The efficiency of drinking water treatment plants in removing immunotoxins

by

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae (M.Sc.) in Immunology in the Department of Medical Bioscience, University of the Western Cape, South Africa.

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Declaration

I declare that the thesis entitled 'The efficiency of drinking water treatment plants in removing immunotoxins' is my work, that it has not been submitted for any degree or examination at any other University, and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Cheryl Malan

Signed: _____

Date: _____

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List of abbreviations

%	percent
°C	degrees Celsius
µl	microlitre
Ab	antibodies
ACTH	adrenocorticotropic hormone
Ag	antigen
ANOVA	one way analysis of variance
APC	antigen presenting cell
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
Cd	cadmium
CD4	helper T cell
CD8	cytotoxic T cell
cGMP	cyclic guanosine monophosphate
CRH	corticotropin-releasing hormone
DAEC	diffusely adhering *E. coli*
DBPs	disinfection by-products
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAEC	enteroaggregative *E. coli*
EDCs	endocrine disrupting chemicals
EHEC	enterohemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
ELISA	Enzyme Linked Immunosorbant Assay
EPEC	enteropathegenic *E. coli*
ER	estrogen receptor
ETEC	enterotoxigenic *E. coli*

EU	European Union
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
H_2SO_4	sulphuric acid
HAA	haloacetic acids
HPT	hypothalamus-pituitary-thyroid axis
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRF	interferon regulating factors
LDH	lactate dehydrogenase
L	litre
LH	leutinizing hormone
LPS	lipopolysaccharide
M	Molar
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MASP	MBL-associated serine protease
MBL	mannan-binding lectin
mg/ml	micrograms per millilitre
mg/ml	milligram per millilitre
MHC	major histocompatibility complex
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
ng/l	nanogram per litre
NK	natural killer
NO	nitric oxide
NO_3	nitrate
OD	optical density

PAMPs	pathogen-associated molecular patterns
PCB	polychlorinated biphenyls
pg/ml	picogram per millilitre
PHA	phytohemagglutinin
PRRs	pattern-recognition receptors
R^2	correlation co-efficient
RPMI-1640	Roswell Park Memorial Institute 1640
SANS	South African National Standards
SD	standard deviation
SDS	sodium dodecyl sulphate
SPE	solid phase extraction
T3	triiodothyronine
T4	tetraiodothyronine
TAP	transporter associated with presentation
TBTC	tributyltin chloride
Tc	T cytotoxic cell
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TGF	transforming growth factors
Th 1	T-helper type 1 cells
Th 2	T-helper type 2 cells
THM	trihalomethanes
TLRs	toll-like receptors
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TRH	thyroid releasing hormone
TSH	thyroid stimulating hormone
USEPA	United States Environmental Protection Agency
WBC	whole blood cultures
WHO	World Health Organisation

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ABSTRACT

The provision of an adequate and safe drinking water supply is a high priority issue for safeguarding the health and well-being of the human population. In Africa however, particularly in the rural areas and smaller towns, the inadequate and/or dysfunctional treatment of drinking water, the lack of sanitary infrastructures and poor hygiene practices leaves many people with poor water quality and an increased risk for water related infections or diseases. The sources of contamination of drinking water include contaminants originating from surface and groundwater, and contaminants used or formed during the treatment and distribution of drinking water. Exposure to these contaminants may modulate the endocrine and immune system resulting in physiological system dysfunction and the development of disease.

The aim of this study was to evaluate the effectiveness of water treatment processes of two drinking water plants to remove immunotoxins and steroid hormones. Raw and treated drinking water was screened for effects on inflammatory activity using the biomarker IL-6, humoral immunity using the biomarker IL-10 and cell mediated immunity using the biomarker IFN-γ. *In vitro* human whole blood culture assays were used in order to elucidate potential immunotoxicity.

