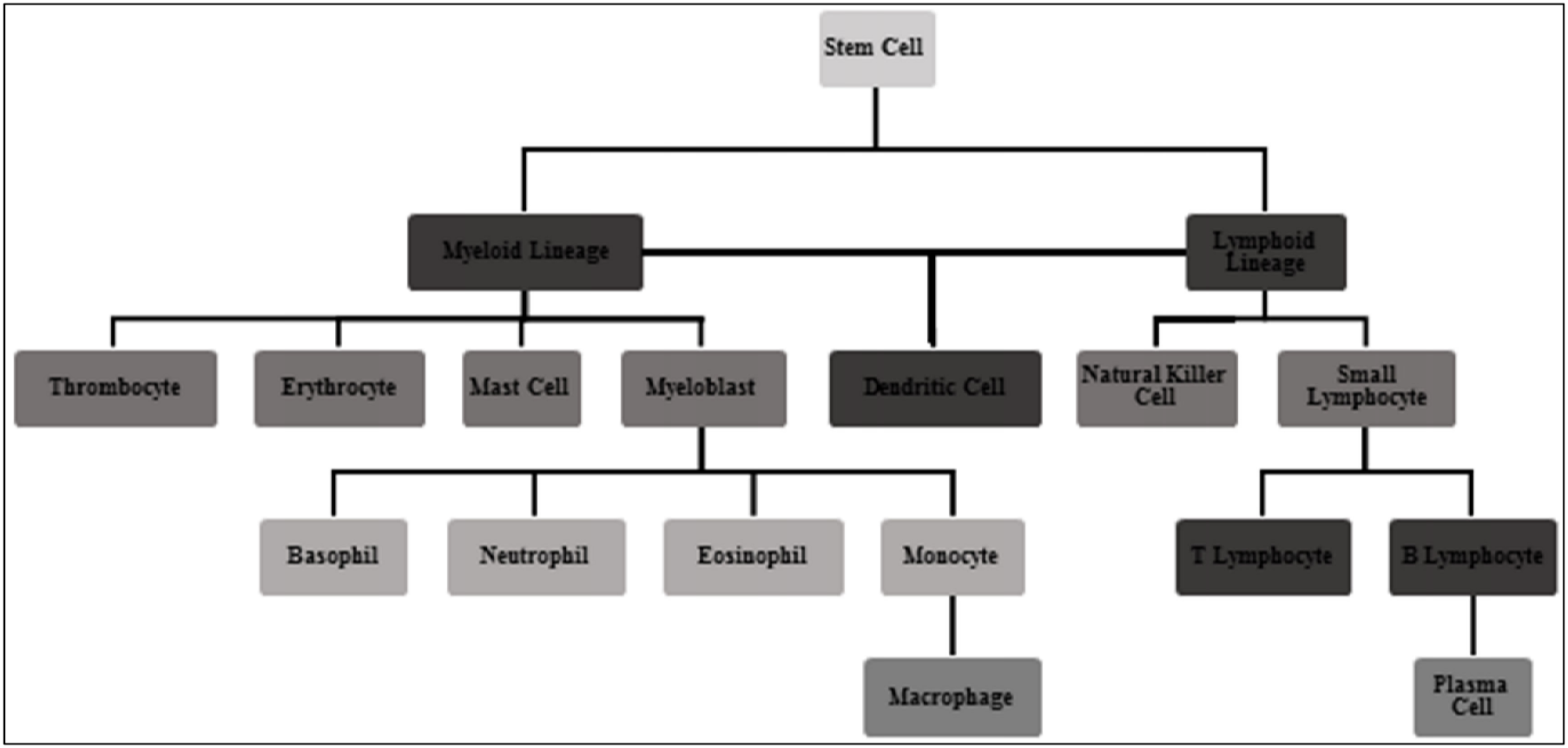

Figure 2.1 Simplified Diagram of the Types and Origin of Leukocytes, Erythrocytes and Thrombocytes (adapted from Murphey and Weaver 2016)

The mucosal epithelium, as is found within for example the respiratory ducts, gastrointestinal tract and oral cavity, secretes and are covered by a layer of mucus (Schleimer et al. 2007; Murphy and Weaver 2016). This viscous liquid functions in defence against pathogens by preventing their adhesion to the epithelium as well as by trapping pathogens. To rid the organism of these pathogens, the mucus and the pathogens contained in it, are continuously being expelled through mucociliary (respiratory surfaces) and peristaltic actions (gastrointestinal tract).

Not all bacteria present on epithelial surfaces are harmful. Healthy epithelial surfaces are populated with non-pathogenic bacteria (microbiota) that competes with pathogens and thus helps prevention of infection (Murphy and Weaver 2016). They produce an assortment of antimicrobial substances such as lactic acid, glycoproteins (mucins) and antimicrobial peptides that fights pathogens as well as stimulate the epithelium to produce antimicrobial peptides.

Epithelial cells and phagocytic cells can eliminate pathogens by producing the antimicrobial enzyme lysozyme (Peterson and Artis 2014). This forms part of the innate immune response and has the ability to recognize and attack specific receptors on bacterial cell walls. It functions by breaking chemical bonds within the cell wall of bacteria and the resultant cellular damage ultimately leads to bacterial death (Callewaert et al. 2017).

The three main antimicrobial peptides produced by epithelial cells and phagocytes are defensins, cathelicidins and histatins (Schleimer et al. 2007; Peterson and Artis 2014; Murphy and Weaver 2016). Epithelial cells secrete these antimicrobial peptides into the mucus lining of the epithelium while it is secreted into tissues by phagocytes. Defensins and cathelicidins act by disrupting the cell membranes of bacteria and fungi and the membrane envelope of some viruses. Histatins are constantly produced by the parotid, sublingual and submandibular glands and acts against pathogenic fungi.

When pathogens manage to bind to or cross over epithelial surfaces, an infection occurs. Damage to epithelial surfaces, like cuts, bites and burns, can allow pathogens to easily enter and infect deeper lying tissues. If not successfully

eliminated, airborne pathogens may infect respiratory tract epithelium while pathogens in food can infect gastrointestinal tract epithelium. In order to maintain its immunological functions, epithelial layers are continuously renewing themselves to ensure intact barriers for protection and other functions.

2.5.2.2 Complement Pathway

When the barrier defence is breached, the complement pathways form the next line of defence. It consists of approximately 30 plasma proteins that collectively or together with antibodies target pathogens (Murphy and Weaver 2016). The complement proteins opsonize pathogens as well as trigger the series of steps involved in the inflammatory process (Janeway et al. 2001). The complement pathways produce three main effectors: 1) proinflammatory anaphylatoxins (C3a and C5a) for activation and attraction of leukocytes; 2) opsonins (e.g. C3b) which attach to pathogens for recognition by phagocytes (opsonisation); and the membrane attack complex (MAC) that lyse opsonised pathogens (Noris and Remuzzi 2013). Complement proteins are synthesized in the liver and it circulates in the blood and other fluids in an inactive form. The complement proteins activates only when in contact with pathogens or antibody-bound pathogens.

Complement pathways acts by killing pathogens either directly, or by facilitating their phagocytosis, and it triggers inflammation utilizing the complement proteins. Three complement pathways exist, all three producing C3 convertases, the role of which will be explained later. They are the classical, alternative and lectin pathways.

The classical pathway is set off when the C1 complement component, containing a recognition protein C1q, recognizes pathogen-associated molecular patterns (PAMPs) on a microbial surface directly, or binds to an antibody-bound pathogen (Cota and Midwinter 2009; Noris and Remuzzi 2013; Ricklin et al. 2016; Murphy and Weaver 2016). The classical pathway is activated by immunoglobulin G (IgG) and IgM, by apoptotic and necrotic cells, and by proteins like C-reactive protein (Noris and Remuzzi 2013).

The lectin pathway, is set off when mannose-binding lectin (MBL) or ficolin recognises specific carbohydrates, or carbohydrate signatures, on pathogens. The MBL, or ficolin, binds these signatures and as a result activates MBL-associated serine proteases (MASPs). The MASPs initiate complement protein cleavage of C4 and C2 which generates the C3 convertase C4bC2a (Murphy and Weaver 2016; Ricklin et al. 2016).

Alternative pathway activation can happen in two ways. In the blood plasma, C3 spontaneously hydrolyses (a process called "tickover") to form C3 (H_2O) and then binds to Factor B. Factor B is cleaved by Factor D into Ba and Bb to form C3 (H_2O)Bb, which is known as short-lived fluid-phase C3 convertase (Murphy and Weaver 2016). The tickover path of the alternative pathway remains primed and active at low levels in a healthy host and can rapidly act against pathogens (Noris and Remuzzi 2013). The second method of activation of the alternative pathway produces the C3 convertase C3bBb. It requires the C3b product of either the classical pathway or lectin pathway to bind to a microbial surface. Factor B then binds to the C3b and is cleaved by Factor D. The cleavage fragment Bb remains bounded to the C3b forming the C3 convertase C3bBb (Murphy and Weaver 2016). The alternative pathway is a signal amplification pathway as the production of C3b leads to the production of C3bBb which in turn produces more C3b when cleaving additional C3.

As mentioned earlier, all three pathways, when interacting with a pathogen, activate C3 convertase enzymes (see Table 2.1). These convertases cleave C3 into C3b and C3a (Ricklin et al. 2010; Noris and Remuzzi 2013; Murphy and Weaver 2016). Opsonisation of pathogens takes place when C3b, acting as an opsin, binds covalently to pathogens (Janeway 2001). The opsonin component is then recognized by phagocytes leading to the phagocytosis of the pathogen. C3b can in turn bind to C3 convertase of the classical and lectin pathways to form C5 convertase. C5 convertase cleaves C5 into C5a and C5b. C5b in turn binds to other complement proteins to form a membrane attack complex (MAC) on pathogens. The MAC results in cell death by causing cell lysis through formation of transmembrane channels in its cell membrane. These transmembrane channels

or pores results in cell lysis through the disruption of the lipid bi-layers (Hadders et al. 2012).

Table 2.1 Convertases of the different complement pathways

Pathway	C3 Convertases	C5 Convertases
Lectin pathway	C4b2a	C4b2a3b
Classical pathway	C4b2a	C4b2a3b
Alternative Pathway	C3bBb	C3bBb3b
Fluid Phase	C3 (H_2O) Bb	

Initiation of phagocytosis takes place when a complement receptor (CR) on the phagocyte recognizes and binds to the C3b opsonin on the pathogen cell membrane and the inflammatory mediator fragment C5a binds to the C5a receptor on the phagocyte (Ricklin et al. 2010; Murphy and Weaver 2016). The C3a and C5a fragments also trigger local inflammatory responses and they recruit antibodies, complement factors and phagocytic cells to infected areas. They result in contraction of smooth muscle, increase vascular permeability, and induce synthesis of adhesion molecules by endothelium of blood vessels. It also activates mast cells to release inflammatory molecules like histamine and cytokine tumor necrosis factor-α (TNF-α). Histamine increases vascular permeability, resulting in more fluid in the infected area which in turn speeds up the movement of pathogen-bearing antigen-presenting cells (APCs) to local lymph nodes. The presence of APCs accelerates the initiation of the acquired immune response (Murphy and Weaver 2016).

The effector functions of the complement pathways have the potential to harm the host itself, if not properly regulated (Noris and Remuzzi 2013). In normal functioning immune systems, complement pathways are prevented from triggering inflammation and cell destruction in non-target areas. This is achieved by rapid inactivation of activated complement components, should they fail to bind to the surface of the pathogen that stimulated their activation (Murphy and Weaver 2016). Also, specific proteins prevent activation of complement components on healthy host cells (Noris and Remuzzi 2013; Murphy and Weaver 2016). However, the complement pathways do rid the body of dying host cells by

triggering phagocytosis of these cells. This prevents release of the cell contents of the dying cells and thus prevents autoimmune responses (Murphy and Weaver 2016). Figure 3.2 provides a summary of the three complement pathways (Murphy and Weaver 2016).

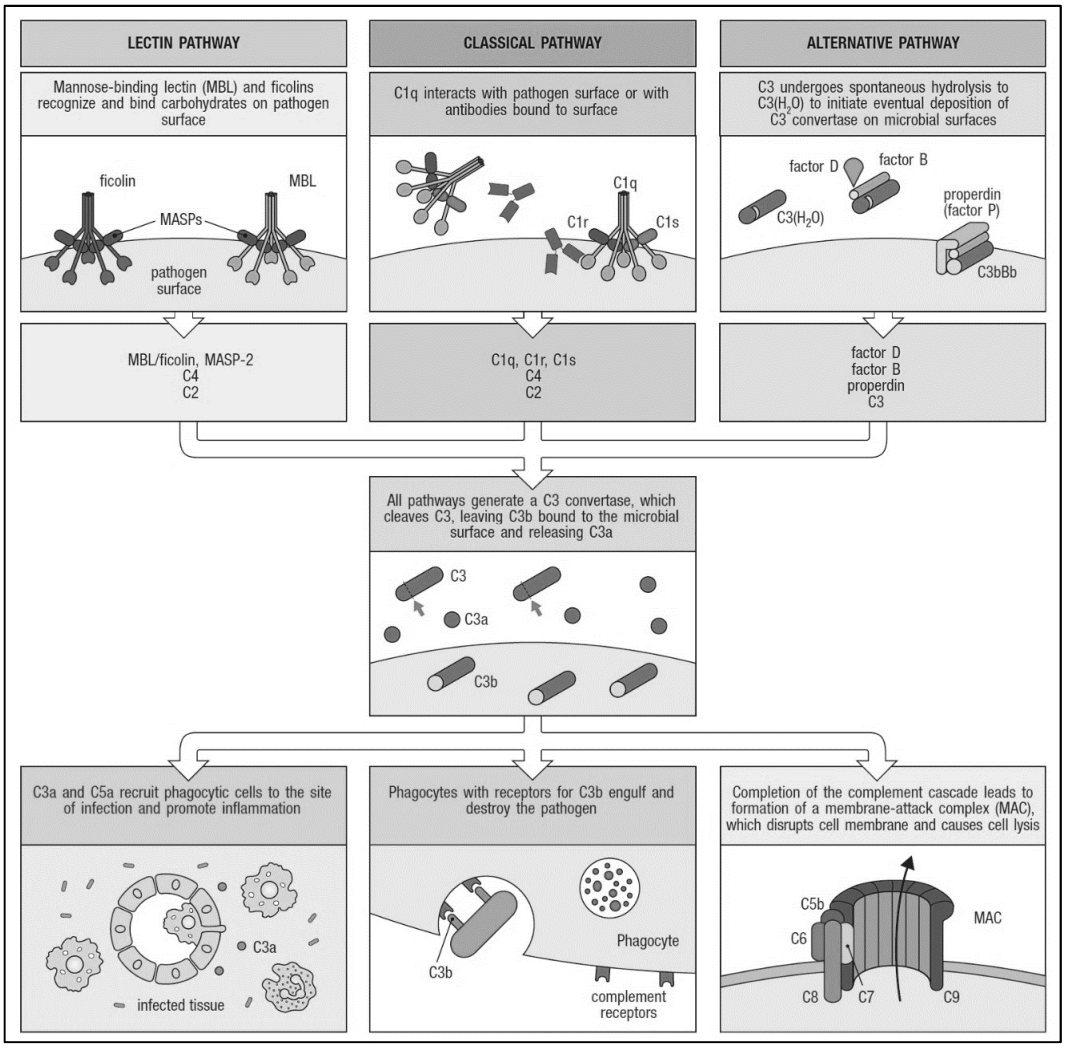

Figure 2.2 The Complement Pathways (from Murphy and Weaver 2016)

2.5.2.3 Phagocytosis

Phagocytosis is the internalization and subsequent destruction of pathogens by phagocytes (Botelho and Grinstein 2011). Phagocytosis occurs either after a chance encounter between a phagocyte and a pathogen or, as was already mentioned in the complement pathways, through receptor-mediated uptake (Murphy and Weaver 2016).

Macrophages, monocytes, granulocytes (mainly neutrophils) and dendritic cells are the four main classes of phagocytic cells (Botelho and Grinstein 2011; Murphy and Weaver 2016). Macrophages are the most abundant phagocytic cells present in healthy tissues. They are formed during embryonic development and then self-renew during life and they can be formed from circulating monocytes (Murphy and Weaver 2016). The latter occurring when monocytes enter tissue from blood vessels during inflammation. The phagocytic granulocytes include neutrophils, eosinophils and basophils. The neutrophils are the major phagocytes of the group as well as the one immediately involved in innate immunity against infection. Granulocytes are present in blood, but not in healthy tissues. Both macrophages and granulocytes recognize, phagocytize and break down pathogens without input from acquired immunity (Murphy and Weaver 2016).

Dendritic cells are found within lymphoid organs and peripheral tissues (skin and mucosal tissues) as immature cells. Dendritic cells ingest and break down pathogens, but conventional dendritic cells (cDCs) can also produce peptide antigens specific to the pathogen processed (Murphy and Weaver 2016). These antigens activate T cells and trigger acquired immunity. The cDCs also produce certain cytokines that play a role in immunity. Another type of dendritic cell, the plasmacytoid dendritic cells (pDCs), produce cytokines called type I interferons which are antiviral interferons and form part of innate immunity.

Phagocytosis of pathogens by phagocytes involves various steps. Pathogen recognition receptors (PRRs) are present on macrophages, neutrophils and dendritic cells and they have the ability to recognize molecular structures specific to pathogens (Greenberg and Grinstein 2002; Botelho and Grinstein 2011; Murphy and Weaver 2016). Once recognized, the phagocyte binds to the pathogen and then internalizes it in its phagosome through a process of pseudopod formation and engulfment. The cell lysosomes fuses with the phagosome to form a phagolysosome and the lysosome contents are released into the phagolysosome. The phagolysosome becomes acidified and acquires antimicrobial peptides and enzymes (from cytoplasmic granules of neutrophils), reactive oxygen species (ROS), and reactive nitrogen species (Botelho and Grinstein 2011; Murphy and

Weaver 2016). All these aid in killing of the pathogens. Acidification reduces the pH to between 3.5 and 4.0 which creates an environment that is either bacteriostatic (stops bacterial reproduction without killing cells) or bactericidal (kills bacterial cells) (Murphy and Weaver 2016). Reactive oxygen species include superoxide, hydrogen peroxide, singlet oxygen (high energy oxygen), hydroxyl radicals and hypohalite while reactive nitrogen includes nitrous oxide. These are efficient microbe killing products, but when released into extracellular environment can cause damage and are toxic to the host cells. The active enzymes released by phagocytes are lysozyme that digest Gram-positive bacteria cell walls and acid hydrolases that break down ingested microbes. In macrophages, antimicrobial peptides that are produced are cathelicidin and macrophage elastase-derived peptides, while neutrophills produce α-defensin, β-defensin, cathelicidin, azurocidin, bacterial permeability inducing protein, and lactoferricin (Botelho and Grinstein 2011; Murphy and Weaver 2016).

2.5.2.4 Inflammatory Response

As mentioned earlier, inflammation is triggered through innate immune system responses to infections or injury (Lilic 2009; Newton and Dixit 2012). The inflammatory response performs three main functions: 1) it increases the number of effector molecules and immune cells at the site of infection and thus speeds up the elimination of pathogens; 2) it prevents spreading of infection in the blood by inducing localized blood clotting, and 3) it enhances repair of damaged tissues (Murphy and Weaver 2016).

Inflamed tissues are typically red, painful, warm and swollen as a result of the changes that occur in localized blood vessels (Newton and Dixit 2012; Murphy and Weaver 2016). These changes are firstly the dilation of blood vessels in the infected area. This result in increased blood flow, albeit at reduced speed, through the area, which in turn increases the temperature in surrounding tissues, as well as give the area the red colour associated with inflammation. Secondly, the endothelial cell lining of blood vessels in the inflamed area start expressing cell-adhesion molecules (Murphy and Weaver 2016). Leukocytes circulating in the blood adhere to these molecules on the endothelial cells and can then migrate over

the epithelium into the infected area, a process known as extravasation. The blood vessels also become more permeable through the slight separation of the usually tightly joined endothelial cells. This endothelial activation allows plasma proteins, like complement and MBL, as well as fluids, to move from the blood into the infected tissue (Murphy and Weaver 2016). This results in swelling (edema), as well as pain, due to increased pressure in the area. Lastly, blood starts clotting inside the microvessels to prevent the spreading of pathogens via blood circulation.

The changes in blood vessels and leukocyte response are brought about by pro-inflammatory proteins called cytokines and chemokines (collectively referred to only as cytokines) that are secreted by immune system cells, like macrophages. Cytokines play an important role in cell signaling (Murphy and Weaver 2016). Some cytokines can be produced by a range of immune system cells while others are produced by very specific cells. Likewise, some cytokines have a wide range of receptor cells, while others have very specific receptors. Receptor specificity is brought about by cytokine specific receptors on target cells.

The main function of cytokines is to amplify an immune response subsequent to exposure to a pathogen, while chemokines are responsible for chemoattraction of leukocytes (chemotaxis) as well as increasing the permeability of endothelium of blood vessels (Murphy and Weaver 2016). Cytokines trigger the endothelial cells to produce more cytokines, thereby accelerating the response to the infectious agent.

The inflammatory response is initiated when initially macrophages and later more leukocytes, recognise pathogens and secrete inflammatory mediators (Murphy and Weaver 2016). These mediators are prostaglandins, leukotrienes and platelet-activating factor (PAF) which are lipid products resulting from enzymatic degradation of membrane phospholipids. Inflammatory mediation is followed by cytokine and chemokine action. It is also during this inflammatory response that the cleaved C5 product, C5a, promotes inflammation by increasing vascular permeability and stimulating mast cells to release histamines, the cytokine TNF-α and cathelicidins as discussed earlier.

Specific transmembrane proteins, called toll-like receptors (TLRs), are found on cell membranes and endocytic vesicles of immune cells of the innate immune system (Newton and Dixit 2012; Murphy and Weaver 2016). TLRs are found mainly in macrophages, mast cells and dendritic cells, although they can also be expressed by other cells like B cells and certain epithelia. Toll-like receptors have the ability to recognize specific molecular patterns on bacteria, fungi and viruses that are not present in vertebrate cells, primarily in the extracellular environment (Murphy and Weaver 2016). Important ones include lipoteichoic acids (Gram-positive bacteria) and lipopolysaccharide (LPS) (Gram-negative bacteria). In turn, Nucleotide-binding oligomerization domain-like receptors or NOD-like receptors (NLRs) are intracellular sensors of microbial products or cellular damage (Murphy and Weaver 2016). Two NLRs, NOD1 and NOD2, are responsible for recognition of bacterial products. The NOD1 recognize γ-glutamyl diaminopimelic acid, a breakdown product of peptidoglycans of Gram negative bacteria and some Gram positive bacteria while NOD2 detect muramyl dipeptide (MDP) found in peptidoglycans of most bacteria. The NOD proteins have the ability to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and pro-inflammatory cytokines.

2.5.3 Acquired Immunity

Systems of innate immunity are the first line of defense against pathogens. When the innate immune system fails at eliminating a pathogen, the acquired immune system is activated. This activation occurs as the pathogen multiplies and specific sensor cells of innate immunity are triggered which in turn activate acquired immunity (Murphy and Weaver 2016).

The main cells of acquired immunity are the T cells and B cells (Cota and Midwinter 2009; Lilic 2009; Murphy and Weaver 2016). Both are lymphocytes which are produced in the bone marrow, but T cells mature within the thymus and B cells in the bone marrow. They are found in the lymphatic system and the T cell subsets, T helper 1 (T_H1) and cytotoxic T cells are involved in cellular immunity (cell-mediated immunity) while Transitional Stage 2 (T2) B cells are involved in humoral immunity (antibody-mediated immunity).

2.5.3.1 The Role of Phagocytosis in Acquired Immunity

In order for T cells and B cells to recognize an infection by a pathogen and act upon it, they have to encounter some form of activator. This is achieved when antigen-specific receptors on T and B cells encounter antigen presenting cells (APCs) that are expressing antigens in a manner that it is recognized by the T and B cell receptors (Lilic 2009; Chaplin 2010; Murphy and Weaver 2016). The APCs are dendritic cells, B-lymphocytes and macrophages that have processed antigens and display them on their membrane surface. Of the APCs the dendritic cells are the most important and they are abundant in areas that are in close contact with surface epithelia such as the skin, lung and intestine, as well as in organs like the heart and kidneys.