Raw and drinking water samples were collected seasonally from the M and Bar treatment plants located in the same catchment system on the Vaal River, Gauteng. The chemical, physical and organoleptic parameters were determined by Rand Water Analytical Services using South African certified protocols. Water samples were assessed for steroid hormones using quantitative testosterone and estrone ELISA's.

The chemical analysis showed that the Bar-plant had higher values for calcium, hardness, magnesium, potassium, sodium, sulphur, chloride, nitrate and sulphate in comparison to the M-plant.

The quantitative steroid hormone ELISA's showed that the raw water from the Bar-plant contained higher levels of testosterone and estrone than the drinking water. The drinking water from the M-plant however, showed higher levels of testosterone than the raw water.

Cytotoxicity was determined by using an LDH assay. The raw and drinking water samples were not cytotoxic. Whole blood culture assays for immunotoxicity were run in the presence and absence of stimuli (LPS and PHA) in addition to the raw and drinking water. The addition of drinking water samples from both the M and Bar-plants to unstimulated cultures, had no significant effect on IL-6, IL-10 and IFN-γ secretion for all the seasons compared to the negative control cultures. The addition of raw water from the Bar-plant to unstimulated cultures induced higher IL-6 secretion during autumn and winter, higher IL-10 secretion during winter and had no effect on IFN-γ secretion compared to the negative control. The addition of raw water from the M-plant to unstimulated WBC had no significant effect on IL-6, IL-10 and IFN-γ secretion. The addition of raw water from both the M and Bar-plants to stimulated WBC induced higher IL-6 secretion during spring and winter compared to the positive control. Additionally, the raw water from the Bar-plant inhibited IL-6 secretion during summer in comparison to the positive control. The addition of raw and drinking water from both plants to stimulated WBC induced higher IL-10 than the control cultures during spring, autumn and winter. The addition of raw water to stimulated WBC induced higher IFN-γ secretion during all the seasons compared to the control.

This study shows that due to the location of the Bar-plant, anthropogenic activities such as mining, wastewater treatment and general urbanisation influenced the chemical quality of the raw water. Also, the raw from the Bar-plant contained high levels of testosterone and estrone. What is encouraging to see is that the use of GAC adsorption as part of the treatment processes in the plant effectively removes these steroid hormones. Additionally, the immunotoxic effects observed seasonally by raw water and drinking water from both plants may be due to the presence or absence of microbial pathogens and toxic chemicals.

We suggest that urgent steps be taken to monitor all water resources intended for domestic, agricultural and recreational use by screening for immunotoxins. To prevent the adverse effects of associated with immunomodulation and endocrine modulation, it is important to implement assays such as steroid hormone ELISA's and whole blood cultures. Steroid hormone ELISA's can successfully quantitate estrone and testosterone levels in water. Whole blood cultures are sensitive in detecting immunotoxins in water. The relative ease of implementing these assays is a definite advantage.

CHAPTER 1: General Introduction

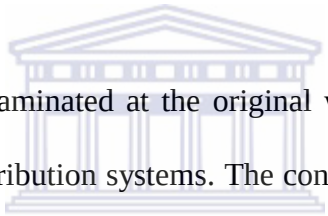
1.1 Background

Water is an important natural resource and without it life on earth would seize to exist (Bates, 2000). A safe and sufficient water supply is a basic human requirement and is therefore essential to the health and well-being of the human population (Bordalo and Savva-Bordalo, 2007). The Constitution of South Africa, Act No. 108 of 1996 (RSA, 1996) states that everyone has the right to have access to an environment that is not harmful to their health or well-being. This includes access to a continuous supply of clean, safe drinking water.

The provision of a safe water supply is one of the major concerns for governments throughout the world. In South Africa however, particularly in the rural areas and smaller towns, citizens do not have access to an adequate and safe drinking water supply due to water quality failures and lack of water quality monitoring (DWAF, 2005). The pollution of water resources is a global problem because of its negative impact on water quality and far reaching consequences to plant, animal and human life.