When APCs phagocytose pathogens as part of the innate immune response, the breakdown products of the pathogens (antigens) are attached to the major histocompatibility complex (MHC) molecules to form an antigen: MHC complex on the cell membrane of the APC (Murphy and Weaver 2016). The APC presents the antigen: MHC complex which is recognized by the T and B cells. However, in order for activation to take place, certain co-stimulatory molecules must also be present on the APC. Co-stimulatory molecules are expressed as a result of pro-inflammatory cytokines that are produced by dendritic cells following intracellular degradation of pathogens (Chaplin 2010; Murphy and Weaver 2016). These cytokines include interleukin (IL) -6, IL-12, IL-18 and interferon (IFN)-α and IFN-β. Thus, the co-stimulatory molecules are required in conjunction with the antigen: MHC complex for activation to occur. This dual trigger requirement prevents activation of T and B cells against self-peptides.

There are two main ways in which dendritic cells handle antigens (Chaplin 2010; Murphy and Weaver 2016):

1) Dendritic cells either phagocytose extracellular antigens through recognition of antigen: antibody complexes (receptor mediated phagocytosis) or take them up nonspecifically through micropinocytosis. These two methods of uptake allow dendritic cells to process and present almost any type of pathogen. Extracellular

antigens taken up in these two ways are processed in the endocytic pathway and presented on MHC class II molecules which is recognized by CD4 T cells (T cells carrying the co-receptor CD4). The CD4 T cells activate and then differentiate into effector T cells.

2) Antigens can directly enter the cytoplasm of dendritic cells as is the case with viral infections. While in the cytoplasm, the virus synthesizes proteins which are degraded within the dendritic cell's proteasome and are subsequently transported into the endoplasmic reticulum. The antigen peptides are then presented on MHC class 1 molecules on the cell membrane of the dendritic cell (or APC). These antigen: MHC 1 complexes specifically activate CD8 T cells (T cells carrying the co-receptor CD8) which differentiate into cytotoxic effector CD8 T cells with the ability to recognize and kill virus-infected cells.

It is possible for dendritic cells to phagocytose extracellular virus particles or virus-infected cells. This will still lead to the formation of antigen: MHC I complexes and activation of cytotoxic effector CD8 T cells. However, viral antigen: MHC II complexes can also be formed that results in activation of naive CD4 T cells. The resultant effector CD4 T cells stimulate antiviral antibody production by B cells and cytokine production responsible for enhancing the immune response (Chaplin 2010; Murphy and Weaver 2016).

The expression of MHC II and co-stimulatory molecules by macrophages result in the amplification of T cell responses where T cells have already been activated by dendritic cells. B cells can present antigens as antigen: MHC II complexes to interact with CD4 T cells already activated by the same antigen. This results in the T cell signaling the B cell to differentiate into antibody producing cells.

2.5.3.2 Cell-Mediated Immunity

After developing in the thymus, T cells move into the bloodstream and are referred to as naive T cells since they have not encountered their specific antigens (Janeway et al 2001). The naive T cells circulate between the secondary lymphoid tissues and the blood until they encounter their specific antigen in the form of the

antigen: MHC complex as well as the required co-stimulatory molecules (Janeway 2001; Chaplin 2010; Murphy and Weaver 2016). The T cells, upon meeting the antigen: MHC complex will then proliferate and differentiate to form effector T cells that contribute to the removal of antigens. Some also differentiate into memory cells specific to the encountered antigen. The differentiation of the T cells into different effector T cells after activation is controlled by cytokines. T cells therefore do not react to the pathogen itself, but rather to the host cells carrying the peptide antigen: MHC complex. These host cells are referred to as target cells. The process leading up to the differentiation of naive T-cells into effector T cells forms part of the innate immune system.

CD8 T Cell Activation

Naive T cells can differentiate into different effector T cells each specialized for different functions (Janeway 2001; Chaplin 2010; Murphy and Weaver 2016). For example, naive CD8 T cells recognize antigen: MHC I complexes and form cytotoxic CD8 T cells which have the ability to recognize and kill infected cells. These cells are important in the defense against intracellular pathogens and especially viruses. They are however very destructive due to their cytotoxic effects and their activation must be carefully controlled and therefore requires more co-stimulatory activity than CD4 T effector cells.

Activation of CD8 cytotoxic T cells can occur in two ways (Janeway 2001; Chaplin 2010; Murphy and Weaver 2016). Firstly, it can for example with virus infections, become activated when dendritic cells induce CD8 T cells to produce interleukin (IL)-2 that will trigger its own differentiation into cytotoxic effector cells. Secondly, its activation can be assisted by CD4 effector T cells that express IL-2 and CD40 ligand. The CD40 ligand binds to CD40 on the dendritic cell and through a few processes provide additional co-stimulation to the naive CD8 T cells. Simultaneously the IL-2 also promotes effector CD8 T-cell differentiation.

CD4 T Cell Activation

CD4 T cells can differentiate into subsets of effector T cells when they recognize antigen: MHC II complexes, each subset having its own immunological role

(Janeway 2001; Chaplin 2010; Murphy and Weaver 2016). The main subsets are T_H1, T_H2, T_H17 and T follicular helper (T_{FH}) subsets with the function of activating target cells, as well as regulatory T cells or T_{reg} cells which inhibit immune activation. These effector T cells can 1) migrate rapidly to inflamed areas where pathogens are present when they get in contact with APCs; or 2) move to B cell zones to generate pathogen-specific antibodies in secondary lymphoid tissues.

The following is brief description of the effector T cell subsets (Janeway 2001; Chaplin 2010; Murphy and Weaver 2016):

T_H1 Cells: In the presence of high levels of the cytokines IFN-γ and IL-12 during initial stages of naive T cell activation, T_H1 cells are produced. In turn T_H1 cells produce the cytokine IFN-γ that plays a part in eliminating infections by microbes that replicate and survive in macrophages (e.g. protozoans, intracellular bacteria and some viruses). The T_H1 cells recognizes and enhances activation of the macrophages containing infections by releasing IFN-γ. This increases the macrophages ability to kill ingested microbes. The release of IFN-γ also reinforce the differentiation of more T_H1 cells (positive feedback).

T_H2 Cells: Cytokine IL-4 stimulates the production of T_H2 cells. T_H2 cells produce IL-4, IL-5 and IL-13 and assist in controlling extracellular parasitic infections (e.g. by helminths). It boosts eosinophil, mast cell and IgE mediated responses. Since the T_H2 cells produce IL-4, they are also a positive feedback system.

T_H17 Cells: T_H17 cells are produced in the presence of high levels of the cytokine IL-6 and transforming growth factor (TGF)-β during initial stages of naive T cell activation. IL-6 and TGF-β are produced by microbial product activated innate immune cells. T_H17 cells produce IL-17A, IL-17F and IL-22. T_H17 cells are induced by, and enhances neutrophilic action against, extracellular bacterial and fungal infections. The cytokines produced by T_H17 cells also activate antimicrobial peptide production in barrier epithelial cells as is found in the gastrointestinal, respiratory and urogenital tracts and the skin. Indirect

reinforcement of T_H17 differentiation occurs when the IL-17, produced by T_H17 cells, enhances IL-6 production by innate immune cells.

T_{FH} cells: The requisites for production of T_{FH} cell differentiation is not yet understood although it seems IL-6 plays an important role. T_{FH} cells produce low levels of cytokines similar to those of the other effector T cell subsets like IFN-γ, IL-4 and IL-17, as well as high levels of IL-21 that assist B cell differentiation into plasma cells that produce antibodies.

T_{reg} cells: In contrast to the previous effector T cells, T_{reg} cells suppress T cell responses and thus limits immune responses. This plays an important role in preventing autoimmunity (immunity against self). The T_{reg} cells are activated when TGF- β is present, but not IL-6. Since IL-6 are produced in the presence of pathogens, T_{reg} cells are produced in the absence of pathogens and presence of TGF- β and thus prevent unwanted immune responses. The T_{reg} cells also produce TGF- β and IL-10, both being immunosuppressive and responsible for suppressing inflammation.

2.5.3.3 Cell Death

The need for T cell-mediated immunity arises from the fact that all viruses and some bacteria enter cells and multiply within cytoplasm (Murphy and Weaver 2016). Here they are out of reach of antibody-mediated elimination, and thus the only way they can be rendered harmless is by total destruction of the host cell. This is achieved mainly by cytotoxic CD8 T cells (Murphy and Weaver 2016).

There are two main types of cell death (Janeway et al. 2001; Wu et al. 2001; Elmore 2007; Murphy and Weaver 2016). The first is necrosis where cells disintegrate as a result of physical or chemical injury. The second type is apoptosis, or programmed cell death, which is also the way in which cytotoxic T cells kills off infected cells. Apoptosis is triggered by one of two pathways: extrinsic pathway of apoptosis and intrinsic (mitochondrial) pathway of apoptosis. Both pathways activate aspartic acid-specified cysteine proteases (caspases) which plays a role in apoptotic cell death. Initiator caspases cleave and activate

other caspases while effector caspases brings about cellular changes in cells which must undergo apoptosis. The latter is achieved by cleaving proteins critical to cellular integrity and by activating enzymes promoting cell death. An example of this is the cleaving of nuclear proteins and activation of endonucleases. Intact nuclear proteins provide structural integrity to the nucleus while endonucleases fragment chromosomal deoxyribonucleic acid (DNA).

The extrinsic pathway of apoptosis occurs when extracellular ligands activates "death receptors" in receptor-bearing cells (Elmore 2007). This means the cell kills itself due to signals from other cells. The extrinsic pathway can be mediated either by a Tumor necrosis factor (TNF) path or by a First apoptosis signal (FAS) path (Elmore 2007; Murphy and Weaver 2016).

The intrinsic pathway of apoptosis occurs in the presence of noxious stimuli or in the absence of growth factors that are important for cell survival (Janeway et al. 2001; Elmore 2007; Murphy and Weaver 2016). The cells therefore kill themselves because the cell itself senses stress. The intrinsic pathway is initiated when mitochondria release cytochrome c which in turn triggers caspases. In the cytoplasm cytochrome c binds to apoptotic protease activating factor-1 and forms the apoptosome. The apoptosome recruit initiator caspase which in turn activate effector caspases. Effector caspases like caspase 3 can activate enzymes which can cleave DNA of target cells which lead to cell death.

Phagocytic cells recognise cells in the process of cell death and respond rapidly to phagocytose these apoptotic cells. Once ingested the cells are degraded completely (Murphy and Weaver 2016). This serves to protect host cells from the release of the contents of apoptotic cells.

In addition to inducing apoptosis, CD8 cytotoxic cells also release the cytokines IFN-γ, TNF-α and Lymphotoxin (LT) -α (Murphy and Weaver 2016). The IFN-γ plays, among others, a role in directly inhibiting viral replication and increases expression of MHC I molecules. The TNF-α and LT-α acts synergistically with IFN-γ to activate macrophages via TNFR-II as well as to kill target cells through interaction with TNFR-I.

2.5.3.4 Humoral Immunity

Humoral immunity is responsible for the protection against extracellular pathogen infections (Hoffman et al. 2015). During the humoral immune response, B cells produce antibodies that protect against extracellular microorganisms and their by-products (Murphy and Weaver 2016).

The B cells are produced in the bone marrow but move to the secondary lymphoid organs, like the spleen, to mature into naive B cells (Murphy and Weaver 2016). Naive B cells, unlike T cells and natural killer cells, have receptors specific to certain antigens on their cell membranes, called B cell receptors (BCRs) (Hoffman et al. 2015). When an antigen binds to the BCR on the naive B cell, it is taken up by the B cell through endocytosis. Inside the B cell it is degraded and the resultant antigenic peptides are presented on the surface of the B cell with MHC-II. The antigen: MHC-II complex can now be recognized by antigen specific T_H cells that originated during the phagocytic process against the same pathogen described earlier (Hoffman et al. 2015; Murphy and Weaver 2016). The T_H cells express co-stimulatory ligands and cytokines, both with the role of helping the B cell to proliferate and differentiate (Hoffman et al. 2015; Murphy and Weaver 2016). Proliferation and differentiation of B cells are required in order to produce antibody-secreting cells and memory B cells, and also to form germinal centers inside lymphoid follicles (Murphy and Weaver 2016). The signals from the T_H cells includes the activation of B cell CD40 by T_{FH} expression of CD40L as well as IL-21, IL-4, IL-6, IFN-γ and TGF-β (Janeway et al. 2001; Hoffman et al. 2015; Murphy and Weaver 2016). Activation of CD40 promotes B cell survival while IL-21 promotes B cell proliferation and differentiation into plasma cells and memory B cells. Interleukin-4, IL-6, IFN-γ and TGF-β play a regulatory role in the type of antibody that will be produced. Some B cells can be activated directly as a response to certain microbial pathogens without the presence of T_H cells. This results in a rapid response to pathogens, but with lower affinity and less functionality than those where T_H cells plays a part (Murphy and Weaver 2016).

Activated B-cells differentiate into plasmablasts (short-lived), plasma cells (long-lived) and memory B cells (Janeway et al. 2001; Hoffman et al. 2015; Murphy

and Weaver 2016). Plasmablasts are formed within the secondary lymphoid organs and immediately produce weak antibodies for a quick response. The antibodies produced are mainly the class IgM. Some activated B cells however enter a lymphoid follicle and from a germinal center. Here, facilitated by T_{FH}, further proliferation, immunoglobulin class switching, and affinity maturation of activated B cells occur to produce long-lived plasma cells and memory B cells. Plasma cells are responsible for secreting a large number of antibodies of the immunoglobulin classes IgA, IgM, IgE, IgD and IgG.

Antibodies fulfil a number of roles including neutralisation of toxins originating from bacteria, preventing microbes to attach to mucosal surfaces, activating the complement pathway of innate immunity, opsonisation of bacteria to promote its phagocytosis, and promotion of cytotoxic killing of cells infected with pathogens. The methods by which these functions are fulfilled include (Janeway et al. 2001; Hoffman et al. 2015; Murphy and Weaver 2016):

- Antibodies binding to antigens to immobilize them and neutralise their toxins.
- Precipitation of antigens by binding to multiple microbes and causing their agglutination.
- Attraction of phagocytes by agglutinated, immobilized and neutralized antigens resulting in their ingestion and degradation.
- Activation of C1 molecules as a result of formation of antigen: antibody complexes which triggers the classic pathway of the complement system.
- C1 molecules aiding in identification of pathogens by macrophages to stimulate phagocytosis.
- Antibodies, specifically IgE inducing inflammation by binding and activating eosinophils, basophils and mast cells.
- Antibody-dependent cell-mediated cytotoxicity (ADCC) occurring when antibodies bound to a cell surface interacts with NK cells. NK cells then recognize IgG1 and IgG3 subclasses and the activated NK cell releases

cytoplasmic granules containing perforin and granzymes that degrade the target cell (pathogen).

2.6 Biomarkers for Immunotoxicity

During the last decade, a number of *in vivo* and *in vitro* biomarkers of immunotoxicity have been investigated. These biomarkers are indicative of the immunosuppressive or immunostimulatory effects of toxicants. Immunosuppression refers to the suppression or dampening of the immune response to antigens and immunostimulation is the upregulation, and in some instances exacerbation, of immune responses.

Biomarkers employed to show the immunomodulatory nature of some xenobiotics have been investigated. Thymus histology in guinea pigs and mice was, for example, used as early as 1973 as a biomarker of 2,3,7,8-Tetrachlorodibenzo-p-dioxin exposure (Vos et al. 1973). In rainbow trout exposed to creosote, pronephros leukocyte respiratory burst, phagocytic index, lymphocyte proliferation and lysozyme activity were investigated (Karrow et al. 1999). IgE and IgG, as well as genes for the cytokines IL-1, IL-4, IL-6 and IL-8, were used as biomarkers in human blood exposed to heavy metals (Marth et al. 2001). Similarly, cytokines like interleukin IL-6 and IL-10 in human whole blood cultures (Faul et al. 2013; Faul et al. 2014) and inflammatory activity in mouse RAW264.7 cells, measured as nitric oxide and IL-6 (Makene and Pool 2015), have been used. Intestinal mucosal immunoglobulins in $NiCl_2$ exposed broiler chickens were determined by Wu et al. (2014). Biomarkers of the cellular immune system include, for example in mussels, hemocyte viability, cellularity, phagocytosis efficiency, NK cell-like cytotoxic activity and lysozyme activity (Farcy et al. 2011).

Immunosuppression results in lowered immunity and can ultimately lead to the development of tumors and cancers (Duramad and Holland 2011). Immunostimulation can lead to immune-mediated diseases like atherosclerosis, asthma and allergy hypersensitivities, and auto-immune diseases like type 1 diabetes and rheumatoid arthritis (Duramad and Holland 2011).

2.7 Conclusion

The large number of natural and anthropogenic EDCs and toxins released into the environment presents a number of health risks to both humans and wildlife. The aquatic environment presents special concern due to the ease of introduction of EDCs through storm water runoff and wastewater streams. Apart from the diverse number of ecosystem effects presented by EDCs, a pressing concern is where EDCs remain extant within potable water sources. Rapid, reliable, and cost-effective methods to determine the presence and effects of EDCs in the environment and specifically water sources are thus important. Cellular, neurological and immunological changes in response to exposure to potentially contaminated water sources presents a number of options for testing for EDC contamination in water. These can either be measured *in vivo* or *in vitro*, each with its own advantages and disadvantages.

The remaining chapters of this thesis investigates the presence of selected EDCs, specifically the natural steroid hormones, and some physiological endpoints related to cytotoxicity, neurotoxicity and immunotoxicity in raw, treated and reclaimed sewage as well as selected untreated surface water sources in Namibia. This is the first study for determining endocrine disrupting potential in these water sources and it is specifically important for the reclaimed water from the Goreangab Water Reclamation Plant supplying the capital of Namibia, Windhoek, with potable water. It is also the first study, world-wide, where proteome profiling is performed as a rapid screen for immunotoxicity biomarkers in exposed human blood culture exposed to reclaimed water.

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Chapter 3

Oestrogen, Testosterone, Cytotoxin and Cholinesterase Inhibitor Removal during Reclamation of Sewage to Drinking Water

FAUL, A.K., JULIES, E. & POOL, E.J. 2013. Oestrogen, testosterone, cytotoxin and cholinesterase inhibitor removal during reclamation of sewage to drinking water. *Water SA*. **39**: 499–506.

3.1 Abstract

Namibia is the driest sub-Saharan country in Africa. Namibia's capital, Windhoek, reclaims sewage water for domestic use at the Goreangab Water Reclamation Plant (GWRP). Risks associated with sewage effluent and reclaimed sewage should be closely monitored; therefore water at the Gammams Sewage Treatment Plant's (GSTP) inlet and outlet, as well as reclaimed water from the GWRP, were assayed using selected bioassays. The water samples collected were analysed using enzyme-linked immunosorbent-assays and chromogenic tests for some selected, natural steroid hormones, neurotoxicity, cytotoxicity and inflammatory activity. The sstradiol level at the sewage treatment inlet was 78 pg/ml and the treated sewage showed an 83% to 95% reduction in this level, while after reclamation the level was below detection limit. The estrone concentrations at the sewage treatment inlet ranged from 10 to 161 pg/ml. The sewage treatment reduced estrone by between 85% and 92%. After reclamation the level of estrone was below detection limit. The testosterone level ranged between 162 and 405 pg/ml at the sewage plant inlet. The sewage treatment removed 96% of the initial testosterone. The residual testosterone was effectively removed by processes in the GWRP and after reclamation no testosterone was detected in the water. The acetylcholinesterase (AChE) inhibition at the sewage treatment inlet was 50% while it was only 27% after sewage treatment. After reclamation, the AChE inhibition was not detected. The water at the sewage inlet, in March and February, showed cytotoxicity. High inflammatory activity was detected at the sewage plant inlet. The sewage treatment reduced inflammatory activity by 64%. After reclamation low inflammatory activity was induced. The treated sewage used for reclamation tested positive for most of the biomarkers and can pose a risk to human health. However, reclamation successfully removed these contaminants. Due to the presence of contaminants in the intake water at the reclamation plant, it is essential to routinely monitor the water produced by the reclamation plant for potential residues that can adversely affect human health.

Keywords: Endocrine disrupting potential, cytotoxicity, neurotoxicity, inflammatory response, steroid hormones, water quality

3.2 Introduction

The protection and management of water resources are becoming increasingly important. The growing human population, linked to its resource needs, puts existing water sources under immense pressure due to unsustainable use and the effects of various environmental pollutants. Namibia has to take special care of its water sources, since it is the driest sub-Saharan African country, with an average rainfall of 360 mm per year. More than half of the surface of this country is considered semi-arid to hyper-arid. This is further aggravated by extremely high evaporation rates of 3 000–3 500 mm/a (Mendelsohn et al. 2009). With a total land surface area of 825 000 km^2 and just over 2 million inhabitants it is one of the most sparsely populated countries in the world (Mendelsohn et al. 2009). Urbanisation has led to more than 10% of the country's population settling in the capital city, Windhoek, resulting in an increased demand for clean water.

Windhoek is a relatively small city and home to most of the 250 000 people of the Khomas Region (Mendelsohn et al. 2009). The current unofficial estimate for Windhoek's population is more than 300 000 inhabitants. In 1969 water shortages led to the upgrade of the conventional water treatment plant in the vicinity of the Goreangab Dam to a fully functional water reclamation plant, with the purpose of reclaiming the final effluent from the city's Gammams Sewage Treatment Plant (GSTP) (Du Pisani 2006). The Goreangab Water Reclamation Plant (GWRP) was the first water reclamation plant of its kind in the world, where the final effluent from a sewage water treatment plant was purified, together with water from the Goreangab Dam, for domestic use and human consumption. In full operation it could deliver up to 4 300 m^3/day and the reclaimed water was mixed with borehole water before being delivered to the city of Windhoek (Du Pisani 2006).

Goreangab Dam later became unfit for reclamation due to increasing organic matter input from expansion of the city as well as informal settlements that lie within the catchment area of the dam. As a result only sewage effluent was reclaimed. In subsequent years the water demand increased further and water had to be sourced from further away. The GWRP underwent several upgrades to finally reach a capacity of between 7 500 and 8 000 m^3/day in 1997 (Du Pisani

2006; Kirchner and Van Wyk 2001). By this time the GWRP reached the end of its viable life and a new, larger plant (also called the Goreangab Water Reclamation Plant or new Goreangab Water Reclamation Plant) was built with a capacity of 21 000 m^3/day (Lahnsteiner and Lempert 2007). Currently it has the potential of supplying up to 30% of the City of Windhoek's water demand, the other 70% being supplied from the S Von Bach Dam, located approximately 60 km from Windhoek, as well as from about 50 boreholes tapping into the Windhoek Aquifer. The new GWRP is managed by the Windhoek Goreangab Operating Company (WINGOC). The old GWRP is still used today to produce semi-purified water that is used for irrigation of Windhoek's parks and sport fields.

Using reclaimed sewage water for domestic purposes has the potential of posing various health threats. This includes the presence of endocrine disrupting compounds (EDCs) that may not be eliminated during the purification process or that may result from malfunctioning of the reclamation plant. Endocrine disrupting compounds include various natural and synthetic chemicals and compounds, the most important being natural and synthetic steroidal oestrogens and heavy metals (Bondegaard and Bjerregaard 2005; Medesani et al. 2004; Rodríguez et al. 2007). These compounds may interfere directly with hormonal systems of various animals by mimicking or antagonising the effects of hormones, altering hormone synthesis and metabolism, and modifying hormone receptor levels (Burkhardt-Holm 2010). Numerous studies have revealed that aquatic systems and their fauna are often very sensitive to the effects of EDCs (Harries et al. 1997; Porte et al. 2006; Tyler and Routledge 1998). The effects on endocrine and neural systems of animals, plants and humans are manifested as adverse effects on growth, development and reproductive success of individuals and these may eventually affect whole ecosystems (Burkhardt-Holm 2010; Gronen et al. 1999; Harries et al. 1997; Tyler and Routledge 1998). The link between EDCs and detrimental effects on the endocrine systems of children, decreased fertility in males, and increased incidence of breast cancer in females has also been suggested by various studies (Burkhardt-Holm 2010; Rogan and Ragan 2007).