The ability to produce adequate and safe drinking water is the chief factor that contributed to the decline in mortality and morbidity rates in developed countries. In 1992, the World Health Organisation (WHO) reported that almost half the population in developing countries suffered from health-related issues associated with water contaminated with microbial pathogens. As time progressed, the identification of potential drinking water contaminants shifted from microbiological to chemical contamination as our knowledge base increased on the hazards of human exposure to chemical compounds (Calderon, 2000).

A decline in surface water quality can be due to many sources and these can be categorized based on their origin (Ritter et al., 2002). The contaminant may enter through non-point source pollution or point source pollution (Calderon, 2000). In non-point source pollution contaminants originate from poorly defined, diffuse sources that typically occur over a large geographical area. Examples of sources in this category include runoff from agricultural activities such as pesticide and fertilizer application as well as storm water and urban runoff (Ritter et al., 2002). In point source pollution, the contaminant may enter the waterway from a single defined location (Ritter et al., 2002) Examples of point source pollution include discharges from sewage treatment plants and municipal storm sewage systems as well as industrial and mining effluent (Ritter et al., 2002).



Drinking water can become contaminated at the original water source, during treatment or through materials used in the distribution systems. The contaminants in drinking water range from microbiological, natural substances that leach from soil and rock, runoff from agricultural activities, effluent discharges from sewage treatment or industrial plants, disinfectants and their by products as well as corrosion or leaching from distribution materials (Calderon, 2000).

It is therefore of utmost importance to monitor water quality from the source to the tap. The National Water Act of South Africa (DWAF, 1998), the South African National Standard (SANS) 241 Drinking Water Specification (SANS 241, 2006), the Drinking Water Quality Framework of South Africa (DWAF, 2005) and the South African Water Quality Guidelines (DWAF, 1996) require drinking water supplies to be monitored and routinely screened for various physical, organoleptic, chemical and microbiological parameters.

Another problem is the emerging issue of endocrine disruptors in water. Endocrine disrupting compounds (EDCs) interfere with the natural action of the endocrine system by mimicking or inhibiting the synthesis, distribution and metabolism of natural hormones. EDCs have been linked to variety of adverse effects in wildlife and humans. The concern is that EDCs have been discovered in surface, ground and even drinking waters (Fawell and Chipman, 2000; Peterson et al., 2001; Benotti et al., 2009). It is therefore important to monitor various hormonal systems for potential impacts of EDCs. There is a need to routinely screen for EDCs in water using simple, rapid techniques. These techniques are currently not readily available, but should be a priority due to the increase in problems adversely affecting normal endocrine function and endocrine activity in water.

Direct contact or consumption of the polluted water can be associated with waterborne diseases, cancers, endocrine disruption and immunotoxicity. The concern is that certain water pollutants are known for their immunotoxic properties and ability to affect specific immune pathways. Very little work however, has been done on the African continent to evaluate the extent of this problem.

1.2 Research problem

The immune system is an interactive network of various cell types, cell products, tissues and organs. Immune system responses fall into two categories, namely the innate and the adaptive responses which have mechanisms that protect the host against invading foreign pathogenic substances. Cytokines, with the aid of other molecular components play an essential role in the induction and regulation of these mechanisms, thus maintaining homeostasis. Exposure to

contaminants via drinking water can potentially cause chronic health effects which in turn modulate the functions of cytokines and other mediators of the immune system, resulting in immune dysfunction and the development of disease in humans and animals.

1.3 Aim of study

The aim of this study is to evaluate the effectiveness of water treatment processes at two drinking water treatment plants in Gauteng to remove steroid hormones and immunotoxins. Gauteng was selected for this study as it is the main industrial centre of South Africa and also due to its high population density. These two factors are key problems resulting in pollution of aquatic environments.

1.4 Study objectives

The first objective of this study was to do a seasonal study on the chemical quality of environmental and drinking water collected from two drinking water treatment plants in Gauteng.

The second objective of this study was to do a seasonal study to assess environmental and drinking water for steroid endocrine disruptors namely estrone and testosterone. These two hormones were selected as representatives of the estrogenic and androgenic hormones.