Potential sources of EDCs are raw and processed sewage that are released into the environment (Jackson and Sutton 2008; Swart and Pool 2007).

One of the most important measures applied to ensure the supply of safe drinking water from the GWRP was to implement and strictly control the separation of industrial influent from domestic influent (Du Pisani 2006). The GSTP feeding the GWRP therefore only receives water from households and small businesses in Windhoek. Furthermore, stringent measures are in place at the GWRP to ensure the highest quality of reclaimed water, and daily testing is performed to ensure that water meets the water quality standards provided by the World Health Organization, the Rand Water Guidelines (South Africa) and the Namibian Guidelines for Group A Water (Lahnsteiner and Lempert 2007). If contamination of the water is detected, water supply from the GWRP is immediately stopped. Currently no published literature is available on the efficiency of the removal of EDCs from raw sewage at the GSTP or the efficiency of the GWRP in removing residual EDCs from the GSTP effluent which serves as the inlet for the GWRP. Furthermore, despite a significant amount of research done worldwide on EDCs over the past two decades, no international limits have been set for permissible steroid hormone levels in various water sources. Currently a useful guideline may be the predicted-no-effect-concentrations (PNECs) for synthetic oestrogen 17-α ethinylestradiol of 0.1 pg/ml, estradiol of 1 pg/ml, and estrone of 3–5 pg/ml, in freshwater ecosystems (Burkhardt-Holm 2010). However, the effects of oestrogens vary significantly from species to species and with different environmental conditions (Shin'ichiro et al. 2003). The synergetic effect of different xenobiotic hormones, and duration of exposure, influence the effects these EDCs may have (Vonier et al. 1996). More research is required to establish safe critical limits. Until these have been established it is difficult to monitor and implement policies regarding hormone levels at the GWRP.

This study is aimed at assessing the effectiveness of some selected EDCs removal in the GSTP and GWRP. Specific EDCs were quantified in water samples from the GSTP inlet (influent) and effluent, as well as from the reclaimed water (effluent) produced at GWRP. Seven different bioassays consisting of both

enzyme-linked immune-sorbent assays (ELISAs) and chromogenic spectrophotometry were used to measure selected steroid hormone levels, immunotoxicity, cytotoxicity and neurotoxicity of the water samples. These bioassays were chosen for their rapid production of reliable test results and their endpoints provide an overview of the efficiency of reduction of potential endocrine toxicity of sewage water in the GSTP and of the reclaimed water from the GWRP. The results of this study are useful for the establishment of biomarkers for routine monitoring of water reclamation plants.

3.3 Materials and Methods

3.3.1 Sample collection

Water samples were collected on 5 different occasions to include various stages of the wet and dry seasonal cycle (Table 3.1). This is important, since a higher organic matter load from increasing runoff into the sewage system is expected during the wet season. Windhoek received exceptionally high rainfall for January and February 2011 with the highest monthly rainfall ever recorded for Windhoek occurring in the month of January (320 mm). The sampling points were (1) the GSTP inlet (raw sewage), (2) the final GSTP maturation pond which also acts as the inlet for the GWRP, (3) the GWRP outlet (Figure 3.1) and (4) the Friedenau Dam, a suspected unpolluted dam close to Windhoek. The Friedenau Dam was included for assay verification purposes.

Table 3.1 Rainfall recorded in Windhoek for the particular sampling months.

Sampling Month	Description	Average rainfall (mm)
March 2010	Towards end of rain season	26.5
September 2010	End of dry season	11.9
November 2010	Beginning of rain season	114
February 2011	Peak of rain season	179.3
April 2011	Towards end of rain season	161.5

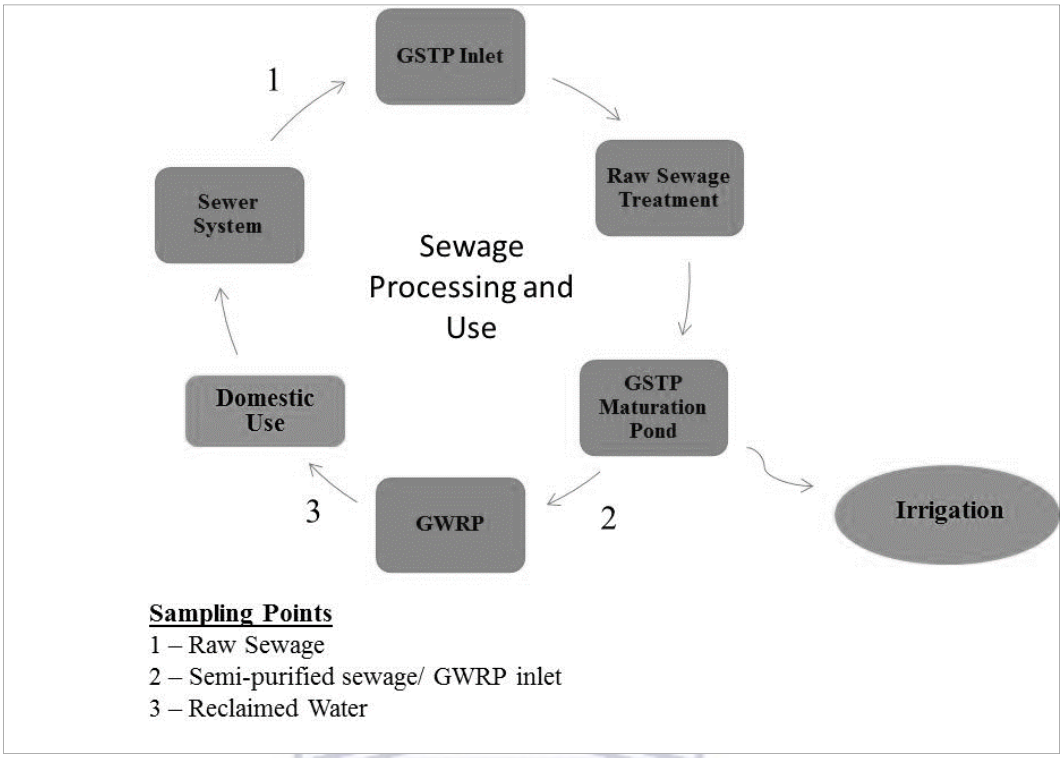

Figure 3.1 Schematic representation of sewage processing and use in the City of Windhoek at the Gammams Sewage Treatment Plant (GSTP) and the Goreangab Water Reclamation Plant (GWRP).

Water was collected in Schott bottles that had been thoroughly cleaned with soap solution and then rinsed with tap water followed by distilled water and finally chemically pure acetone. At the sampling point any residual acetone was rinsed from the bottles with the source water. The samples were transported on ice and frozen in the laboratory until solid-phase extraction was performed. For each sample 3 aliquots of 1 ml each were frozen in SureLock tubes for toxicity testing. At all sampling points 1 sample was taken, except during September 2010 when more replicates were taken for assay verification purposes.

3.3.2 Solid-phase extraction

Frozen samples were left at room temperature to thaw before organics were extracted using DSC-18 solid-phase extraction (SPE) columns (Supelco, Sigma-Aldrich). A modified version of the extraction procedure followed by Pool and Magcwebeba (2009) was used. In short, the column was first washed with 2 ml of methanol followed by 2 ml of solvent mixture (40% v/v hexane, 45% v/v

methanol, and 15% v/v *2*-propanol). It was then washed with 2 ml of methanol and the column was filled with distilled water, after which the samples were applied. Once the samples had passed through, the columns were dried under vacuum for at least 1 h. The hydrophobic molecules were then eluted from the SPE columns into glass vials by applying two 1 ml volumes of solvent mixture. The eluate was dried under hot air before being reconstituted in dimethyl sulphoxide (DMSO) to a final volume of 0.1% of the original sample volume.

3.3.3 Steroid hormones

Steroid hormone levels were determined using the Estradiol, Estrone and Testosterone ELISA kits (Sigma, Germany). The assay ranges of the kits are: 9.7–2 000 pg/ml for estradiol, 2.21– 1 000 pg/ml for estrone and 83–16 000 pg/ml for testosterone. For estradiol and estrone a 100-fold dilution, and for testosterone a 50-fold dilution of the extracts were made using diluted wash solution. All samples were applied to the ELISA microplate in duplicate and the assay procedure of the ELISA kit was followed. In short: standards, controls and sample extracts were applied to the microplate wells. Enzyme conjugate was added and the plates were incubated at room temperature. After incubation the plates were washed thoroughly and substrate solution was added. After a short incubation period stop solution was added and the absorbance was measured at 450 nm.

The inter- and intra-assay variation of the steroid hormone ELISAs is minimal (Swart and Pool 2007) and therefore eliminates the need for expensive replication of samples. A once-off estradiol verification assay was performed using 3 replicates of each sample taken during September 2010 to determine intra-sample variation. To determine inter-sample variation 6 replicates of 1 sample from the GSTP inlet were analysed.

3.3.4 Blood collection

Blood was collected at the University Health Centre from a healthy male volunteer that was not on any medication for the 3-month period prior to collection. Blood was collected in sterile heparin vacutainer tubes (Lasec, South

Africa) and stored at room temperature. Blood samples were used for experiments within 18 h of collection.

3.3.5 Neurotoxicity

Water samples were screened for general neurotoxicity using an acetylcholinesterase inhibition assay. 6 µl of the water extracts was diluted with 54 µl of 0.1 M phosphate buffer. As positive control, 6 µl of 1 nM chloropyrifos in 6 µl DMSO, was used. The negative control consisted of 6 µl DMSO. Both positive and negative controls were diluted to 60 µl with 0.1 M phosphate buffer. The positive control was also used to construct a 4× dilution range. An acetylcholinesterase extract was prepared by mixing heparinised human blood, distilled water and 0.1 M phosphate buffer (in a ratio of 3:20:97). 25 µl of each sample was added in duplicate to a storage microplate followed by 25 µl of blood solution. The plate was left to incubate for 2 h after which 50 µl of substrate was added. The substrate consisted of 100 µl of 0.01 M 5,5-dithiobis (2-nitro-benzoic acid) (DTNB) that was pre-mixed with 3 ml of 0.1 M phosphate buffer. 20 µl of 0.075 M acetylthiocholine iodide (ATCI) was then added to the DNTB-phosphate buffer mix. Optical density of each sample was immediately determined at 405 nm and then every 30 min for 2 hours.

3.3.6 Whole blood culture assays

All culture assays were performed under sterile conditions. Water from each sample was pipetted into duplicate wells (20 µl/well) of a tissue culture plate (Nunc^{TM}, Denmark). Sterile distilled water was used as a negative control. Heparinised blood (5 ml) was added to 45 ml Dulbecco's Modified Eagle's Medium (BioWhitakker) and 200 µl of this mixture was added to each well. The plate was covered and incubated overnight at 37 °C, after which the supernatant was collected for lactate dehydrogenase (LDH), interleukin-6 (IL-6) and interleukin-10 (IL-10) analysis.

3.3.7 Cytotoxicity assays

Lactate dehydrogenase leakage from damaged cells (i.e. cell death) into the plasma was monitored as an indication of cytotoxicity. Lactate dehydrogenase was measured using a commercially available chromogenic LDH assay kit (Sigma, Germany). Diluted blood (200 µl) was mixed with 20 µl 10% Tween 20 detergent (Sigma, Germany) to result in complete lysis of the cells. The lysate was used as a 100% cytotoxicity control. Whole blood culture supernatants or dilutions of the positive control (10 µl/well) were transferred into wells of a 96-well plate. Lactate dehydrogenase substrate was prepared according to the manufacturer's instructions and 200 µl of substrate was added to each well. Optical density was immediately determined at 450 nM and then at intervals of 10 min. Between measurements the plate was incubated in the dark and at room temperature. The LDH concentrations were determined using a standard curve constructed from dilutions of the 100% cytotoxicity control.

3.3.8 Cytokine assays

Double antibody sandwich ELISAs (e-Bioscience, Germany) were used to determine IL-6 and IL-10 concentrations in the whole blood culture supernatants collected. The kit's instructions were used with minor modifications. In brief: Nunc-Immuno microplates (Nunc™, Denmark) were coated with 50 µl of capture antibody in coating buffer and sealed and incubated overnight at 4°C. Plates were washed 5 times with wash buffer and blotted dry on absorbent paper. Wells were blocked with 100 µl of 1× assay diluent for 1 h at room temperature after which they were washed and blotted dry. A 2-fold dilution range of the standard (50 µl/well) was included on all plates. The culture supernatants were diluted 2-fold with 1× assay diluent and 50 µl of each sample was added in duplicate wells of the plate. The plates were covered and left to incubate for 2 h at room temperature. The plates were washed 5 times and blotted dry. Detection antibody (50 µl/well) diluted in 1× assay diluent was added to wells after which the plates were sealed and incubated for 1 h at room temperature. The plates were then washed 5 times, blotted dry, and 50 µl of Avidin-Horseradish Peroxidase (Avidin-

HRP) was added to each well. The sealed plates were left to incubate for 30 min at room temperature, after which they were washed 7 times and blotted dry. Then 50 µl of substrate solution was added per well and left to incubate for 15 min, after which 25 µl of stop solution was added. Optical densities were determined at 450 nM and IL-6 and IL-10 concentrations were determined from standard curves.

3.4 Results

3.4.1 Assay verification

The measuring range of the estradiol ELISA is between 9.7 pg/ml and 2 000 pg/ml. The intra-sample variation is shown for a negative control, water samples from the Friedenau Dam, the GSTP maturation pond and the GSTP inlet (Table 3.2). The inter-sample variation is shown for 6 replicates produced from a sample taken at the GSTP inlet.

Table 3.2 Intra- and inter-sample variation for the estradiol ELISA as validation of the steroid hormone ELISAs.

	Sample Site	N	Estradiol Concentration (pg/ml)	SD	% SD
Intra-sample variation	Negative control	3	2.0	0.1	3.6
	Friedenau Dam	3	2.5	0.1	3.9
	GSTP maturation pond	3	12.8	0.9	7.2
	GSTP inlet	3	80.8	0.3	0.3
Inter-sample variation	GSTP inlet	6	80.2	0.6	0.8

3.4.2 Steroid hormone concentrations

The highest concentrations of estradiol were found in the GSTP inlet and the GSTP maturation pond. The maturation pond revealed significant removal of estradiol during the purification process (Figure 3.2). The concentrations of estradiol in the GWRP outlet (the reclaimed water) were below detection limit. The highest estradiol concentrations in the GSTP inlet were recorded for the months of September 2010 and April 2011. Estrone concentrations in the GSTP inlet increased significantly at the start of the rainy season, from below 20 pg/ml to 161 pg/ml in November 2010 (Figure 3.3). Subsequent months showed a gradual decrease in estrone levels as the rainy season continued. Significantly reduced estrone concentrations were measured in the GSTP maturation pond with the highest concentrations measured in November 2010 (19.5 pg/ml). The estrone levels for the GWRP were below detection limit.

High testosterone levels, ranging from 150 to approximately 400 pg/ml, were detected in the GSTP inlet for all of the months sampled (Figure 3.4). March 2010 and April 2011 show the lowest levels, of 162 and 159 pg/ml respectively, while the highest value, of 405 pg/ml, was detected for November 2010, at the start of the rainy season. Testosterone removal is highly effective during the sewage water treatment process, since testosterone was less than the detection limit in all samples from the GSTP maturation pond and GWRP outlet.

3.4.3 Neurotoxicity

Acetylcholinesterase inhibition was high in the GSTP inlet for all months, except February 2011, when no inhibition was recorded, and April 2011 when low inhibition was recorded (Figure 3.5). The highest inhibition was recorded in November when total AChE inhibition was 94%. The GSTP maturation pond shows 22 ±7% AChE inhibition for March and September 2010 and April 2011. No inhibition was measured for November 2010 and February 2011. The GWRP outlet shows no AChE inhibition, except for March 2010 when a 6% AChE inhibition was detected.

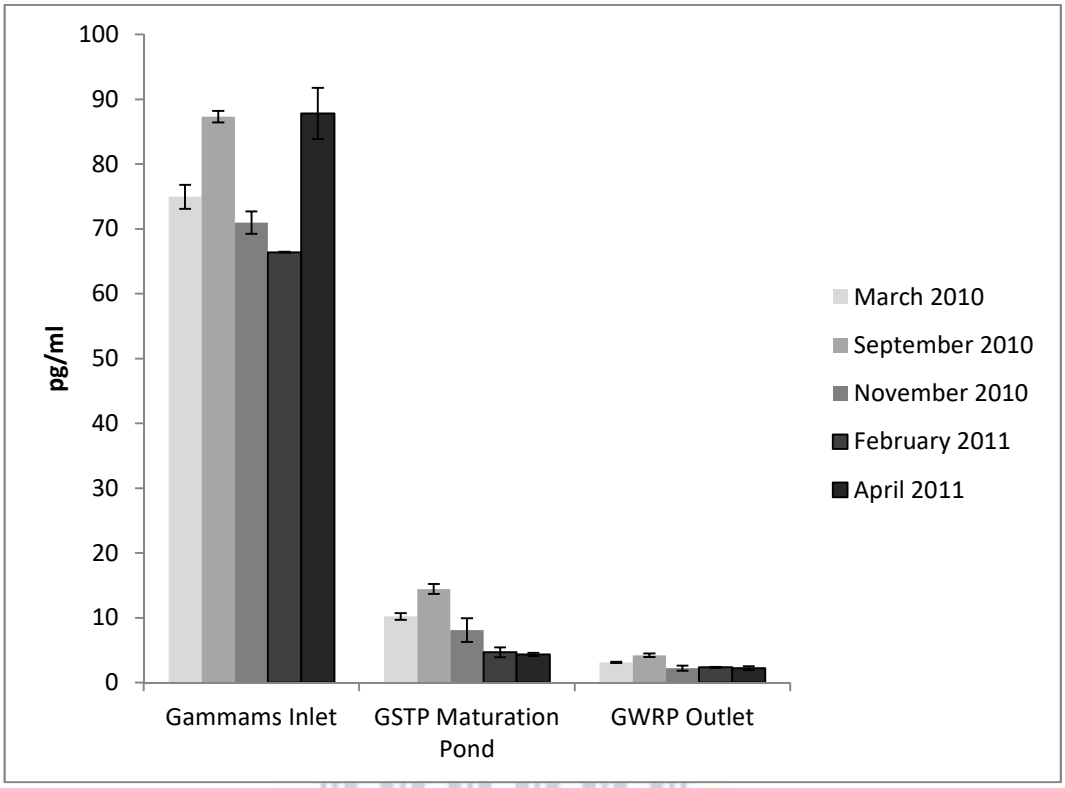

Figure 3.2 Estradiol concentrations (pg/ml) for the months of March, September, November 2010 and February and April 2011.

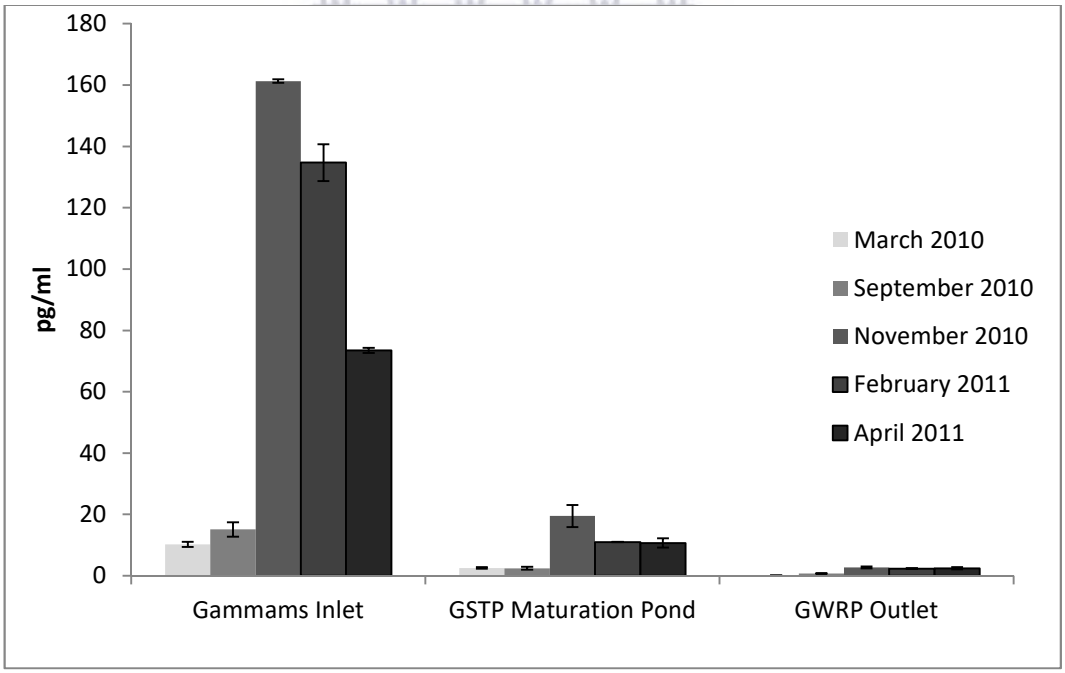

Figure 3.3 Estrone concentrations (pg/ml) for the months of March, September, November 2010 and February and April 2011.

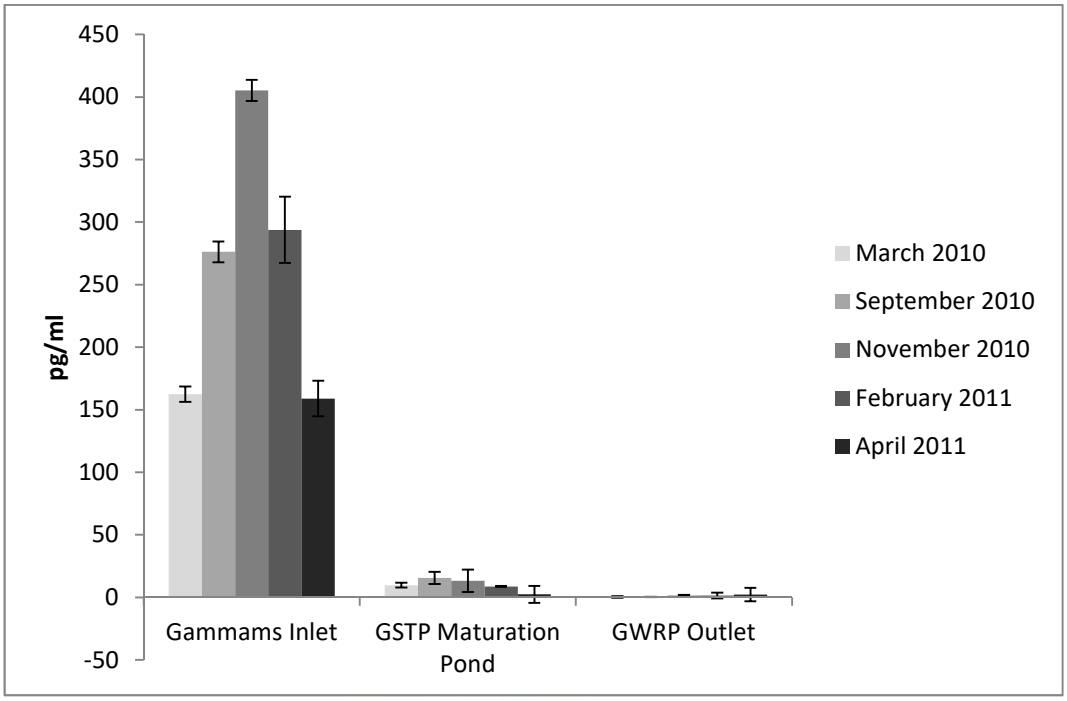

Figure 3.4 Testosterone concentrations (pg/ml) for the months of March, September, November 2010 and February and April 2011.

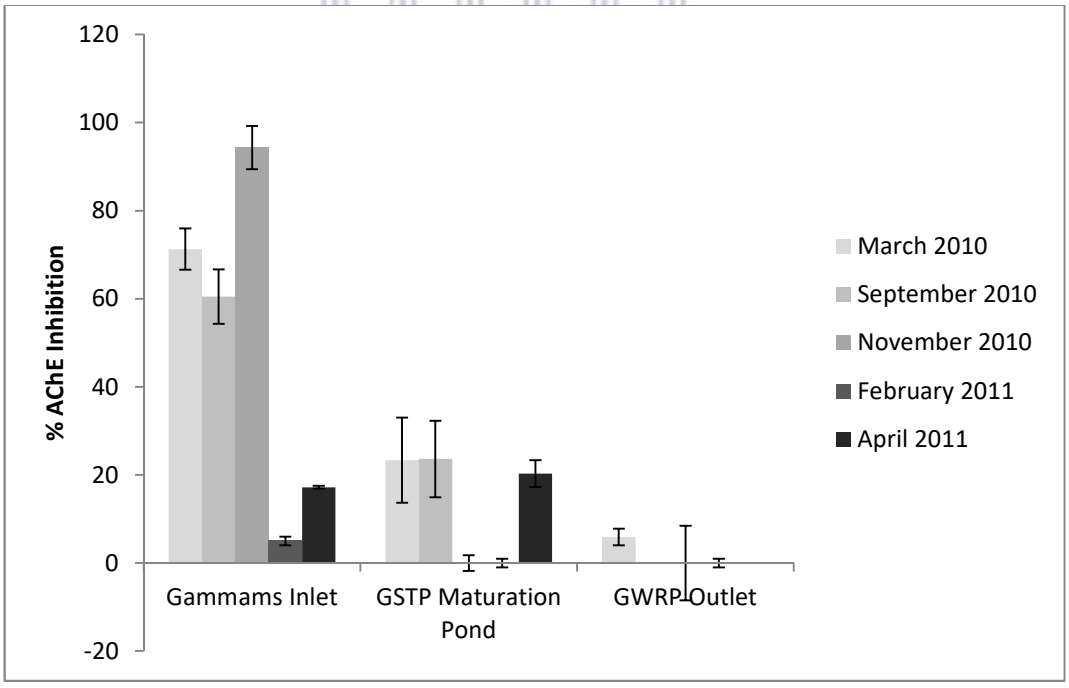

Figure 3.5 Human blood acetylcholinesterase (AChE) inhibition by water samples collected at the GSTP inlet, GSTP maturation pond and GWRP outlet. AChE inhibition is presented as a percentage of the AChE activity of the negative control.

3.4.4 Inflammatory activity for cytotoxicity and cytokine analysis

Cytotoxicity was only detected in the March 2010 (13%) and February 2011 (25%) samples of the GSTP inlet (Figure 3.6). All other samples for the GSTP inlet as well as the maturation pond and GWRP outlet were negative.

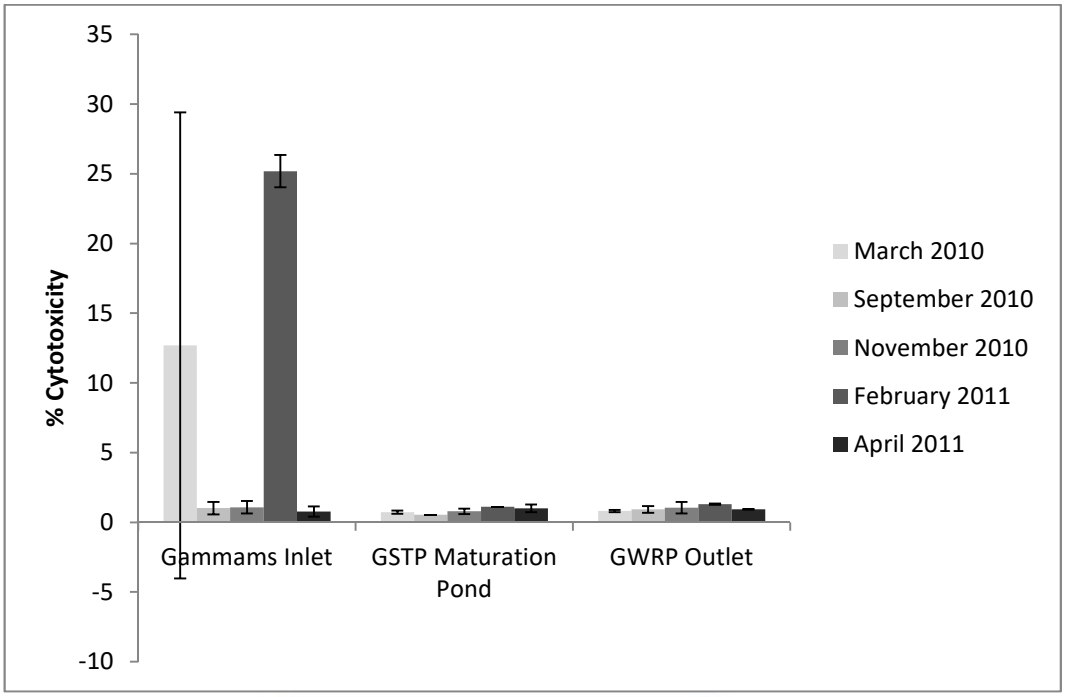

Figure 3.6 Percentage cytotoxicity of water extracts from the GSTP inlet, GSTP maturation pond and GWRP outlet measured as concentrations of lactate dehydrogenase in supernatants of human blood cultures.

High blood cytokine IL-6 levels were recorded after exposure of whole blood cultures to water extracts collected at the GSTP inlet (Figure 3.7). The lowest concentration was recorded in March 2010 (745 pg/ml) and the highest in April 2011 (1 421 pg/ml). During September and November 2010, and February 2011, concentrations ranged between 900 and 1100 pg/ml. Water extracts from the GSTP maturation pond show reduced inflammatory activity with IL-6 levels reducing to between 500 and 800 pg/ml (average 618 pg/ml). Water collected at the GWRP outlet induced very low IL-6 levels (average 52 pg/ml) compared to the GSTP inlet and GSTP maturation pond samples. This translates into a 38 ± 12% reduction during sewage treatment. After reclamation only 9 ± 8% of the initial IL-6 inducing activity was detected in the samples.

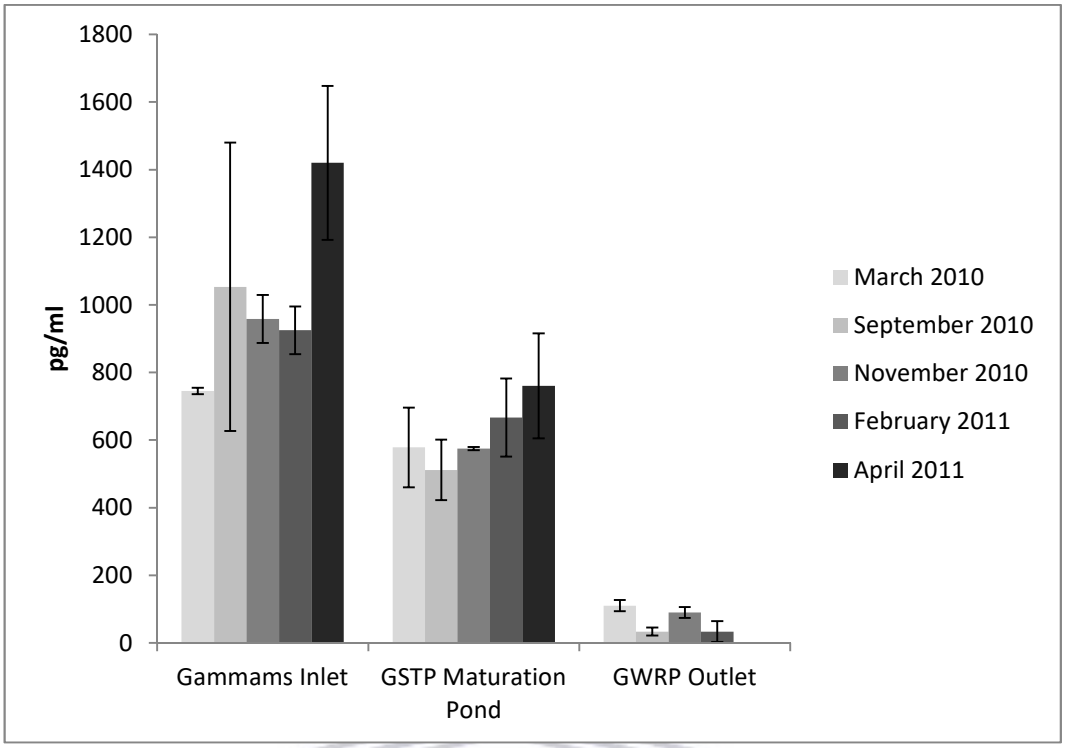

Figure 3.7 IL-6 concentrations in blood culture supernatants after overnight exposure to the extracts of water from the GSTP inlet, GSTP maturation pond and GWRP outlet.

The IL-10 levels in the culture supernatants ranged between 150 and 250 pg/ml for the months of March, September and November 2010 as well as February 2011 (Figure 8). During April 2011 a significant increase in IL-10 production was detected in both the GSTP inlet and maturation pond, with the inlet reaching 527 pg/ml. Water treatment in the GSTP resulted in a significant reduction in IL-10 production. The GWRP outlet tested negative for IL-10 induction in all samples.

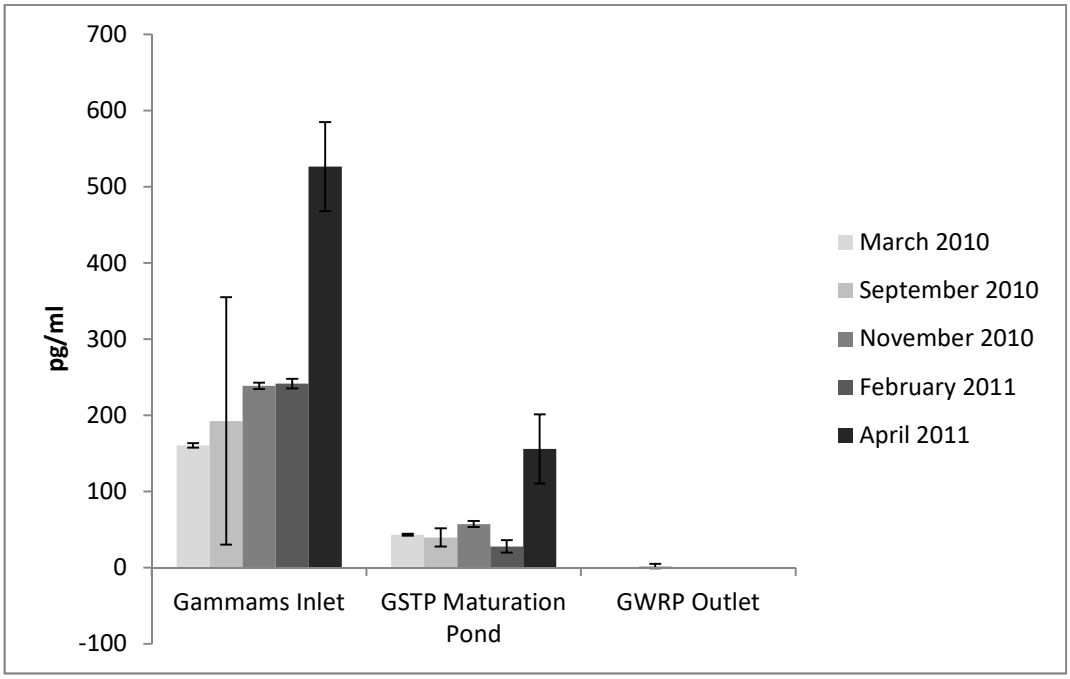

Figure 3.8 IL-10 concentrations in blood in blood culture supernatants after overnight exposure to the extracts of water from the GSTP inlet, GSTP maturation pond and GWRP outlet.

3.5 Discussion

The GWRP in Windhoek reclaims a significant proportion of treated wastewater to supplement the domestic water supply. Therefore, there are concerns regarding the presence and potential effects of EDCs, although the main concern of the public still remains microbial pollution and algal toxicity. EDCs and their presence in the environment are still not well known to the general Namibian public.

Relatively high levels of steroid hormones are present in the raw sewage entering the GSTP. However, during processing at the GSTP and the GWRP steroid hormones and neurotoxic, cytotoxic and immunotoxic chemicals present in raw sewage are effectively removed. Endocrine disrupting chemicals can be removed through various steps in the sewage treatment process. Biodegradation and sorption by activated sludge seems to be the most efficient and most often used step for the removal of hormones, especially estradiol and testosterone (Janex-

Habibi et al. 2009; Leusch et al. 2006). However, for other EDCs, their removal is reported to be limited (Chang et al. 2009).

The estradiol concentrations in the GSTP inlet were relatively constant throughout the sampling period (78 ± 9 pg/ml). This seems to be, on average, a 4 to 5 times higher concentration than for other sewage plants; values of 1.6 pg/ml in Spain (Carballa et al. 2008), 21 pg/ml in Brazil (Ternes et al. 1999), 15 pg/ml in Germany (Ternes et al. 1999), and 15.6 pg/ml in Canada (Servos et al. 2005; Ternes et al. 1999) have been measured. The dissimilarities between measurements might be due to, amongst others, the sampling regimen, differences in sewage influent properties, differences in the treatment processes, differences in the accuracy, and/or precision of the analytical test methods used. Estradiol is efficiently removed in the GSTP, with between 83% and 95% removal. This removal efficiency is similar to that in other countries (Ternes et al. 1999; Servos et al. 2005; Carballa et al. 2008). The water that enters the GWRP is that from the GSTP maturation pond, and all remaining estradiol that was detected in the pond was successfully removed at the GWRP. In line with research done by Kolpin et al. (2004) on the dilution effect on organic wastewater contaminants, lower estradiol concentrations during March and November 2010 and February 2011 can be explained by the dilution effect of runoff rainwater, since these samples were collected during or soon after rain events that resulted in increased runoff into the sewage system. In contrast, during September 2010 the long dry winter resulted in a higher concentration of chemicals in the raw sewage. However, the higher estradiol concentration during April 2011 was measured at the end of a long intense rainy season and one would have expected it to be lower as well. The reason for this increase in concentration is not known, but may have been as a result of raw sewage runoff in the catchment. To our knowledge no information on steroidal hormone levels in storm-water runoff exists.

The presence of estrone showed a different pattern compared to estradiol, with much more seasonal variation in the raw sewage. Initial concentrations for post-wet (March 2010) and post-dry months (September 2010) were low (13 pg/ml). This was followed by a 12-fold increase in estrone (to 161 pg/ml) after the first

precipitation in November 2010. As the wet season continued the estrone levels decreased to 135 pg/ml in February and 74 pg/ml in April 2011. These findings suggest high levels of estrone build-up within the catchment of the GSTP during the dry season, and initial high levels due to runoff during the rainy season. The amount of estrone in raw sewage in comparable studies was 40 pg/ml for Brazil, 27 pg/ml for Germany and 49 pg/ml for Canada (Ternes et al. 1999). The removal efficiency in Brazil and Canada ranged from 98% to instances where estrone levels in the effluent were even higher than in the raw sewage (Ternes et al. 1999). In this study, estrone removal in the GSTP was 100% during March and September 2010 when initial estrone concentrations were low. During the subsequent months, estrone removal was 85% to 92% and the remaining estrone in the maturation pond was completely removed in the GWRP. Estradiol is readily oxidised to estrone under aerobic conditions (Ternes et al. 1999) and studies have shown that estrone may, as a result of this, be found in higher concentrations in the final effluent of sewage treatment plants (Chang et al. 2011; Swart and Pool 2007). This was however not the case in the GSTP where the estrone and estradiol concentrations in the effluent were very similar (9.2 ± 1.2 pg/ml and 8.4 ± 4 pg/ml, respectively).

The testosterone concentrations were, on average, much higher than those of estradiol and estrone. This is in agreement with the excretion concentrations of the natural steroid hormones by humans and animals (Shore and Shemesh 2003), and is similar to trends shown by Leusch et al. (2006) for sewage treatment plants in Australia and New Zealand. Similarly to estrone, testosterone also peaked in November 2010 at the start of the rainy season (405 pg/ml). Overall, testosterone concentrations showed greater variation between the different samples with the lowest concentration, of 162 and 159 pg/ml, occurring at the end of the rainy season (March 2010 and April 2011), probably as a result of dilution. The testosterone levels measured correspond well with those measured by Stalter et al. (2011) in Switzerland and Germany (21 to 400 pg/ml), while Chang et al. (2011) observed much lower concentrations (21 to 76.7 pg/ml) in China. Measurements at the GSTP are also within the range of testosterone concentrations measured in Australia and New Zealand (Leusch et al. 2006); however the variation in

concentrations measured by Leusch et al. (2006) is much more extreme (113 to 4 300 pg/ml). Testosterone was almost completely removed in all of the samples after treatment at the GSTP, with only 10 ± 5 pg/ml remaining. This represents a 96% removal efficiency of testosterone, which is the same as that observed in China by Chang et al. (2011).

Although the measured steroid hormone concentrations were drastically reduced in the effluent, it may still pose an environmental and health risk. A multitude of studies have shown that the presence of steroid hormones in effluents have adverse effects on wildlife, including, among others, reduced fertility, abnormal development of male and female secondary sex characteristics, alteration in sex ratio, feminisation of males and alteration of behaviour (Sharpe 1998; Tyler and Routledge 1998; Rodríguez et al. 2007; Saaristo et al. 2009). Thus, where effluent is used for irrigation purposes or excess sewage effluent enters rivers or dams it poses health threats to both animals and humans. In Windhoek, excess sewage effluent ends up in the Goreangab Dam, currently used for recreational purposes (no swimming or fishing allowed). In addition, the effluent is reclaimed at the GWRP to supplement Windhoek's domestic water supply. However, EDC removal in the GWRP was 100% effective.

The toxicity of raw and treated wastewater has been demonstrated in previous research (Farcy et al. 2011; Gagné et al. 2011; Macova et al. 2011). Persistent organic pollutants (POPs) like polychlorinated biphenyls, organochlorine pesticides, and polycyclic aromatic hydrocarbons are typically responsible for many of the toxic effects of wastewater and are characterised by persistence by resisting chemical and biological degradation. The most effective means of removal seems to be dissolved organic carbon (DOC), powder activated carbon (PAC) and ozonation (Bolong et al. 2009; Katsoyiannis and Samara 2007a; Stalter et al. 2011). At the GSTP the raw sewage sample showed neurotoxicity, with 50% inhibition of AChE. It was only in February 2011, at the peak of the rainy season, that no inhibition was detected. This was probably due to the dilution effect of the continued rain in the catchment area. The highest AChE inhibition, of 94%, was detected in November 2010 after the first rains, when toxins washed in from the

city environment. Neurotoxicity was reduced by 73% during the sewage treatment process and 100% reduction was achieved at the end of the GWRP. This reduction efficiency corresponds well to the study of Macova et al. (2011) on reclamation plants in Australia. Cytotoxicity was low overall and only recorded in the GSTP inlet for March 2010 and February 2011 (13% and 25% respectively). Sewage effluent showed no cytoxicity, and this corresponds to studies elsewhere (Smital et al. 2011). Toxicity testing should, however, be done using a wide array of tests to include toxic chemicals of varying characteristics, and to accurately determine toxicity in a wide range of organisms and under different conditions, as suggested by Dizer et al. (2002).

Pro-inflammatory IL-6 levels were very high in all of the raw sewage samples, indicating high microbial activity or microbial breakdown products (Pool et al. 2000) or high steroid hormone presence, especially estradiol (Ansar Ahmed 2000). The IL-6 level induced by the March 2011 sample was the lowest, at 745 pg/ml, and for April 2011 the highest, at 1 421 pg/ml. Average IL-6 levels were 1 020 ± 250 pg/ml. High levels are to be expected since it is raw domestic sewage containing faecal bacteria, and the highest IL-6 production also corresponded with the highest estradiol levels in the GSTP inlet. On average the effluent from the GSTP resulted in 38% less IL-6 production, indicating that there is still relatively high microbial activity in the effluent. This may pose health risks where the effluent is used for irrigation purposes or if the reclamation process at the GWRP malfunctions. During this study, the reclaimed water had negligible effects on IL-6 production. Anti-inflammatory IL-10 production was lower than that of IL-6 with an average of 272 ± 146 pg/ml for the raw sewage. Similar to IL-6 the production of IL-10 was also the lowest for March 2010 and the highest for April 2011.

From this study it is evident that the steroid hormone concentrations and neurotoxicity are higher towards the start of the rainy season, and cytotoxicity and immunotoxicity higher towards the end of the rainy season. Neurotoxicity is commonly caused by many anthropogenic chemicals, such as organophosphates that accumulate in the catchment during the dry season and then reach the plant

after the first rains. In contrast, cytotoxicity and immunotoxicity are mostly caused by microorganisms that need time to proliferate in polluted water before reaching levels where adverse effects are expressed. Since the rivers in the catchment are all seasonal rivers they are dry throughout the winter. With initial rains the water is expected to have relatively low microbial content. However, after longer periods of rain, and with accumulation of stagnant pools, there will be increased microbial activity and therefore increased cyto- and immunotoxicity. This may explain the higher cytotoxicity in March and April and higher immunotoxicity in April.

Results from this study indicated that high rainfall decreases the general quality of influent water to the wastewater treatment plant, since the concentration of hormones such as estrone, estradiol and testosterone increases. The increase in the various hormones does not occur simultaneously. Water quality was worst at the beginning of the rainy season due to increased runoff bringing in all the accumulated pollutants from the streets and buildings. During the last, and more intense, part of the rainy season a dilution effect on sewage, and therefore lower endocrine disrupting potential, exists. This study revealed that the GSTP is not entirely successful in the removal of all EDCs, but achieves relatively good results and compares well with other sewage treatment plants, even when the load on the systems drastically increases, as was the case with the exceptionally high rainfall of the 2010/11 rainy season. It also indicates that remaining EDCs are successfully removed by the GWRP. The results of this study form a baseline for further EDC studies on the City of Windhoek's potable water supply. Total oestrogenic activity and androgenic activity, as well as the bioavailability of EDCs, remain to be investigated. Furthermore, EDC presence in the surface and subterranean water sources of Windhoek's water supply should be determined.

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Chapter 4

Steroid hormone concentrations and physiological toxicity of water from selected dams in Namibia

FAUL, A.K., JULIES, E. & POOL, E.J. 2014. Steroid hormone concentrations and physiological toxicity of water from selected dams in Namibia. *African J. Aquat. Sci.* **39**: 37–41.

4.1 Abstract

Namibia is a semi-arid to arid country and has most of its surface water in dams built on ephemeral rivers. Whilst water quality is often measured in terms of bacterial contamination and general physico-chemical characteristics, this study extends water quality assessment to include steroid hormone presence and potential physiological toxicity. This is the first study to determine these parameters in dams in Namibia at various stages of the seasons. Seven bioassays were used to determine estradiol (E2), estrone (E1) and testosterone (T) concentrations, as well as neurotoxicity, cytotoxicity and immunotoxicity, in water sampled during 2010 and 2011. Estradiol and E1 concentrations of up to 7.2 pg/ml and 7.6 pg/ml, respectively, were recorded. Testosterone concentrations measured up to 19/pg ml. No cytotoxic effects were detected, while acetylcholinesterase (AChE) inhibition assays indicated low neurotoxic effects in Goreangab Dam (18% AChE inhibition) and no neurotoxic effects in other samples. The immune system biomarker interleukin-6 was high in all samples (457 pg/ml), with interleukin-10 being high only at Avis (46 pg/ml), Goreangab (74 pg/ml) and Swakoppoort (81 pg/ml) dams. The results suggest that water from Goreangab and Swakoppoort dams may have the potential to modulate endocrine systems, and shows physiological toxicity.

Keywords: cytokines, cytotoxicity, endocrine disrupting chemicals, ephemeral rivers, inflammatory response, neurotoxicity, steroid hormones, water quality

4.2 Introduction

Rapid increase in the global human population over the last two centuries has increased the demand for potable water, as well as its use in industry, mining and agriculture. Simultaneously, the adverse effects of these stressors threaten the world's water resources in terms of various forms of pollutants being released into the environment. It is therefore of utmost importance that the world's water resources are constantly monitored and protected from various forms of pollution.