The third objective of this study was to do a seasonal study to assess the impact of environmental water on specific immune pathways.

The fourth objective of this study was to do a seasonal study to assess the impact of drinking water on specific immune pathways.

1.5 Site description

An agreement was made with the water supplier that the names of the plants would be abbreviated. The two drinking water treatment (DWT) plants namely the M and Bar-plants, are located in the same catchment system on the Vaal River. The M-plant is situated upstream from the Bar-plant. The M-plant therefore receives its raw water from are relatively "clean" site while the Bar-plant receives raw water that may be contaminated due to human activity. The sampling points represent the raw water and treated water from each treatment plant. The DWT plants employ conventional water treatment processes, such as coagulation, flocculation, sedimentation, filtration and disinfection. Coagulation is a chemical process in which coagulant salts and polymers are added to water to destabilise colloidal particles and allow them to stick together (Vulliet et al., 2009). After mixing, the water enters a flocculation basin where the destabilised particles collide and aggregate into large clusters called floc (Amuda and Amoo, 2007). This is followed by sedimentation, a solid-liquid separation process, in which the floc settles on the bottom of the basin under the force of gravity (USEPA, 1999). The clear water then passes through different filters containing sand and/or granular activated carbon (GAC) to retain fine particles (Vulliet et al., 2009). The water is then exposed to ultra violet (UV) light for primary disinfection which inactivates pathogenic microorganisms (LeChavallier and Au, 2004). This is followed by the addition of chlorine for secondary disinfection. Secondary disinfection processes maintain the water quality achieved at the treatment plant throughout the distribution system (LeChevallier and Au, 2004).

The Bar-plant utilises all the above mentioned treatment processes. Figure 1.1 illustrates a schematic diagram of the treatment process in the Bar-plant. The M-plant however, makes use of all the above processes apart from GAC adsorption and UV disinfection. Figure 1.2 illustrates a schematic diagram of the treatment processes in the M-plant.