One of the major water contaminants that came under scrutiny in the last few decades is a group referred to as endocrine disrupting compounds (EDCs) (Tyler and Routledge 1998; Van Der Kraak 1998; Gadd et al. 2005; Pojana et al. 2007; Jackson and Sutton 2008). These are chemicals that have the ability to modulate the endocrine systems of animals and therefore have the potential to affect various physiological processes adversely. Natural and synthetic steroid hormones and heavy metals are environmental contaminants recognised as EDCs, most frequently encountered in aquatic ecosystems (Medesani et al. 2004; Bondegaard and Bjerregaard 2005; Rodríguez et al. 2007). In addition, other recognised EDCs include alkylphenols, polychlorinated biphenyls (PCBs), chlorinated pesticides, herbicides and petroleum hydrocarbons (Pojana et al. 2007; Rodríguez et al. 2007; Swart and Pool 2007; McKinlay et al. 2008). Previous studies have demonstrated that EDCs regularly occur in aquatic systems and impact wildlife when contamination with raw and semi-purified wastewater from various industries and sewage treatment works (STWs) occurs (Harries et al. 1997; Tyler and Routledge 1998; Van Der Kraak 1998; Gadd et al. 2005; Pojana et al. 2007; Jackson and Sutton 2008). Adverse effects on wildlife reproduction include reduced fertility, abnormal development of male and female secondary sex characteristics, alteration in sex ratio, feminisation of males, masculinisation of females, intersex and alteration of behaviour (Sharpe 1998; Tyler and Routledge 1998; Rodríguez et al. 2007; Saaristo et al. 2009). These effects were in the past typically associated with the presence of steroidal oestrogens in the environment, but more recently the role of chemicals with anti-androgenic properties in rivers receiving effluent from wastewater treatment plants has been highlighted as another major

potential cause (Jobling et al. 2009). Apart from reproductive effects, EDCs may also influence other endocrine systems by having neurotoxic, cytotoxic and immunotoxic effects, such as acetylcholinesterase (AChE) inhibition in *Xenopus laevis* larvae (Colombo et al. 2005), immune system modulation (Jin et al. 2010; Rogers et al. 2013), and cytotoxic effects of EDCs in mussels (Parolini et al. 2011).

Namibia has a low population density, with 823 680 km^2 of land being inhabited by a population of about 2.1 million in 2011 (National Planning Commission 2012). It is a predominantly hyper-arid to semi-arid country with an average annual rainfall of 360 mm (Mendelsohn et al. 2009). There are only seven perennial rivers, six of which are situated on the borders of the country. Consequently, the largest part of Namibia relies on water from boreholes or surface water in dams for human consumption, agriculture and industry. However Windhoek, the capital of Namibia, also relies on the reclamation of sewage water to supplement the potable water supply, and it is especially here that the monitoring for EDCs becomes critical (du Pisani 2006; Faul et al. 2013). A large proportion of the country is hyper-arid and the majority of the population is concentrated in more densely populated towns, usually located close to water sources. This exerts additional pressure on the existing water sources and may result in EDC introduction into its available water sources. In the rest of Namibia, the relatively low population density probably has minor impacts on the water resources.

To date, no published data exists on the evaluation of EDCs in the natural or impounded water sources in Namibia. Therefore, this study aimed to determine the endocrine modulating potential of water from nine selected dams acting as major surface water sources in Namibia.

4.3 Materials and methods

4.3.1 Sample collection

The grab water samples were collected every two to three months throughout the year, so as to include different times of the wet and dry seasons, between March 2010 and April 2011 from nine dams. Tables 4.1 and 4.2 show the dams sampled, their main uses, the sampling dates and related rainfall information. Since weather stations were present at only some of the dams sampled, or in their catchments, nearby weather stations in the areas were selected to provide an overview of rainfall patterns for the sampling months (Figure 4.1).

One 400 ml water sample was collected in a Schott glass bottle from 10 cm below the surface at each location. Prior to collection the bottles were thoroughly washed with soap solution, rinsed with reverse osmosis water and then with chemically pure acetone. The bottles were rinsed with water from the sample site before collection to get rid of any residual acetone. All water samples were transported on ice. Three 2 ml aliquots of each sample were frozen in SureLock tubes for cytotoxicity and immunotoxicity analysis. The remaining water was frozen until extractions were made for steroid hormone and neurotoxicity analysis.

The water samples were screened for the female hormones 17ß-estradiol (E2) and estrone (E1) (also a metabolite of E2) and the male hormone testosterone (T). Bioassays to evaluate physiological toxicity, using whole blood cultures to assess immunotoxic, cytotoxic and neurotoxic activity in the selected water sources, were also conducted.

Table 4.1 Namibian dams selected for water analysis and the main uses of the water.

Name	Domestic Supply	Irrigation	Mining	Augmen-tation	Recreation
Omatako				●[a]	
S von Bach	●				●
Swakoppoort	●		●	●[a]	●
Goreangab					●
Avis					●[b]
Friedenau			●		●[b]
Oanob	●	●			●
Hardap	●	●			●
Naute	●	●			●[b]

a Supplement S von Bach Dam b Limited Recreation – No watercraft with petrol engines allowed

Table 4.2 Total monthly rainfall data (mm) for selected weather stations in Namibia.

Station	Mar '10	Sep '10	Nov '10	Feb '11	Apr '11
Hochfeld[a]	166.2	0	47.4	155.1	256.6
Windhoek[b]	26.5	11.9	114.0	179.3	161.5
Rehoboth[b]	23.5	0	40.0	207.5	82.3
Stampriet[a]	14.0	0	0	152.2	55.0
Keetmanshoop[b]	9.5	0	3.6	158.5	14.7
Canon Roadhouse[a]	17.5	0.3	1.0	8.2	11.9

a Data obtained from the Namibia Weather Network (www.namibiaweather.info).

b Data obtained from the Namibia Meteorological Service (www.meteona.com).

Shaded areas indicate the months with the lowest and highest rainfall recorded.

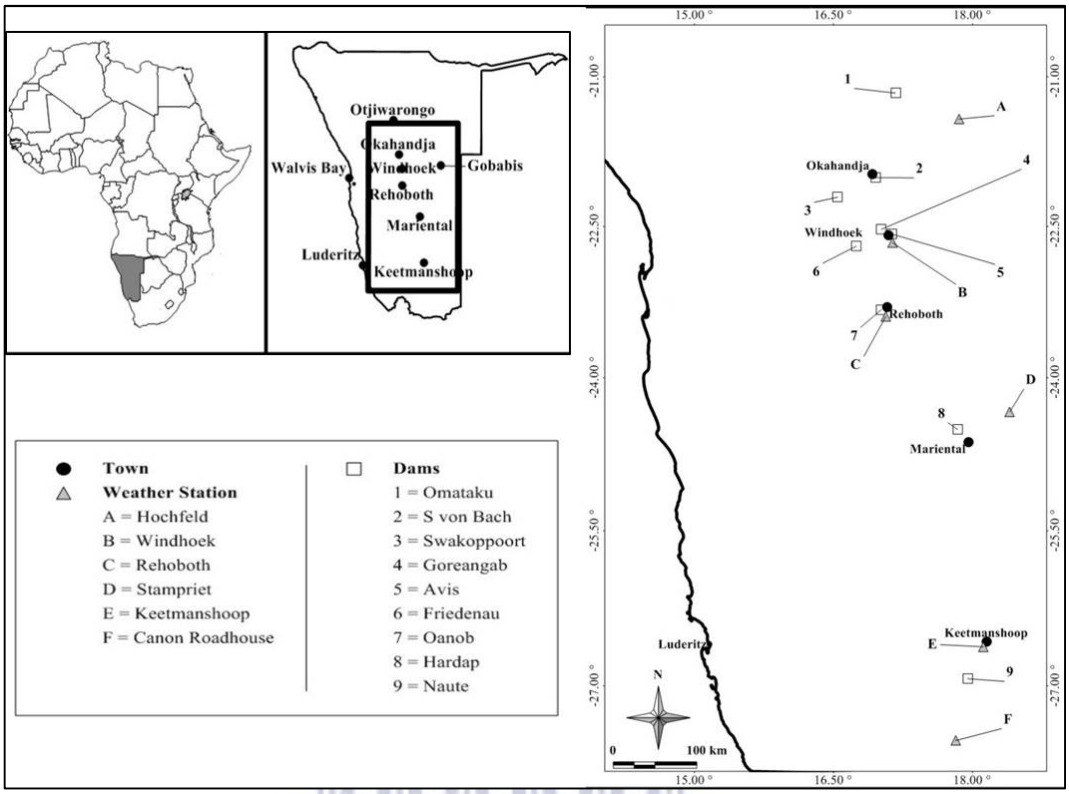

Figure 4.1 Locations of dams sampled and weather stations used to obtain rainfall data, in relation to the major towns in Namibia.

4.3.2 Solid phase extraction

Extraction of organic compounds was done using DSC-18 solid phase extraction (SPE) columns (Supelco, Sigma-Aldrich). Frozen samples were thawed at room temperature, and a modified version of the extraction procedure followed by Pool and Magcwebeba (2009) was used. The column was charged by washing with 2 ml of methanol followed by 2 ml of solvent mixture (40% v/v hexane, 45% v/v methanol and 15% v/v 2-propanol). It was then washed with 2 ml of methanol and filled with distilled water, after which 100 ml of the unfiltered samples were applied. Once the samples had passed through, the columns were dried under vacuum for at least 1 h. The hydrophobic molecules were eluted from the SPE columns into glass vials by applying two 1 ml volumes of solvent mixture. The eluate was dried with a hairdryer placed 30 cm above the samples and inside a fume hood. The dried samples were reconstituted in dimethyl sulphoxide (DMSO)

to a final volume of 0.1% of the original sample volume extracted. All extracts were therefore a 1 000× concentrate of the original sample.

4.3.3 Steroid hormones

The estradiol, E1 and T concentrations were determined using enzyme-linked immunosorbent assay (ELISA) kits (Sigma, Germany). Inter- and intra-assay variation for steroid hormone ELISAs are negligible, as shown by Swart and Pool (2007) who determined inter-assay variation at 5.6% (n = 3) and intra-assay variation between 0.6% and 2.5% (n = 3). This was repeated by Faul et al. (2013), who measured inter-assay variation at 0.8% (n = 6) and intra-assay variation between 0.3% and 7.2% (n = 3). Thus, the precision of the ELISAs reduces the need for expensive and time-consuming replication and provides for a rapid screen of a number of samples.

All procedures provided in the ELISA kits were followed. All samples were applied to the ELISA microplates in duplicate. For E2 and E1 a 100-fold dilution, and for T a 50-fold dilution, of the 1 000× concentrated extracts were made using wash solution. Thus, the E1 and E2 samples were applied to the ELISA microplates 10× concentrated, and the T samples were applied 20× concentrated. For most of the samples this allowed the concentration of hormones to fall within the dynamic ranges of the assays and above the lower limit of quantification (LOQ). Afterwards, the results of the ELISAs were adjusted to represent the true concentration of hormones in the environment. The dynamic ranges of the assays, as provided by the supplier, were: 9.7–2 000 pg/ml for E2; 2.21–1 000 pg/ml for E1; and 83–16 000 pg/ml for T. After factoring in the concentrated samples that were applied to the ELISAs, the effective lower LOQ for each was reduced to 0.97 pg/ml for E2, 0.22 pg/ml for E1 and 4.15 pg/ml for T.

4.3.4 Blood collection

Blood from three healthy male volunteers, who had not used any medication for a three-month period, was collected at the University of the Western Cape Health

Centre. Sterile heparin Vacutainer® tubes (Lasec, South Africa) were used for collection, and the blood was stored at room temperature and used within 18 h.

4.3.5 Neurotoxicity

Water samples were screened for general neurotoxicity by measuring acetylcholinesterase (AChE) inhibition in heparinised human blood. Tenfold dilutions of 6 µl extracts were prepared using 0.1 M phosphate buffer. Six millilitres of 1 nM chloropyrifos were mixed with 6 µl DMSO for the positive control. Six microlitres of DMSO was used as negative control and both positive and negative controls were diluted to 60 µl using 0.1 M phosphate buffer. The positive control was used for a 4× dilution range. Samples in volumes of 25 µl were applied in duplicate to a storage microplate. An AChE extract was prepared by mixing the collected blood, distilled water and 0.1 M phosphate buffer in a ratio of 3:20:97. Thereafter, 25 µl of the diluted blood was added to each well on the microplate and left to incubate for 2 h. Substrate was prepared by mixing 100 µl of 0.01 M 5,5-dithiobis (2-nitro-benzoic acid) (DTNB) with a solution of 3 ml 0.1 M phosphate buffer and 20 µl 0.075 M acetylthiocholine iodide (ATCI). After incubation, 50 µl of substrate was added to each well and the optical density of each sample was immediately determined at 405 nm, and thereafter at 30 min intervals for 2 h.

4.3.6 Whole blood culture assays

The collected healthy heparinised human blood was used to initiate an inflammatory response. Under sterile conditions, 20 µl of unextracted water from each sample was pipetted in duplicate into the wells of a tissue culture plate (NuncTM, Denmark). Sterile distilled water was used as negative control. A 10× dilution of heparinised human blood in Dulbecco's modified Eagle's medium (BioWhittaker) was prepared and 200 µl of the blood-medium mixture was added to each well. The plate was covered and, after an overnight incubation at 37 °C, the supernatants were collected for lactate dehydrogenase (LDH), interleukin-6 (IL-6) and interleukin-10 (IL-10) analysis.

4.3.7 Cytotoxicity assays

Lactate dehydrogenase concentrations in the plasma were measured as an indication of cytotoxicity of water samples using a commercially available chromogenic LDH assay kit (Sigma, Germany). A 100% cytotoxicity control was prepared by mixing 20 µl 10% Tween 20 detergent (Sigma, Germany) with 200 µl of diluted blood and allowing for complete lysis of the cells. Ten micro litres of the whole blood culture supernatants and a dilution range of the positive control were transferred into a 96-well storage plate; 200 µl of LDH cytotoxicity assay kit WST-8 substrate was diluted in 10 ml of LDH assay buffer and 50 µl of the dilution was added to each well. Optical density was immediately determined at 450 nm and thereafter at intervals of 10 min. Between measurements, the plate was incubated at room temperature in the dark. The LDH concentrations were determined using a standard curve from the dilutions of the 100% cytotoxicity control.

4.3.8 Cytokine assays

Bi-directional interaction exists between endocrine and immune systems. The increased production of cytokines has been linked to exposure to EDCs (Lee et al. 2003). To determine the immunotoxicity of the water samples, the supernatants collected were analysed for IL-6 and IL-10 concentrations using double antibody sandwich ELISAs (e-Bioscience, Germany). The ELISAs were performed with minor modifications to the supplier's instructions. Nunc-Immuno microplates (Nunc™, Denmark) were coated with 50 µl of capture antibody in coating buffer then sealed and incubated overnight at 4 °C. Plates were washed five times with wash buffer and blotted dry on absorbent paper. Wells were blocked with 100 µl of 1× assay diluent for 1 h at room temperature after which they were washed and blotted dry. Fifty microlitres of the standards were added and a 2-fold dilution was performed to create a standard curve. The supernatants were diluted 2-fold with 1× assay diluent and 50 µl of each sample was added in duplicate to the plate. The plates were covered and left to incubate for 2 h at room temperature. The plates were washed five times and blotted dry. Fifty microlitres of detection antibody in

1× assay diluent was added, the plates sealed and incubated for 1 h at room temperature. The plates were washed, blotted dry, and 50 µl of Avidin-Horseradish Peroxidase (Avidin-HRP) was added to each well. The sealed plates were left to incubate for 30 min at room temperature after which they were washed seven times and blotted dry; 50 µl of substrate solution was added and the plates were left to incubate for 15 min, after which 25 µl of stop solution was added. Optical densities were determined at 450 nm and IL-6 and IL-10 concentrations were determined from standard curves. The LOQ for both ELISAs was 15.6 pg/ml.

4.3.9 Statistical analysis

Descriptive statistics were used to represent data. The Kruskal–Wallis test was used to determine significant differences between data. Where significant differences existed, the Mann–Whitney test was used for post hoc analysis. OpenStat 2013 and Microsoft Excel 2010 were used for statistical analysis.

4.4 Results

4.4.1 Steroid hormone concentrations

The results of the ELISAs for the female steroid hormone E2 are presented in Figure 4.2. The observed levels of E2 in all the samples were higher than the lower LOQ of the test and exceeded the predicted no effect concentration (PNEC) values for fish (1 pg/ml) as proposed by Young et al. (2004) and Burkhardt-Holm (2010). The highest recorded E2values were 7.2 pg/ml and 5.5 pg/ml for the Oanob and Goreangab dams, respectively, for September 2010, which was also the month with a statistically significant higher average E2 concentration (4.6 pg/ml) than all the other months ($p < 0.05$). All E1 concentrations were above the lower LOQ. The highest concentrations being 6.1 pg/ml in Avis Dam in November 2010, and 7.6 pg/ml in Goreangab Dam in April 2011 (Figure 4.3). November 2010 had the highest average E1 concentration (4.4 pg/ml), and the concentrations were significantly higher than those in March and September 2010 ($p < 0.05$), but no difference was detected between November 2010 and February

and April 2011 ($p > 0.05$). On average, the estrone levels in Avis, Friedenau, Goreangab and Swakoppoort dams were above the PNEC values for fish given by Burkhardt-Holm (2010). Testosterone concentrations were mostly above the lower LOQ (Figure 4.4). The highest values were recorded in November 2010, with the maximum concentration of 19 pg/ml being recorded in Friedenau Dam. The average T concentration of 10 pg/ml in November was significantly higher than the February and April 2011 concentrations ($p < 0.05$).

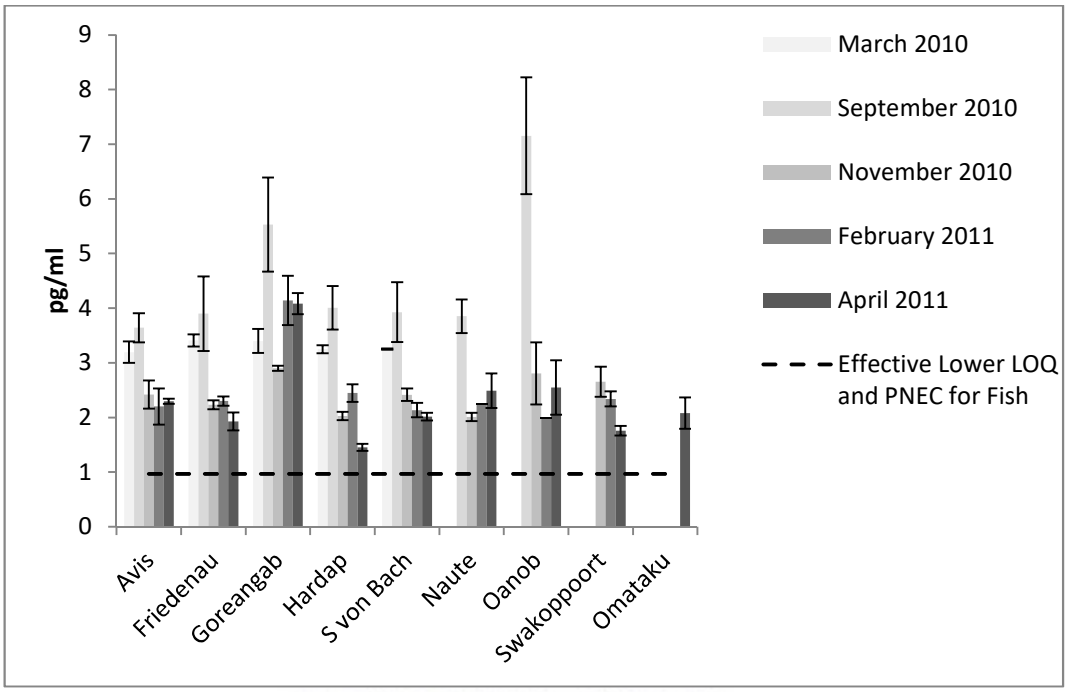

Figure 4.2 Estradiol (E2) concentrations (pg/ml) in water from selected Namibian dams in March 2010–April 2011 ($p < 0.05$ for monthly variation). Effective lower limit of quantification (LOQ) and predicted no effect concentration (PNEC) for fish (Burkhardt-Holm 2010) are indicated. Error bars denote SD; n = 2.

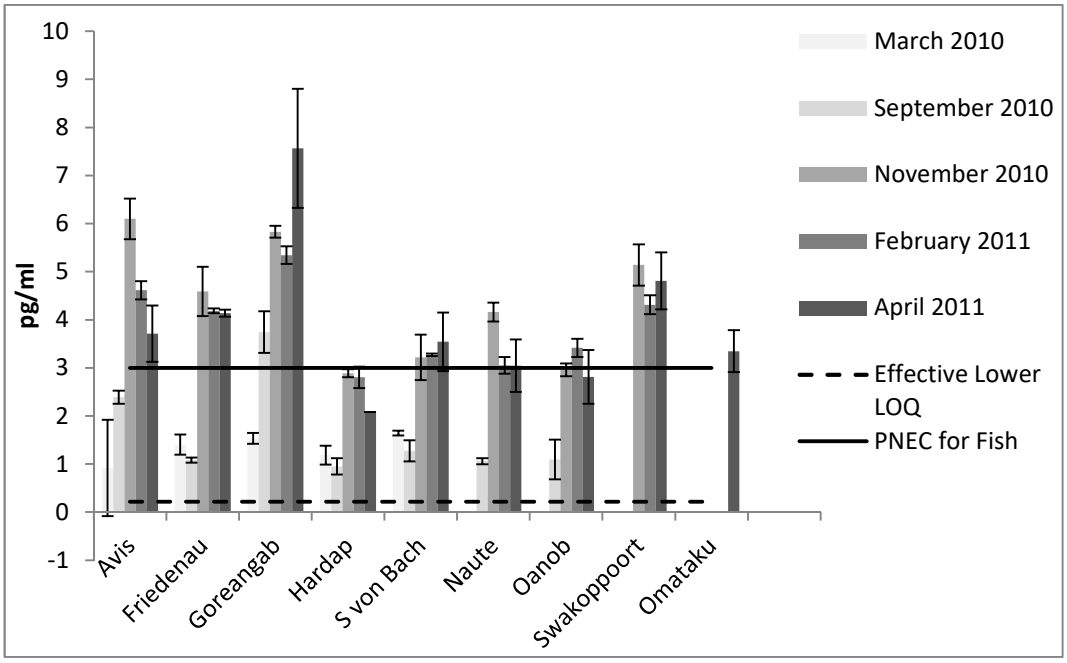

Figure 4.3 Estrone concentrations (pg/ml) in surface water from selected dams in Namibia for the months of March, September, November 2010 and February and April 2011 ($p < 0.05$ for monthly variation). The effective lower limit of quantification (LOQ) and predicted no effect concentration (PNEC) for fish as provided in Burkhardt-Holm 2010 are also indicated. Error bars denote SD; n = 2.

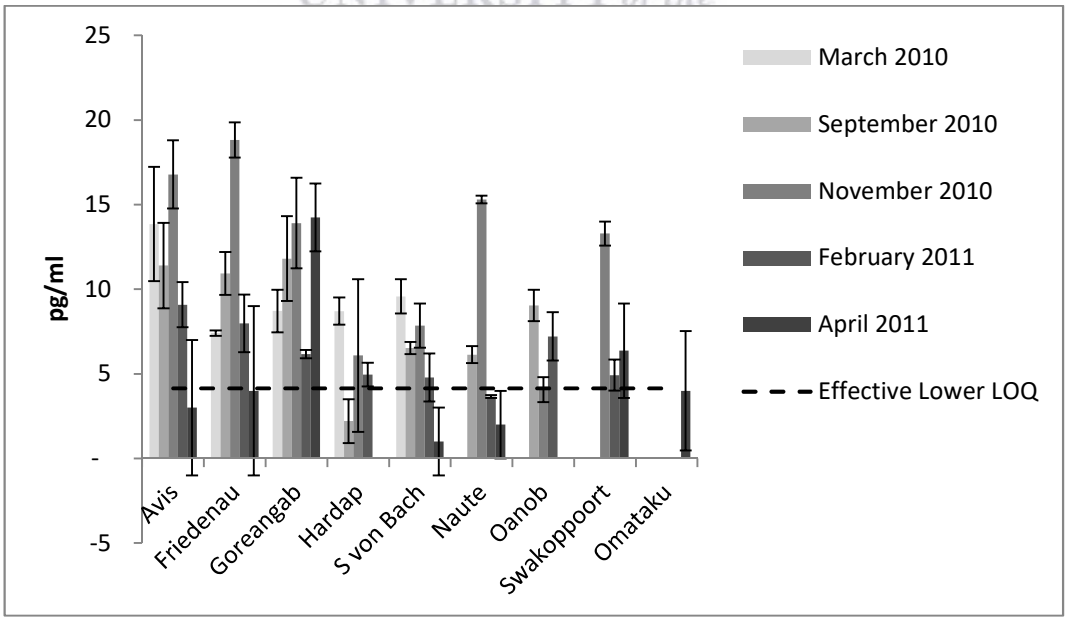

Figure 4.4 Testosterone concentrations (pg/ml) in surface water from selected dams in Namibia for the months of March, September, November 2010 and

February and April 2011 ($p < 0.05$ for monthly variation). The effective lower limit of quantification (LOQ) is also indicated. Error bars denote SD; n = 2.

4.4.2 Neurotoxicity

The acetylcholinesterase inhibition is indicated in Figure 4.5. Water sampled from the Goreangab Dam exhibited the highest AChE inhibition during February 2011 (26%). The month with the highest AChE inhibition for all dams (8%; SD 2%) was April 2011, which was statistically significantly higher than in all other months ($p < 0.05$).

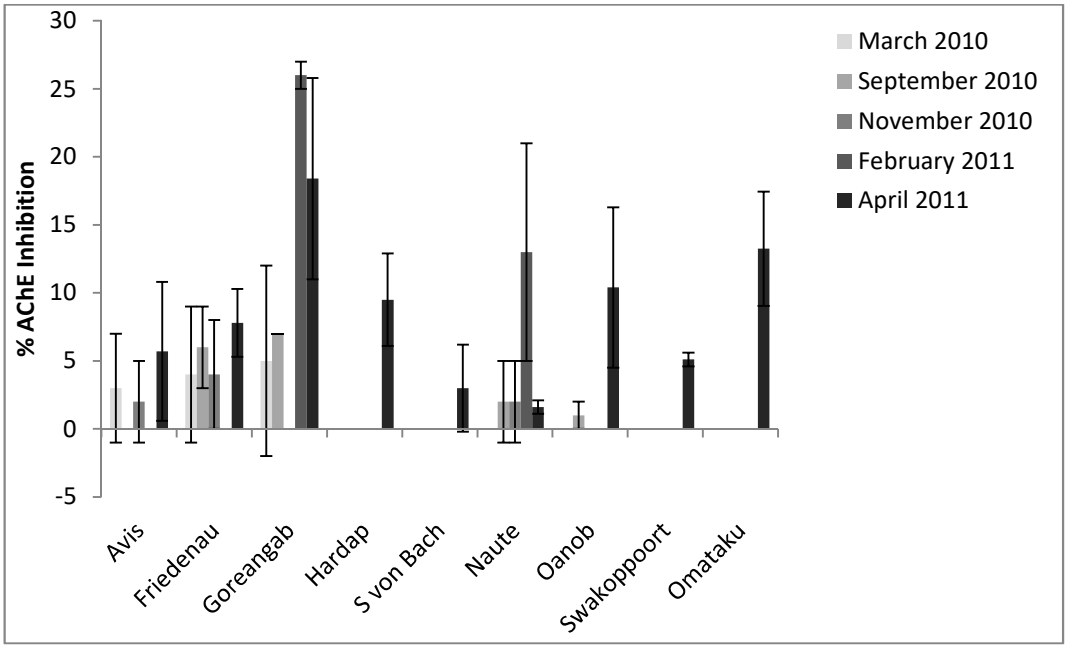

Figure 4.5 Acetylcholinesterase inhibition in human blood spiked with water samples collected from selected dams in Namibia for the months of March, September, November 2010 and February and April 2011 ($p < 0.05$ for monthly variation) (February 2011 data for Goreangab dam not available). Error bars denote SD; n = 2.

4.4.3 Inflammatory activity for cytotoxicity and cytokine analysis

Lactate dehydrogenase was not detected in plasma indicating that none of the corresponding water samples were cytotoxic (1%; SD 0.4%). Interleukin-6 production was high throughout the sampling period in all the dams, averaging

457 pg/ml (SD 54), and no significant difference was detected between the different months ($p > 0.05$) (Figure 4.6). Typically, for healthy blood, IL-6 values of less than 15 pg/ml would be expected. The highest IL-6 concentrations were found in water samples from the Swakoppoort (710 pg/ml; SD 90) and Goreangab (673 pg/ml; SD 60) dams, while the lowest concentrations were in the water samples from the Omatako (131 pg/ml; SD 83) and Naute (207 pg/ml; SD 28) dams. Maximum IL-6 concentrations were observed during February 2011 (651 pg/ml; SD 56) and November 2010 (515 pg/ml; SD 39), while minimum concentrations were observed during April 2011 (323 pg/ml; SD 62) and March 2010 (376 pg/ml; SD 31).

Interleukin-10 concentrations were much lower than those of IL-6 (Figure 4.7). The significantly higher concentrations were in water samples from the Swakoppoort (81 pg/ml; SD 16), Goreangab (74 pg/ml; SD 17) and Avis (46 pg/ml; SD 6) dams, while concentrations in water from the Naute, Hardap, S von Bach, Friedenau and Omatako dams were all below the detection limit ($p < 0.05$). Typically, for healthy blood, IL-10 values of less than 15 pg/ml would be expected. No significant differences were detected ($p > 0.05$) between the monthly concentrations for the water samples from any of the dams.

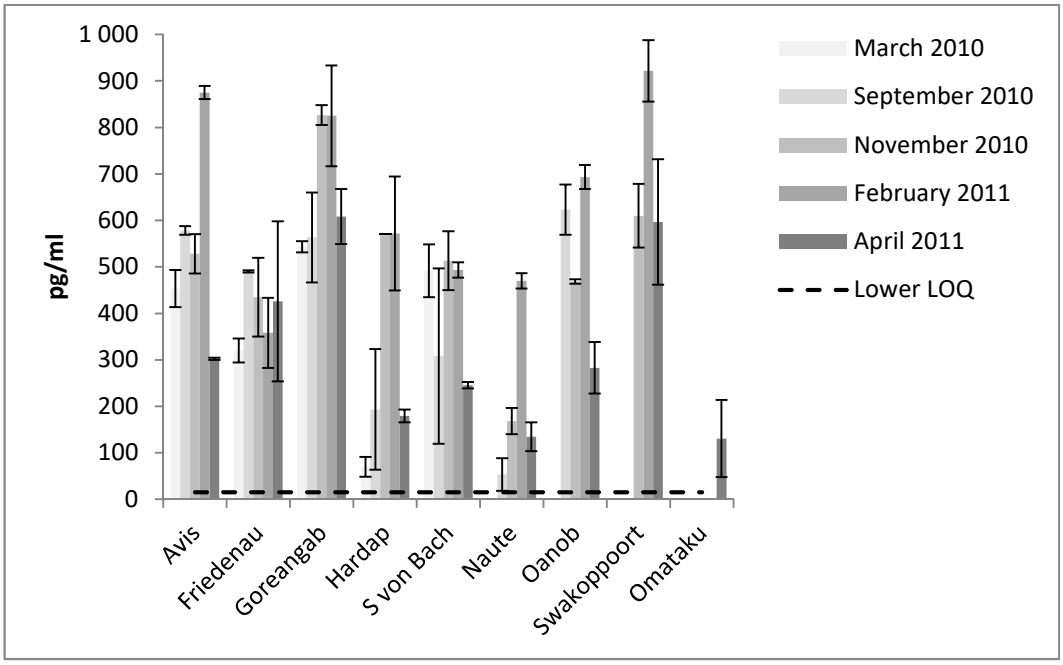

Figure 4.6 Interleukin-6 concentrations (pg/ml) of blood plasma of uncontaminated human blood after overnight exposure to the extracts of water were elevated and showed no significant differences between the selected dams ($p > 0.05$ for monthly variation). Error bars denote SD; n = 2.

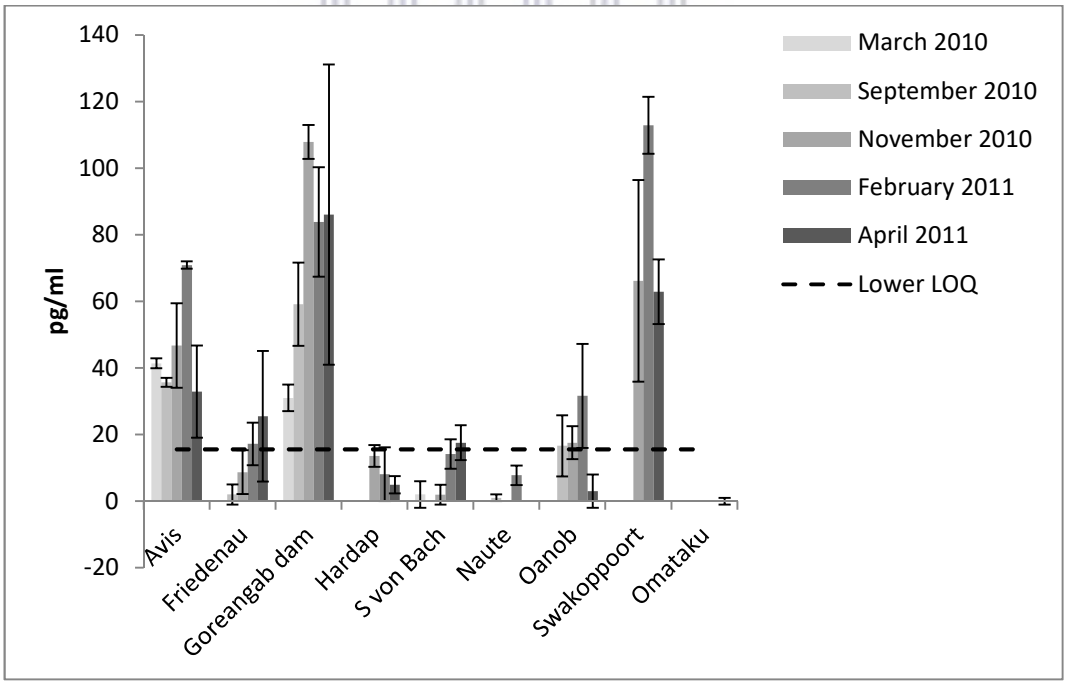

Figure 4.7 Interleukin-10 concentrations (pg/ml) of blood plasma of uncontaminated human blood after overnight exposure to the extracts of water from the selected dams were significantly higher for Avis, Goreangab

and Swakoppoort dams ($p < 0.05$ for monthly variation) while being mostly below the lower LOQ for the other dams. Error bars denote SD; n = 2.

4.5 Discussion

In Namibia, most of the water available for humans comes either from subterranean aquifers or from dams built on ephemeral rivers throughout the country. Human activities and water quality in the catchments of dams may adversely affect the quality of stored water and the ecosystems of these dams. Since Namibia is the driest sub-Saharan country in Africa (Heyns 2005; Baker et al. 2007), it is crucial that surface water sources and underground aquifers are protected from pollutants.

Oestrogenic steroid hormone levels as low as 1–10 pg/ml have been shown to induce reproductive abnormalities in fish (Young et al. 2004). The concentrations of both E2 and E1 in all surface water samples tested in this study were higher than the proposed PNEC values (1 and 3 pg/ml, respectively). No reports exist, locally or elsewhere, on steroid hormone presence in dams built on ephemeral rivers.

However, some reports exist for dams built on perennial rivers. Jafari et al. (2009) measured E2 and E1 concentrations below 7 pg/ml during 2006 and 2007 in Ekbatan Dam, Iran. This is comparable to the concentrations measured in the present study. Globally, more data exist on the oestrogenicity of surface water from perennial streams and rivers than for dam water. Examples include Austria (Hohenblum et al. 2004), Brazil (Sodré et al. 2010), the Netherlands (Belfroid et al. 1999) and France (Vulliet & Cren-Olivé 2011). In these studies, E2 concentrations were determined using either gas or liquid chromatography techniques and results were generally low (<5 pg/ml), while only one sample from Brazil had an E1 concentration of 39 pg/ml.

In general, the testosterone concentrations in this study are comparable to, or lower than, those found by Vulliet and Cren-Olivé (2011) for surface waters in France (0.3–26.3 pg/ml), whereas Kolpin et al. (2002) reported values as high as

214 (average 116) pg/ml for streams in the USA. The higher testosterone concentrations measured during November 2010 in the Friedenau, Avis, Naute and Goreangab dam samples can possibly be explained by the onset of that rainy season causing testosterone in agricultural runoff to be washed from their catchments, since all these dams, except Goreangab, lie within livestock farming areas. Towards the end of the rainy season the sluices of most of the dams were opened for long periods of time, due to high rainfall. This may have resulted in the reduced testosterone concentrations (average concentration below effective LOQ of 4.15 pg/ml) observed in April 2011 due to the dilution effect of a continued in- and outflow of rainwater. One natural degradation process of testosterone is by exposure to ultraviolet light (Vulliet et al. 2010). This suggests that, with long-term exposure of dam water to sunlight, without additional influxes, one would expect a reduction in testosterone concentrations. The dam with the highest testosterone concentrations throughout the sampling period was Goreangab. This dam's catchment consists of a mixture of informal settlements and residential and industrial areas of the city of Windhoek. Furthermore, part of the effluent from the Gammams sewage treatment plant (GSTP) is released into this dam. This effluent contains testosterone concentrations exceeding 10 pg/ml (Faul et al. 2013).

The steroid hormone levels reported in this study are comparable to measurements made elsewhere (Jafari et al. 2009; Vulliet and Cren-Olivé 2011), although research results on water from artificial reservoirs on ephemeral rivers, such as those in Namibia, are scarce. This study further indicates that, of the dams investigated, Goreangab Dam generally has the highest steroid hormone levels. This is most likely as a result of the inflow of sewage effluent, the catchment including the city of Windhoek, and the presence of informal settlements around the dam.

All the dams, except Goreangab, have their catchments outside urban or agricultural areas, where intensive use of pesticides or other chemicals may occur. This explains why negligible or no neurotoxic effects are present in the surface waters of these dams. However, the possibility of chemicals with neurotoxic

characteristics occurring in the sediments needs to be investigated, since organic hydrophobic pollutants will accumulate in sediments, as suggested by Xue et al. (2005).

The water sampled from Goreangab Dam shows neurotoxic effects, possibly because its catchment lies in the city of Windhoek. This includes a large informal settlement on the immediate northern side of the dam. Persistent neurotoxins can therefore be introduced into the dam from upstream. Furthermore, excess sewage effluent from the GSTP, which receives stormwater runoff and domestic sewage, is also released into this dam. This effluent showed AChE inhibition of up to 22% (Faul et al. 2013). Comparable studies indicated that sewage effluent and stormwater runoff can be a source of chemicals (e.g. organophosphate compounds) with potential neurotoxic effects (Martínez-Carballo et al. 2007; Regnery and Püttmann 2010). Apart from anthropogenic chemicals, algal blooms containing microcystins can also have potential neurotoxic and endocrine disrupting effects (De Figueiredo et al. 2004; Osswald et al. 2007; Rogers et al. 2011). Recently, Rogers et al. (2011) showed that algal blooms release compounds with endocrine disrupting effects that are not related to microcystins. Although no published data exist, previous water quality monitoring at the Swakoppoort and Goreangab dams indicated that these dams have high microbial activity as well as eutrophication leading to algae, mostly cyanobacteria, blooms. One of the main reasons why the Goreangab Dam wtare source is considered unfit for purification as drinking water is the large amount of algae present in the dam, creating problems with the maintenance of filters. The occurrence of cyanobacteria, specifically *Microcystis*, with its link to neurotoxic effects, in Goreangab Dam requires further investigation. Similarly, concern about the high algae content in the Swakoppoort Dam should also be closely monitored, because this dam supplements the Windhoek water supply.

The results of the current study indicate that the tested surface water sources for human consumption are free from neurotoxic effects. This does not imply that substances such as polychlorinated biphenyls, organochlorines, organophosphates and polycyclic aromatic hydrocarbons, as well as mycrocystins, are not present. It

may be that these substances are diluted or degraded to such an extent that they were not detected by the assay methods employed, or their concentrations were below the assay quantification limits. Should farming practices or land use in these catchments change to the extensive use of pesticides and fertilisers, the current water quality situation might change, and therefore regular monitoring is advisable.

No cytotoxicity (cell death) was detected in any of the surface water sources sampled. Similar results were found for selected rivers in the Western Cape, South Africa (Pool & Magcwebeba 2009), while variable levels of cytotoxicity were detected in water from rivers in studies done in Tasmania and Slovenia (Khalil & Winder 2008; Žegura et al. 2009). The present bioassay of the cytotoxic properties of water indicates that the tested Namibian surface water sources are of good water quality and are probably unpolluted. However, as suggested by Pool and Magcwebeba (2009), cell death as a measure of cytotoxicity does not exclude possible non-destructive physiological effects on cells.

Pro-inflammatory IL-6 production was high for all the surface water samples, with maximum production being measured during November 2011 and February 2010. The lowest concentrations were detected in water sampled in April 2011 and March 2010, in each case towards the end of the rainy season. This suggests that microbial pollution and its breakdown products increase during the dry season when the water is essentially stagnant, whereas, when the runoff from the first rain water enters the dams from their respective catchments, microbial activity increases significantly. This may be as a result of a high organic carbon load carried from the catchment, since microbes tend to proliferate in high dissolved organic carbon environments (Hamata & Chinsembu 2012). Reduced IL-6 concentrations in water sampled in April 2011 occurred after good rainfall, and the sluices of all the dams in the study area had been open for long periods, and the water in them had largely been replaced. Water samples from the Goreangab and Swakoppoort dams showed the highest IL-6 production throughout the study period. This supports the observations made that these two dams contain high levels of cyanobacteria and microbial activity. The exact cause

of high IL-6 production should be investigated, since the available literature shows that, in the case of cyanobacteria exposure, both suppression and activation of the immune system can be experienced, depending on the type of species and its toxins (Shen et al. 2003; Dogo et al. 2011).

The highest IL-10 productions were measured in Water samples from the Avis, Goreangab and Swakoppoort dams. These dams also had high IL-6 concentrations. The inflammation regulatory cytokine IL-10 is probably high in response to the high IL-6 concentrations, since IL-6 is pro-inflammatory and IL-10 is an inflammation regulatory cytokine. A similar trend was observed by Faul et al. (2013) for cytokine production in response to exposure to sewage water extracts. Relatively high IL-10 concentrations in Avis Dam are also linked to high IL-6 concentrations. Avis Dam also supports periodically increased algal growth, which may induce these types of immune responses.

This study is the first to have used a combination of assays to screen endocrine disrupting potential in dams built on ephemeral rivers in Namibia. It is evident that raw surface water from dams can have possible adverse immune system effects and, where the water is used for domestic purposes, proper purification is required. However, with the exception of Goreangab and to a lesser degree Swakoppoort dams, the overall water quality of the dams studied is good. Low oestrogenicity, testosterone levels, neurotoxicity, cytotoxicity and immunotoxicity were recorded. Currently, water from the Goreangab and Swakoppoort dams may pose the highest risk of eliciting significant immune responses. Although Goreangab dam is not used as a potable water source, the quality of water from the Swakoppoort Dam is of concern. Part of the catchment of the Swakoppoort dam lies in the Windhoek district, and this might be a possible source of pollutants. The presence of algal blooms may be one of the main contributing factors reducing water quality in many of the dams, and therefore the source, impacts and remediation of this should be investigated.

The current study provides baseline information for future research in this field in water from the dams in Namibia.

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Chapter 5

Multiple Biomarker Screening and Proteome Profiling of Whole Blood Culture Supernatants Exposed to Reclaimed Sewage

5.1 Abstract

The Goreangab Water Reclamation Plant in Windhoek, Namibia, was for a long time the only reclamation plant for direct potable reuse of treated sewage in the world. Direct potable reuse of reclaimed sewage raises concerns for potential health impacts on consumers. To date, limited research has been conducted on the potential adverse effects on the immune system caused by reclaimed water produced by the Goreangab Water Reclamation Plant. This study is aimed at determining the immunomodulatory potential of the reclaimed sewage by culturing blood obtained from healthy individuals in the presence of source (treated sewage/influent) or reclaimed (effluent) water samples. Cell viability was determined using a lactate dehydrogenase test and interleukin (IL)-6, macrophage inflammatory protein (MIP)-1β, IL-10 and interferon (IFN)-γ concentrations were determined using enzyme-linked immunosorbent-assays. Proteome profiling was performed to screen for a range of cytokines. Cell cultures were viable after exposure to treated sewage and reclaimed water. The treated sewage sample increased the IL-6 (2 160 pg/ml), MIP-1β (6 008 pg/ml) and IL-10 (67 pg/ml) production significantly. Assay results for the reclaimed water sample were all similar to the negative control. The proteome profile indicated production of IL-1ra, Monocyte Chemoattractant Protein (MCP)-1, MIP-1α/MIP-1β, IL-6 and IL-1β by blood cultures exposed to treated sewage and the lippopolysccharide positive control. These proteins were not produced by blood cultures exposed to the negative control or reclaimed water. The results of the study are indicative of the successful removal of immunomodulatory chemicals from the treated sewage during this reclamation treatment process. It further demonstrates the usefulness of immune system biomarkers for toxicity testing of treated and reclaimed water.

Key Words: Direct Potable Reclamation; Immunomodulatory Potential; Endocrine Disrupting Chemicals, Interleukin, Interferon, Macrophage Inflammatory Protein, Proteome Profile

5.2 Introduction

The City of Windhoek in Namibia has, since 1969, been at the forefront of water reclamation by becoming the first city in the world to directly reclaim domestic sewage effluent for direct potable reuse (Du Pisani 2006). Although indirect potable reuse, by first allowing reclaimed water to pass through an environmental buffer, is a more common practice, it is only recently that other countries started direct potable reuse similar to Namibia (Khan 2013). Initially reclamation in Windhoek was performed at the Old Goreangab Water Reclamation Plant, originally constructed to treat water from the Goreangab Dam as well as the sewage effluent from the Gammams Sewage Treatment Plant (GSTP). Following a number of upgrades, a new Goreangab Water Reclamation Plant (GWRP) was constructed in 2002, which continues to contribute towards the city's water supply, and importantly so, during times of drought. Today, due to high pollution levels, water from the Goreangab Dam is no longer utilized for reclamation. Thus, the GWRP reclaims 100% treated effluent from the GSTP, which receives mainly domestic raw sewage from the City of Windhoek.

In Namibia, direct potable reuse was necessitated by the relative scarcity of water in a mostly semi-arid environment characterized by low and erratic rainfall (Mendelsohn et al. 2009). The process steps followed by the GWRP are coagulation, dissolved air flotation, rapid gravity sand filtration, ozonation, biological activated carbon filtration, granular activated carbon filtration, ultra-filtration, disinfection, and stabilisation using chlorine and sodium hydroxide (Pers. Comm. Siegfried Mueller). As a health and safety measure, the GWRP contributes not more than 35% to the total volume of potable water used in Windhoek (Pers. Comm. Siegfried Mueller). The remaining 65% being sourced from the S. von Bach Dam and the Windhoek aquifer via boreholes. The S. von Bach is part of a three–dam system whereby the S. von Bach Dam is augmented by the Swakoppoort and Omatako Dams.

Direct potable reuse of reclaimed sewage effluent pose potential health risks to consumers due to the vast array of microbes, endocrine disrupting chemicals (EDCs) and toxins contained in the sewage influent. These include infectious

agents such as viruses, bacteria and parasites (Naidoo & Olaniran 2013), chemicals such as pesticides (Campo et al. 2013), pharmaceutical products (Leung et al. 2012), heavy metals such as cadmium and lead (Rule et al. 2006), synthetic organic chemicals like polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs) (Khan 2010), volatile organic compounds (Khan 2010), and hormones (Swart and Pool 2007). EDCs, if present in the environment and in the domestic water supply, may potentially result in adverse effects in animals and humans by disrupting endocrine systems. This may include cytotoxic, neurotoxic, immunotoxic and reproductive effects (Sharpe 1998; Tyler and Routledge 1998; Colombo et al. 2005; Rodríguez et al. 2007; Saaristo et al. 2009; Jin et al. 2010; Parolini et al. 2011; Rogers et al. 2013).