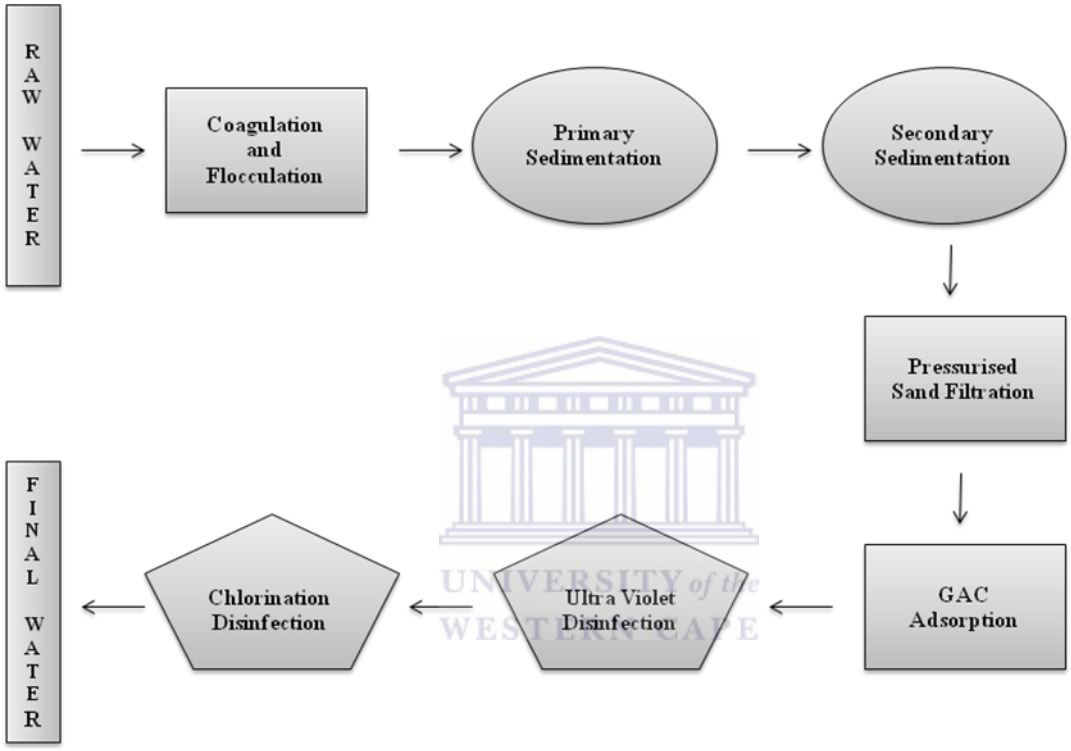


Figure 1.1: Schematic diagram of treatment processes in the Bar-plant.

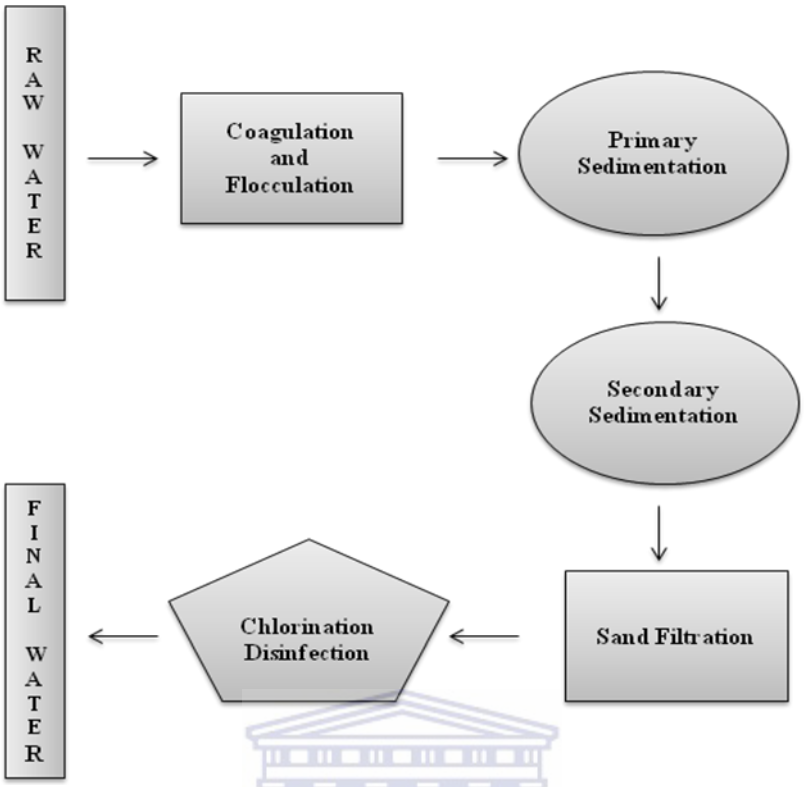


Figure 1.2: Schematic diagram of treatment processes in the M-plant.

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CHAPTER 2: Drinking water contaminants and their impact on health

2.1 Introduction

Drinking water is mainly derived from two sources, namely ground and surface water but may also be obtained from the sea through desalination (Bates, 2000). Surface water includes rivers, reservoirs, natural lakes and streams. Groundwater is generally sourced from aquifers (USEPA, 1999). In nature, all water sources contain natural and anthropogenic contaminants. Naturally occurring contaminants are primarily formed by inorganic compounds derived from natural mineral formations and a few natural organic compounds (Calderon, 2000). Contaminants from anthropogenic activities enter surface and groundwater through point and non-point sources of pollution (Ritter et al., 2002). Contaminants may also arise from chemicals used in treatment processes or through the leaching of materials used in the distribution system (Calderon, 2000; Van Leeuwen, 2000). The safety of drinking water is established by national standards and international guidelines, which specify acceptable concentrations and limits for various microbiological, chemical and physical parameters (Davies and Mazumder, 2003). The standards and guidelines relevant to this project are the South African National Standard (SANS) 241 (SANS 241, 2006) and the WHO Guidelines for Drinking Water Quality (WHO, 1993). These documents are revised on a regular basis as determined by ongoing research.