Contaminated water, like treated sewage effluent, has been shown to result in the modulation of the immune system in animals (Hébert et al. 2008; Farcy et al. 2011). As a result, immunological biomarkers are actively researched as indicators of the presence of contaminants such as EDCs in the environment. This includes the stimulation or suppression of a variety of cytokines (Langezaal et al. 2001; Kindinger 2005; Makene and Pool 2015). Interleukin-6 (IL-6), interleukin-10 (IL-10), and interferon-γ (IFN-γ) have been employed as biomarkers for toxicity testing of water (Pool et al. 2000; Pool and Magcwebeba 2009; Faul et al. 2013; Adebayo et al. 2014; Faul et al. 2014; Makene and Pool 2015; Ohkouchi et al. 2015, Makene et al. 2016). However, only one study, as far as could be discerned, has been conducted to determine the potential immunotoxicological effects of sewage reclaimed for direct potable reuse water (Faul et al. 2013). This study (Faul et al. 2013) indicated that the GWRP successfully eliminated the immunotoxic effects of the treated sewage effluent from the GSTP, measured as IL-6 and IL-10 cytokines in exposed whole blood cultures.

The current study aims at expanding the immunological biomarker range tested *in vitro* as indicators of the successful reclamation of treated sewage effluent at the GWRP. It considers both the stimulation and suppression of immune responses. The cytotoxicity of water samples were determined with a lactate dehydrogenase (LDH) assay. The concentrations of IL-6, IL-10, IFN-γ and the macrophage

inflammatory protein MIP-1β were determined in the culture supernatants of whole blood cultures exposed to the GSTP effluent and the GWRP reclaimed water using enzyme linked immunosorbent assays (ELISAs). A proteome profile was also performed as a broad spectrum, although less sensitive, screen for a wide range of cytokines and chemokines.

5.3 Methods and Materials

5.3.1 Water Collection

Three grab water samples were collected in sterile tubes at each of the GSTP final maturation pond (source for the GWRP) and the GWRP outlet (reclaimed water) on the 15th of May 2017. The samples were stored at -80 °C until used.

5.3.2 Microbial and Chemical Analysis

To ensure the safety and quality of reclaimed water, the Scientific Services division of the City of Windhoek performs regular microbial and chemical analysis on both the source water (treated sewage from the GSTP) and the final product water of the GWRP. The results of these analysis were obtained from the City of Windhoek for inclusion in this study. The results were for water samples collected on the 15th of May 2017, the same day of water collection for the immunotoxicity assays.

5.3.3 Blood collection

Blood was collected from healthy male volunteers that were not on any medication for a three month period prior to collection. Blood was collected in sterile vacutainer tubes (Vacuette, Greiner Bio-One, Germany) containing 3.2% sodium citrate as anti-coagulant and stored at room temperature. Blood samples were used for experiments within 8 hours of collection. Ethical clearance was granted under the project title: The use of whole blood cell cultures as a model for assessing immunotoxicity (Registration no: 10/9/43).

5.3.4 Whole Blood Culture Assays

All culture assays were performed under sterile conditions. Blood was divided into three tubes to prepare unstimulated, lipopolysaccharide (LPS) stimulated, and phytohaemagglutinin (PHA) stimulated whole blood cultures. To prepare the unstimulated whole blood culture, a volume of Roswell Parks Memorial Institute (RPMI) 1640 medium (Sigma, Germany) equal to 25% of the volume of blood, was mixed in a heparinized vacutube (Vacuette, Greiner Bio-One, Germany) and added to the blood. The blood was then diluted to a final dilution of one in ten with RPMI medium. For the LPS and PHA stimulated cultures the same procedure as above was used, but LPS (Sigma, Germany) was added to a final concentration of 0.1 µg LPS/ml of culture medium and PHA (Sigma, Germany) was added to give a final concentration of 16 µg PHA/ml of culture medium. Both LPS and PHA were added to the culture mediums immediately before being applied to a culture plate.

Water from each sample was filtered by passing it through a 0.22 µm syringe filter (Starlab Scientific, China) after which 100 µl was applied in duplicate to a 48-well flat bottom tissue culture plate (BD Falcon, USA). The Roswell Parks Memorial Institute 1640 medium containing 1% Tween 20 (Merck, Germany), was added as positive toxicity control and RPMI medium only was used as negative control.

The unstimulated, LPS-stimulated and PHA-stimulated whole blood in culture medium (900 µl/well) were added to the samples on the culture plate. The plate was covered and incubated overnight at 37 °C after which supernatants were transferred to clean plates and kept at -80 °C until the cytotoxicity and cytokine determination as well as the proteome profiling could be performed.

5.3.5 Cytotoxicity Assay

The cytotoxicity of the water samples was determined using a Lactate Dehydrogenase (LDH) Cytotoxicity Colorimetric Assay Kit II (Biovision, USA). The unstimulated supernatants were centrifuged at 12 100 RPM for 5 minutes after which 10 µl of each sample was transferred to a flat bottom plate (Greiner

Bio-One, Germany). For the positive control (1% Tween 20 exposure) a double dilution was prepared by adding 1 × phosphate buffered saline (PBS) to the supernatant to have final supernatant concentrations ranging from 50% to 0%. Lactate dehydrogenase reaction mixture (100 µl/well) was added and the plate was incubated in the dark for 30 min at room temperature. The reaction was stopped by adding 10 µl stop solution and the optical density was determined at 450 nm in a multi-plate reader (Multskan EX, Thermo Fischer Scientific).

5.3.6 Cytokine Assays

The cytokine concentrations were determined using double antibody sandwich ELISAs (e-Bioscience, Germany, for IL-6, IL-10 and INF-γ and R&D Systems, USA, for MIP-1β). The procedures used were as prescribed in the kit, but were followed with slight modifications. In brief: Flat bottom plates (Nunc Maxisorp^{TM}, Denmark) were coated overnight at 4 °C with 50 µl capture antibody in coating buffer. After incubation the plates were washed five times in wash buffer and tapped dry. The wells were then blocked for an hour with 100 µl assay diluent per well, except for the MIP-1β plate which was blocked with 1% bovine serum albumin in PBS. After blocking, the plates were washed five times and tapped dry before adding 50 µl of the blood culture supernatants. For each cytokine ELISA a standard range was also included as provided in the kits. The plates were incubated for two hours before washing five times and then adding 50 µl detecting antibody. After another two hour incubation followed by a five times wash procedure, 50 µl avidin horseradish peroxidase (HRP) was added to each well. A 20 minute incubation was then followed by washing seven times after which 50 µl of tetramethylbenzidine substrate was added and the plates were again incubated for 20 minutes. The process was stopped and the optical density was determined at 450 nm. For MIP-1β the optical density was also determined at 540 nm as specified in the instructions in order to correct for optical imperfections on the plate.

5.3.7 Proteome Profiling

Proteome profiling was performed using the Proteome Profiler™ Human Cytokine Array (R&D Systems, USA). All instructions as provided in the kit were followed. In brief: The membranes were blocked for one hour in Array Buffer 4. One sample of each of the control, GSTP- and GWRP-unstimulated and LPS-stimulated supernatants were thawed on ice and centrifuged at 12 100 rpm. A volume of 500 µl of each of the sample, Array Buffer 4 and Array Buffer 5 were mixed and 15 µl reconstituted detection antibody was added. The prepared samples were incubated for an hour at room temperature. After one hour, the array buffer was aspirated from the membranes and the prepared samples were added to the membranes. The 4-well multi-dish was covered and left to incubate overnight in the fridge. The membranes were then washed three times for 15 minutes each with 1 × wash buffer. Subsequently 2 ml of diluted Streptavidin-HRP was added to each membrane, which were then incubated for 30 minutes at room temperature. After washing the membrane five times for 15 minutes each with wash buffer, tetramethylbenzidine substrate was added to the membranes to visualise the cytokines detected. Photographs of the developed membranes were taken for analysis.

5.3.8 Data Analysis

All data analysis for the ELISA results were performed using Excel (Microsoft Office Professional 2010) and SigmaPlot 12. One-way Analysis of Variance was used to test for statistically significant differences between groups at $P < 0.05$. Where significant differences were detected, post-hoc analysis were performed using the Holm-Sidak method with statistical significance at $P < 0.01$.

5.4 Results

5.4.1 Microbial and Chemical Analysis

The results of the microbial and chemical analysis of water samples as obtained from the City of Windhoek are presented in Table 5.1. The test results indicated

that all the microbial contaminants that were tested for, were successfully removed in the reclaimed water.

Table 5.1 Microbial and chemical analysis of source (semi-purified sewage) and final reclaimed water samples sampled on 15 May 2017 in comparison to the Namibian (NamWater), South African and US EPA standards/limits.

Measure	Units	Source Water (GSTP)	Final Product (GWRP)	Namibia*	South Africa	US EPA
Heterotrophic plate count	cfu/1 ml	59,500	0	10,000	1000	
Total coliform	cfu/100 ml	5,250	0	100	<10	
Faecal coliform	cfu/100 ml	695	0	50	0	
Faecal streptococci	cfu/100 ml	26	0			
Pseudomonas aeroginosa	cfu/100 ml	1,400	0			
Clostridium spores	cfu/100 ml	3,050	0			
Clostridium viable	cfu/100 ml	4,500	0			
Somatic coliphage	PFU/100 ml	530	0			
Free chlorine	mg/l	-	1.43	0.1-5		
pH	-	8.16	7.81	4.0-11		6.5-8.5
Temperature	°C	13.7	10.9			
Conductivity	mS/m 25°C	125	140	400	170	
Total dissolved solids	mg/l	838	938		1,200	500
Turbidity	NTU	1.31	0.169	10	5	
Total Alkalinity	mg/l $CaCO_3$	228.14	190.7			
Total hardness ($CaCO_3$)	mg/l $CaCO_3$	195	190	1,300		
Nitrate (NO_3^- as N)	mg/l as N	4.6	5.7	40	11	10
Nitrite (NO_2^- as N)	mg/l as N	0.16	<0.1		0.9	1
Potassium (K)	mg/l	28.6	28.6	800		
Sodium (Na)	mg/l	170.9	194.6	800	200	
Calcium hardness ($CaCO_3$)	mg/l $CaCO_3$	120	120	1000		
Magnesium hardness ($MgCO_3$)	mg/l $MgCO_3$	74	72	840		
Ammonia (NH_3-N)	mg/l as N	0.43	<0.15	4	1.5	
Orthophosphate	mg/l	1.2	0.23			
Total Kjeldahl nitrogen (TKN)	mg/l as N	2.4	0.5			
Dissolved organic carbon	mg/l	5.63	1.06			
Chemical oxygen demand	mg/l	20	5			
Absorption (UV 254)	abs/cm	0.174	0.016			
Chlorophyll A concentration	µg/l	12.12	0			

* The limits provided are for water considered to have a low health risk and attention should be given to the problem, although the situation is not critical yet.

5.4.2 Cytotoxicity Assay and Cytokine Analysis

No LDH was detected in culture supernatants for all the samples analysed, thus no apparent cytotoxicity was detected in any of the GSTP or GWRP exposed whole blood cultures (data not shown). Optical densities as determined with the assay were 0.703 for the positive control, 0.135 for the negative control, and between 0.146 and 0.167 for all the samples. Thus, correcting for background absorbance by subtracting the negative control optical density from the samples, this equates to approximately sample optical densities being approximately 2% of the positive control.

The results of the IL-6 ELISA are presented in Figure 5.1. The IL-6 concentration in the LPS stimulated control (2 734 ± 75 pg/ml) was significantly higher ($p < 0.001$) than the IL-6 concentration of the unstimulated control (25 ± 0 pg/ml). The IL-6 concentration in the GSTP-exposed cultures (2 160 ± 140 pg/ml) was significantly higher ($P < 0.001$) than both the unstimulated control (25 ± 0 pg/ml) and the GWRP (24 ± 4 pg/ml) exposures. There were no differences ($p > 0.01$) between the GSTP LPS-stimulated (2 707 ± 136 pg/ml), GWRP LPS-stimulated (2 500 ± 42 pg/ml), and the LPS-stimulated control (2 734 pg/ml). All three sample test results were higher ($P < 0.001$) than the unstimulated control (25 ± 0 pg/ml).

The results of the MIP-1β ELISA are presented in Figure 5.2. The macrophage inflammatory protein-1β concentration in the LPS-stimulated control (7 425 ± 575 pg/ml) was significantly upregulated ($p < 0.001$) compared to the unstimulated control (63 ± 12 pg/ml). In GSTP-exposed cultures the MIP-1β concentration (6 008 ± 954 pg/ml) was significantly higher ($P < 0.001$) than both the unstimulated control (63 ± 12 pg/ml) and the GWRP (54 ± 10 pg/ml) exposures. The macrophage inflammatory protein-1β concentration in the GSTP LPS-stimulated (5 849 ± 241 pg/ml) and in the GWRP LPS-stimulated (5 928 ± 323 pg/ml) cultures were significantly higher ($P < 0.001$) than in the unstimulated control (63 ± 12 pg/ml), but were not different ($P > 0.01$) from that in the LPS-stimulated control (7 425 ± 575 pg/ml).

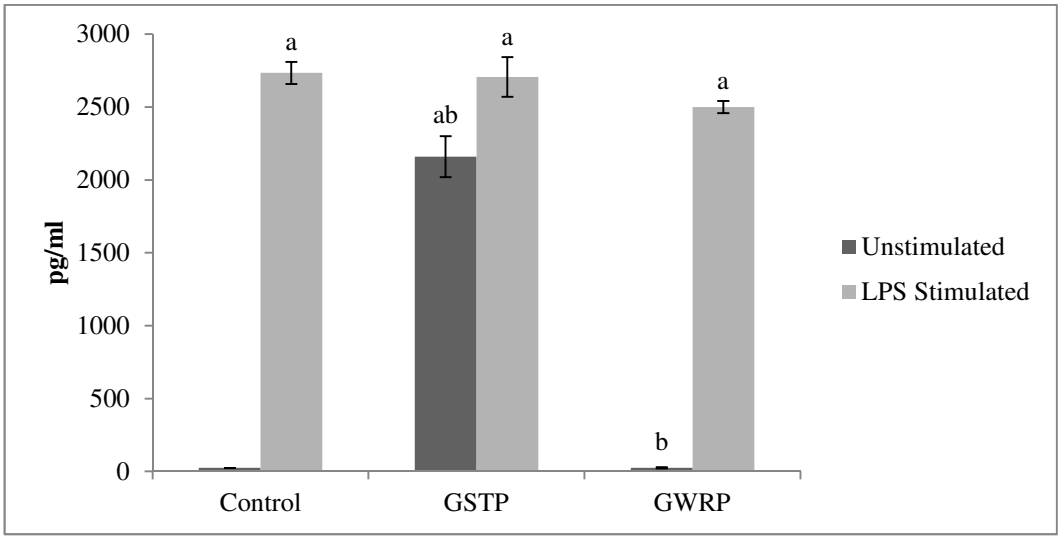


Figure 5.1 Interleukin-6 concentrations, determined using an ELISA, in unstimulated and LPS-stimulated blood cultures after overnight exposure to GSTP and GWRP water samples. Significant differences ($p < 0.01$) are indicated with an "a" (significantly different from unstimulated control) and/or "b" (significantly different from LPS-stimulated control). Error bars denote standard deviation.

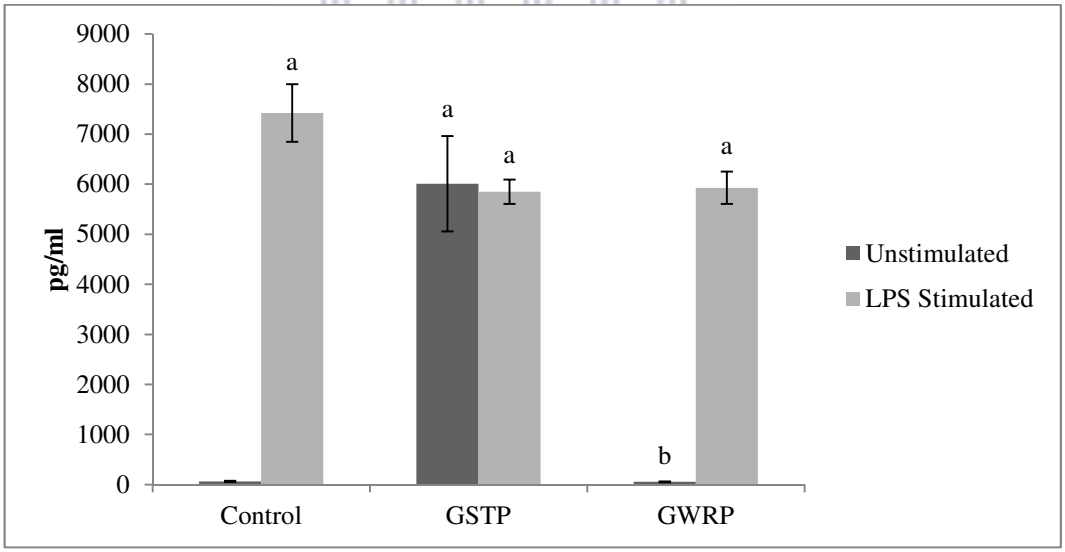


Figure 5.2 Macrophage inflammatory protein-1β concentrations determined using an ELISA, in unstimulated and LPS-stimulated blood cultures after overnight exposure to GSTP and GWRP water samples. Significant differences ($p < 0.01$) are indicated with an "a" (significantly different from unstimulated control) and/or "b" (significantly different from LPS-stimulated control). Error bars denote standard deviation.

The results of the IL-10 ELISA are presented in Figure 5.3. The interleukin-10 concentration in the PHA stimulated control (102 ± 3 pg/ml) was significantly higher ($p < 0.001$) than in the unstimulated control (0 pg/ml). The interleukin-10 in the GSTP-exposed culture (67 ± 14 pg/ml) was higher ($P < 0.001$) than in both the unstimulated control (0 pg/ml) and GWRP (0 pg/ml) exposures. The interleukin-10 concentration in the GSTP PHA-stimulated (146 ± 3 pg/ml) and in the GWRP PHA-stimulated (148 ± 5 pg/ml) cultures were higher ($P < 0.001$) than in the PHA-stimulated control (102 ± 3 pg/ml).

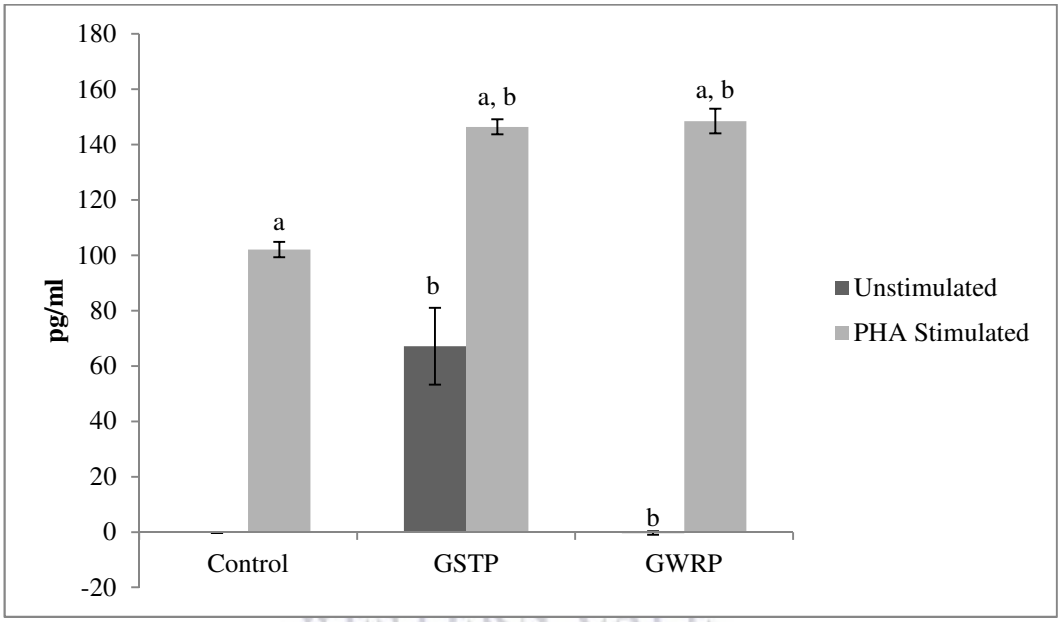


Figure 5.3 Interleukin-10 concentrations determined using an ELISA, in unstimulated and LPS-stimulated blood cultures after overnight exposure to GSTP and GWRP water samples. Significant differences ($p < 0.01$) are indicated with an "a" (significantly different from unstimulated control) and/or "b" (significantly different from PHA-stimulated control). Error bars denote standard deviation.

The results of the IFN-γ ELISA are presented in Figure 5.4. The IFN-γ concentration in the PHA-stimulated control (676 ± 18 pg/ml) was higher ($p < 0.001$) than in the unstimulated control (7 ± 1 pg/ml). The IFN-γ in the GSTP-exposed culture (7 ± 3 pg/ml) was not different ($P > 0.01$) from that in the unstimulated control (7 ± 1 pg/ml) and GWRP (4 ± 1 pg/ml) exposures. The IFN-γ in the GSTP PHA-stimulated (580 ± 8 pg/ml) and the GWRP PHA-

stimulated (585 ± 17 pg/ml) cultures were lower (P < 0.001) than that in the PHA-stimulated control (676 ± 18 pg/ml).

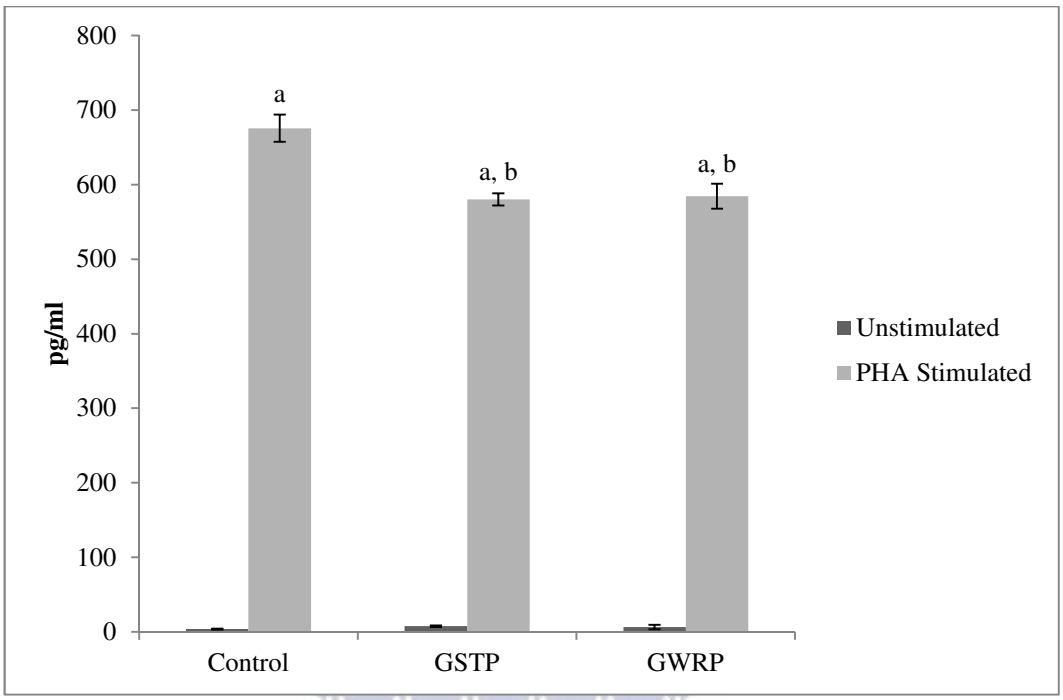


Figure 5.4 IFN-γ concentrations determined using an ELISA, in unstimulated and LPS-stimulated blood cultures after overnight exposure to GSTP and GWRP water samples. Significant differences (p<0.01) are indicated with an "a" (significantly different from unstimulated control) and/or "b" (significantly different from PHA-stimulated control). Error bars denote standard deviation.

5.4.3 Proteome Profiling

As indicated in Figures 5.5 and 5.6 and Table 6.2, the CCL5/RANTES (Chemokine (C-C motif ligand 5 / / regulated on activation, normal T cell expressed and secreted), Macrophage migration inhibitory factor (MIF), serine protease inhibitors (Serpin) E1 and Intercellular Adhesion Molecule (ICAM) -1 show the same trend in all samples. CCL2, MIP-1α/MIP-1β, IL-1 β, IL-1ra, IL-6 and IL-8 are present in both the LPS-stimulated control and the GSTP samples. The LPS-stimulated control also produced CXCL1 which is not present in any of the other samples.

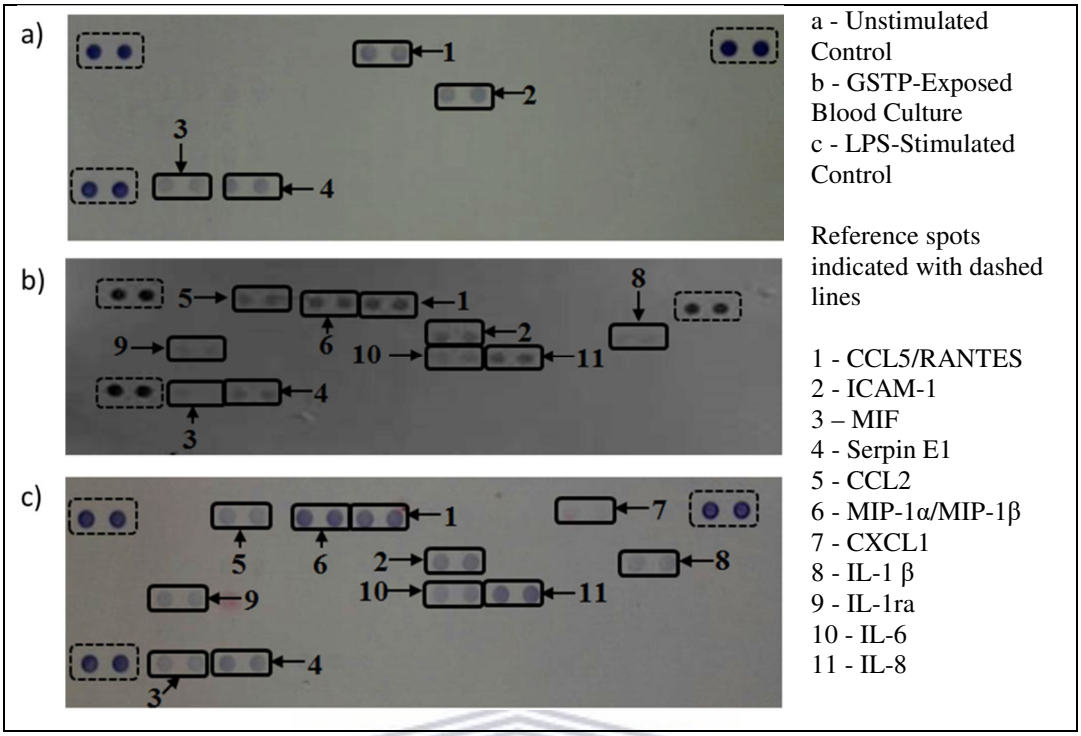
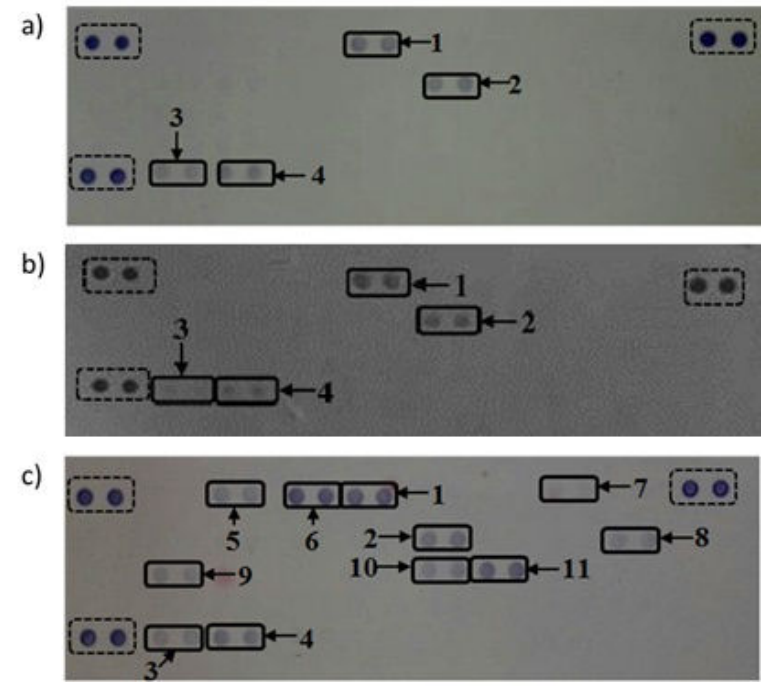


Figure 5.5 Proteome membrane profiles for a) unstimulated control, b) GSTP-exposed, and c) LPS-stimulated control human blood cultures to present an overview of the immunotoxicological potential of the treated source water (GSTP) of the GWRP.



a - Unstimulated Control
b - GWRP-Exposed Blood Culture
c - LPS-Stimulated Control

Reference spots indicated with dashed lines

1 - CCL5/RANTES
2 - ICAM-1
3 – MIF
4 - Serpin E1
5 - CCL2
6 - MIP-1α/MIP-1β
7 - CXCL1
8 - IL-1 β
9 - IL-1ra
10 - IL-6
11 - IL-8

Figure 5.6 Proteome membrane profiles for the a) unstimulated control, b) GWRP-exposed, and c) LPS-stimulated control human blood cultures to present an overview of the immunotoxicological potential of the reclaimed sewage.

Table 5.2 Summary of visual scoring of proteome profiler data for various protein biomarkers. – indicates not detected, + indicates detected.

Biomarker	Water Sample			
	Negative Control	LPS-Stimulated Control	GSTP	GWRP
CCL5/RANTES	+	+	+	+
ICAM-1	+	+	+	+
MIF	+	+	+	+
Serpin E1	+	+	+	+
CCL2	-	+	+	-
MIP-1α/MIP-1β	-	+	+	-
CXCL1	-	+	-	-
IL-1β	-	+	+	-
IL-1ra	-	+	+	-
IL-6	-	+	+	-
IL-8	-	+	+	-

5.5 Discussion

The City of Windhoek relies on the reclamation of sewage effluent for direct potable reuse to augment its water supply. This is achieved at the GWRP where treated sewage effluent from Windhoek's domestic waste water treatment plant (GSTP) is processed. The reclamation of sewage for direct potable reuse does present potential risks where the reclamation process is insufficient or fails. The GWRP thus employs a multi-barrier approach in order to maximize plant efficiency. Due to the vast array of chemical contaminants used daily, it is impossible to analyse for the presence of all potential chemical contaminants that can remain in the reclaimed water. The GWRP employs monitoring mechanisms for the microbial contamination and aesthetic quality of the final product water. These do not however provide any indication of the potential risks of reclaimed water on biological systems such as the endocrine system, immune system, neural

system, etc. The i*n vitro* bioassays can achieve this and the results include synergistic and antagonistic interactions between chemicals (Nelson et al. 2007) and they target the entire toxicity pathway (Escher et al. 2014). The i*n vitro* assays using human blood cultures are relatively quick and easy and they eliminate the need for animal-based studies.

The residual pollutants persisting in treated sewage effluents may have immunomodulatory effects which can include the suppression of the immune system, triggering of inflammation and allergic disease, and the overstimulation of immune responses that can cause autoimmune disease (Kuo et al. 2012). The biomarkers of immunity, for example the expression of cytokines and chemokines, have potential as indicators of pollutants in treated sewage effluent as well as in reclaimed water. These types of biomarkers and the use of proteome profiling have successfully been used to determine the effects of specific chemicals or products on blood cultures. Examples include exposing RAW264.7 mouse macrophages to a Chinese herbal formula, "Zuojin Pill" (Wang et al. 2012) and compounds from Bi-Qi capsules (Wang et al. 2011). Both studies showed that the tested products can up-regulate and down-regulate a number of cytokines. However, despite the successful use of these biomarkers, these methods have never before been employed to determine the immunomodulatory potential of reclaimed water used for direct potable use.

In this study, the whole blood cell cultures remained viable for the control, the GSTP and the GWRP exposures with no cytotoxicity detected. This does however not conclusively rule out the possibility of the presence of products or chemicals in the samples that can modulate physiological processes. Cytotoxic effects can still be contained at the intracellular level, without occurrence of plasma membrane damage, and thus LDH leakage that will be detected in the serum. The viability of all exposed whole blood cultures increases the comparability of the results of the immunological assays.

Interleukin-6 is a pro-inflammatory cytokine that is secreted by dendritic cells and macrophages in response to bacterial products at the sites of inflammation (Pool et al. 2000; Gabay 2006). The secreted IL-6 stimulates monocyte and granulocyte

production and induces acute-phase protein production (Pool et al. 200; Murphy and Weaver 2016). The acute-phase proteins include C-reactive protein and mannose-binding lectin (MBL) which are pathogen-recognition molecules that can opsonize bacteria and activate the complement cascade (Murphy and Weaver 2016). Given the presence of bacteria and breakdown products in the treated sewage effluent from the GSTP (Table 5.1), it is expected that IL-6 production will be stimulated in exposed whole blood cultures. The stimulation of IL-6 production by treated sewage (GSTP) exposed unstimulated blood cultures in this study is thus expected. However, the GWRP samples did not stimulate IL-6 production, indicating the successful removal of bacteria and bacterial breakdown products by the reclamation processes. This result is in line with IL-6 assays conducted during 2010 and 2011 on these two sources, which also indicated the successful removal of IL-6 stimulatory products by the GWRP (Faul et al. 2013). The results of the LPS stimulated GSTP and GWRP exposures showed no differences from the LPS control, indicating that the GSTP and the GWRP water samples had no IL-6 suppressive effects (Figure 5.1). The results of the proteome profile supported the results of the ELISAs with the LPS control and the GSTP exposed samples presenting IL-6 on the proteome profile membranes (Figure 6).

Macrophage inflammatory protein-1β is a chemotactic cytokine (chemokine) responsible for the attraction of anti-inflammatory cells to sites of injury or infection (McManus et al. 1998). MIP-1β is among others produced by monocytes, macrophages, neutrophils and endothelium in response to bacterial endotoxins (Chaisavaneeyakorn 2003; Murphy and Weaver 2016). In comparison to the MIP-1β concentration in the negative control, the MIP-1β concentration in the GSTP-exposed unstimulated blood cultures were significantly upregulated, while no upregulation occurred in GWRP-exposed unstimulated samples (Figure 5.2). No stimulation or suppression of MIP-1β took place in LPS-stimulated GSTP and LPS-stimulated GWRP exposures when compared to the positive control. The results of the proteome profile confirmed the presence of MIP in the GSTP-exposed samples (Figure 5.5 and Table 5.2).

IL-10 is mainly produced by T helper type 2 (T_H2) cells, but also by B cells and innate immune cells like macrophages, mast cells, dendritic cells, neutrophils. It was previously referred to as a human cytokine synthesis inhibitory factor due to its role in supressing immune responses (Saraiva and O'Garra 2010; Murphy and Weaver 2016). It is an anti-inflammatory cytokine with the important role of preventing inflammatory and autoimmune diseases (Saraiva and O'Garra 2010). It also stimulates immunoglobulin production by B cells and mast cell proliferation as well as an increase in the cytotoxicity of NK cells (Oral et al. 2006). Due to its anti-inflammatory function, IL-10 is expected to be present in blood cultures where an inflammatory response was activated. This was true for the GSTP-exposed unstimulated blood culture with an IL-10 concentration of 67 pg/ml, which also had high IL-6 levels and thus an inflammatory response (Figure 5.3). This result is in line with the IL-10 assays conducted during 2010 and 2011 on these two sources, which also indicated the successful removal of IL-10 stimulatory products by the GWRP (Faul et al. 2013). Interleukin-10 concentrations in both GSTP- and GWRP-exposed PHA stimulated blood cultures were significantly elevated above the PHA stimulated control. Although IL-10 was present in GSTP exposed blood cultures as determined with the ELISA, it was not detected by the proteome profile (Figure 5.6). This is likely as a result of the very low concentrations of the interleukin in the samples. The ELISA thus remains a more sensitive, although more time consuming, method for determining IL-10 presence.

To determine the reason for the upregulation of IL-10 in PHA-stimulated exposed blood cultures additional research is required. One possibility is the presence of calcium which has been shown to signal the production of IL-10 by T_H2 cells (Rafiq et al. 2001; Murphy and Weaver 2016). Cytoplasmic calcium binds to calmodulin which in turn activates the protein phosphatase calcineurin. Calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT) and nuclear transporters move the NFAT into the cell nucleus. Gene activation then occurs which allows for T-cell activation and IL-10 production (Rafiq et al. 2001; Murphy and Weaver 2016). Since IL-10 has a very strong inhibitory function on pro-inflammatory cytokines like IL-12 and IL-23, it prevents these cytokines from

stimulating the activation and differentiation of T_H1 cells (Murphy and Weaver 2016). This could possibly explain why suppression of IFN-γ was seen in the PHA-stimulated cultures where IL-10 was upregulated.

In innate immunity, NK cells produce IFN-γ when stimulated by IL-12 and IL-18 produced by activated macrophages (Murphy and Weaver 2016). IFN-γ in turn increases the macrophages' ability to kill pathogens as part of innate immunity as well as stimulating the differentiation of CD4 T cells into T_H1 cells. In the acquired immune response, IFN-γ is produced by $T_H1$cells and acts in the defence against viruses and intracellular pathogens (Murphy and Weaver 2016). Water from both the GSTP and GWRP did not stimulate IFN-γ production in unstimulated blood cultures (Figure 5.4). IFN-γ production in the PHA stimulated cultures were supressed in both the GSTP- and the GWRP-exposed samples compared to the PHA-stimulated control.

Interleukin-8 is a chemo-attractant cytokine with specificity for neutrophils and naïve T cells (Bickel 1993; Murphy and Weaver 2016). It is produced by monocytes, macrophages, fibroblasts, epithelial and endothelial cells and plays a part in the inflammatory response by attracting, among others, neutrophils to the sites of infection (Murphy and Weaver 2016). Interleukin-8 was synthesized by both LPS- and GSTP-exposed blood cultures. The cause of this inflammatory response was however successfully eliminated in the GWRP water.

Interleukin-1β is produced by macrophages and epithelial cells and results in fever as well as T cell and macrophage activation (Murphy and Weaver 2016). Interleukin-1β was upregulated in the LPS-exposed control with very slight upregulation in GSTP-exposed cultures. The reclaimed water did not upregulate IL-1β and thus did not activate a fever-producing response.

The chemokine CCL2, also known as monocyte chemo-attractant protein 1 (MCP-1), is responsible for the attraction of monocytes, memory T cells and dendritic cells to inflamed areas (Deshmane et al. 2009; Murphy and Weaver 2016). It is mainly produced by monocytes/macrophages and it also activates monocytes which differentiates into macrophages in order to phagocytose

pathogens as part of the innate immune response (Deshmane et al. 2009). The presence of CCL 2 in GSTP-exposed blood indicates the inflammatory potential of treated sewage, likely as a result of the presence of endotoxins and persistent chemicals. The absence of CCL2 in GWRP-exposed blood again confirms the efficiency of the GWRP process in eliminating inflammation-causing substances.

The CCL5 chemokine, also known as RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted), attracts various leukocytes to the sites of inflammation (Lv et al. 2013). It is produced by T cells, endothelium and platelets and it plays a role in activating T cells, degranulation of basophils, as well as in chronic inflammation (Murphy and Weaver 2016). CCL5 occurs in healthy blood at levels in the 5 to 6 ng/ml range (Kleiner et al. 2013) and its presence is thus expected in the negative control. The chemokine (C-C motif) ligand 5 was present in the negative control, the LPS stimulated control, the GSTP and the GWRP culture supernatants.

Serpin E1, or Human Plasminogen Activator Inhibitor-1 (PAI-1), is a serine protease inhibitor that plays a regulatory role in the fibrinolytic system (Fay et al. 1997). It thus regulates the breakdown of blood clots at sites of tissue damage and inflammation during the healing process. Serpin E1 is naturally present in healthy human blood and is thus expected to be present in all blood exposures (Cesari et al. 2010). The proteome profile indicates the presence of Serpin E1 in the negative control, positive control, GSTP- and GWRP-exposed culture supernatants.

Interleukin-1ra is an IL-1 antagonist and thus plays an important role as an anti-inflammatory protein regulating the inflammatory response of IL-1 (Arend and Guthridge 2000). It is produced by monocytes, macrophages, neutrophils and hepatocytes (Murphy and Weaver 2016). Expression of IL-1ra occurred in both the LPS-stimulated and the GSTP-exposed blood cultures, indicating the inflammatory potential of the GSTP water. This inflammatory potential was however eliminated during reclamation.

Macrophage migration inhibitory factor produced by the T cell and the pituitary cells is an inflammatory cytokine that inhibits macrophage migration, but it

stimulates macrophage activation and induces steroid resistance (Calandra and Roger 2003; Murphy and Weaver 2016). Macrophage migration inhibitory factor was present in both the negative and LPS-stimulated positive controls indicating that MIF was already present in the blood. Macrophage migration inhibitory factor was also present in the GSTP- and the GWRP-exposed culture supernatants.

Intercellular Adhesion Molecule 1 is a transmembrane glycoprotein that facilitates the transendothelial migration of immune cells to the sites of inflammation (Roy et al. 2001 and Usami et al. 2013). It is expressed at basal levels by epithelium and immune cells and its expression is upregulated by inflammatory mediators (Usami et al. 2013). Intercellular Adhesion Molecule 1 is thus expected in healthy blood and it was present in the negative control, LPS stimulated positive control, GSTP- and GWRP-exposed culture supernatants.

Of the cytokines, IL-6 most frequently has been used to determine the inflammatory potential of various contaminated water sources. Pool et al. (2000) tested the inflammatory potential of water from the Eerste River in the Western Cape, South Africa, using IL-6 production in whole blood cultures. Results indicated that as river water quality deteriorates along a linear gradient its inflammatory potential increases. A second study on the Eerste River also indicated IL-6 inflammatory responses downstream of the upper reaches of the river (Pool and Magcwebeba 2009). The efficiency of pro-inflammatory responses measured as IL-6 production in mononuclear leukocytes in raw and treated water from three dams in the greater Pretoria region, South Africa, were determined by Adebayo et al. (2014). In two of the three water treatment facilities, treatment did reduce the IL-6 inflammatory responses. Interleukin-6 in RAW 264.7 cells exposed to sewage collected at various stages of a sewage treatment plant (Makene and Pool et al. 2015) and human whole blood cultures exposed to raw and final effluent from sewage treatment plants (Hendricks 2011) also indicated a reduced inflammatory response after treatment processes. These studies showed the usefulness of using IL-6 as a biomarker for determining the efficiency of waste water treatment to reduce the inflammatory potential of water.

The study on the Eerste River in South Africa, by Pool and Magcwebeba (2009) also assessed IFN-γ and IL-10 responses of whole blood cultures. Similar to the current study, in the Eerste River study, unstimulated whole blood cultures exposed to water samples produced no IFN-γ and in PHA-stimulated samples IFN-γ production was downregulated. However, in contrast to the present study IL-10 was also downregulated. Thus, while in the GSTP and GWRP samples there was a shift in the T_H1/T_H2 balance towards the T_H2 cells side, both T_H1 and T_H2 production of IFN-γ and IL-10 were supressed in the Eerste River water samples. No studies were found that assessed the stimulation or suppression of MIP1-β by raw and treated wastewater or which employed proteome profiles for screening water for immunotoxicological effects.

5.6 Conclusion

The ability of the GWRP to successfully eliminate pollutants with cytotoxic, neurotoxic and immuno-modulatory potential was previously determined using a small set of biomarkers (Faul et al. 2013). The current study explored a much larger set of immune system biomarkers to further validate the GWRP's effectiveness and to test the usefulness of utilising proteome profiling as a rapid and reliable screen for water quality. The results of the study confirmed the GWRPs ability to successfully eliminate pollutants that may have the ability to modulate immune systems in consumers of the product water. Although less sensitive, the proteome profile could potentially be employed as a reliable and rapid technique for determining a wide range of immune system proteins. The usefulness of the proteome profile as a quick screen for water quality assessment can further be explored. Due to the sensitivity of ELISAs it is recommended that where inflammatory responses are detected in reclaimed or other sources of potable water, selected ELISA's should be performed to better determine the magnitude of the immunomodulatory potential of such water.

5.7 Acknowledgements

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Chapter 6

Summary and Future Perspectives

With the world's population fast approaching eight billion, the pressure on its available resources are becoming increasingly severe. Water, being essential for life, is no exception. Apart from it becoming increasingly scarce, water is also under threat from the vast array of anthropogenic and natural pollutants that goes hand in hand with the increasing human population and its activities and resource requirements. The presence and effects of pollutants in rivers, oceans and other water sources have been demonstrated in numerous scientific journals. Severely polluted water can lead to mortality of organisms, but many water sources have low levels of contaminants that result in adverse effects at, for example, the physiological or biochemical level, without any obvious signs of distress. In the long term however, it can lead to significant biodiversity impacts as well as human health impacts. Examples include reproductive, immunological and neurological abnormalities.

Many pollutants have the potential to adversely affect endocrine systems and are referred to as endocrine disruptors or endocrine disrupting chemicals (EDCs). EDCs are often present in aquatic environments such as rivers due to pollutant presence in storm water runoff and the release of treated sewage into the environment.

During the last three decades toxicologists focussed strongly on EDC research. In Namibia however, very little toxicological research has been performed on water sources and almost no EDC related research has been conducted. With Windhoek, the capital city, reclaiming treated sewage for direct potable reuse for more than four decades, there is a definite need to assess the endocrine disrupting potential of the reclaimed water as well as other sources of potable water for Windhoek and the country as a whole.

The research conducted and presented in this thesis aimed to assess the endocrine disrupting potential of reclaimed treated sewage as well as surface water from nine water storage dams in the country of Namibia. The water was assessed for steroid hormone presence in the form of estradiol, estrone and testosterone. The water's cytotoxic, neurotoxic and immunotoxic potential were also determined by measuring lactate dehydrogenase (LDH) leakage from cells, acetylcholinesterase

(AChE) inhibition, and stimulation or suppression of cytokine production by cells exposed to the source water. Steroid hormones and cytokine (interleukin-6, interleukin-10, macrophage inhibitory protein-1β and interferon-γ) production were determined using enzyme-linked immune-sorbent assays, while cytotoxicity and neurotoxicity was determined with calorimetric assays.

For the first time ever, a proteome profile was performed on culture supernatants exposed to reclaimed treated sewage to screen for stimulation or suppression of the production of 36 different cytokines and chemokines as a broad indicator of immunomodulatory potential of the water.

The results of the research indicated that the reclamation process employed in Windhoek successfully removed contaminants with endocrine disrupting traits. All steroid hormones as well as cytotoxic, neurotoxic and immunomodulatory effects detected in raw and treated sewage were absent from the reclaimed water.

In terms of dam water, the results indicated that, with the exception of Goreangab and Swakoppoort dams, surface water contained in these dams, are generally of good quality with low to no endocrine disrupting potential. The Goreangab dam is however known to be polluted and the raw water is currently not being utilised for human consumption. The Swakoppoort dam contributes to the Windhoek water supply scheme and should thus be monitored.

This study indicated the usefulness of physiological biomarkers as relatively cheap and quick *in vitro* indicators of the endocrine disrupting potential of water sources. Specifically, it shows the potential use of proteome profiling for quick screening of water for immunomodulatory effects. This procedure may prove to be invaluable where treated sewage is reclaimed for direct potable re-use as is the case in Windhoek, Namibia. However, the *in vitro* indicators of toxicity do have their limitations and extrapolation of results to whole organism effects may be less reliable. This is mainly because the i*n vitro* cultures often behave differently from the *in vivo* systems.

Since potentially unsafe reclaimed treated sewage for direct potable reuse may result in adverse health effects, a holistic *in vivo* monitoring system for reclamation plants may need to be developed and/or implemented. Such a system will have to provide continuous monitoring options that provide rapid, cost-effective results. In Windhoek, the use of fish, for example *Oreochromis mossambicus*, or crabs, for example *Potamonautes perlatus*, may be considered as candidates for the *in vivo* studies. Similar biomarkers of cytotoxicity, neurotoxicity and immunotoxicity, as used in this study on blood cultures, may be employed without the need to sacrifice the test organisms. Future research should thus include the development of a set of *in vivo* biomarkers for reclamation plants.