2.2 Health effects of contaminants in drinking water

Toxic levels of pollutants in drinking water may either cause acute or chronic health effects in consumers (USEPA, 1999). Acute effects occur within hours or days after the consumer

ingests a chemical. This normally follows a single exposure to abnormally high amounts of any chemical, such as a chemical spill. Persons with weakened immune systems due to HIV/AIDS and chemotherapy are especially at risk when exposed to high enough levels of contaminants. The health effects include nausea, vomiting, lung irritation, dizziness, skin irritations and possible fatalities (USEPA, 1999). Chronic effects however, occur long after repeated exposure to small amounts of chemical. These adverse effects have the ability to target organs and physiological systems. The health effects include different cancers, damage to the liver or kidneys, nervous and reproductive system disorders as well as damage to the immune system (USEPA, 1999). Toxic pollutants have the ability to exert their action by modulating the endocrine mechanism that regulates the organ or physiological mechanism.

2.3 Microbial contaminants in drinking water

2.3.1 *Cryptosporidium*

Cryptosporidium parvum is an enteric protozoan that has been associated with contaminated food and drinking water supplies (Keegan et al., 2008). These parasites are recognized as health hazards because they cause severe gastroenteritis in humans and can be potentially life threatening to immunocompromised individuals (infection with HIV/AIDS, cancer and transplant patients using immunosuppressive drugs). Symptoms could include diarrhoea, nausea, vomiting and abdominal cramping (USEPA, 1999). *Cryptosporidium* may enter water supplies through faecal contamination from human or animal sources. The major risk factors for infection include swallowing water while swimming, drinking untreated tap water and contact with recreational water (Carmena et al., 2007). *Cryptosporidium* survives in the environment as an oocyst and is highly resistant to chlorine disinfection during water

treatment (Carmena et al., 2007). The South African water quality guideline for this protozoan parasite is <1 oocyst/10 L (SANS 241, 2006).

2.3.2 Giardia

Giardia lamblia is a protozoan parasite and has been identified as an important cause of waterborne disease. This parasite is the causative agent for gastroenteritis in humans and its cysts are transmitted via the faecal-oral route with the consumption contaminated drinking water (Slifko et al., 2000). *Giardia* may enter waterways through human and animal faecal waste. *Giardia* cysts are ubiquitous in the environment and remain viable for several months under appropriate environmental conditions (Smith et al., 1995). Symptoms include diarrhoea, abdominal cramps, vomiting, bloating and flatulence (Plutzer et al., 2010). In addition, *Giardia* remains a threat to human health due to its high resistance to disinfectants used in conventional drinking water treatment plants. The South African water quality guideline for this protozoan parasite is <1 cyst/10 L (SANS 241, 2006).

2.3.3 Enteric viruses

The enteric viruses such as noroviruses, rotaviruses, astroviruses and adenoviruses are responsible for viral gastroenteritis in humans (Wilhelmi et al., 2003). These viruses are transmitted via the faecal-oral route from contaminated drinking water. The rotavirus is the leading cause of severe dehydrating diarrhoea in infants and young children (Albert et al., 1999; McIver et al., 2001; Bereciartu et al., 2002). This virus replicates in the epithelial cells of the villus in the small intestine, causing cell death and subsequent malabsorption and osmotic diarrhoea (Prescott et al., 2005). The enteric viruses are persistent in the environment

due to their high resistance to disinfectants and their ability to adapt to physical conditions (Payment, 1999; Gulati et al., 2001; Sair et al., 2002). The contamination of drinking water may be caused by sewage or surface water, the breakdown of the treatment process, a cross connection between the water supply and an irrigation system or leakages in the distribution system (Hafliger et al., 2000). Recent studies by Hafliger et al. (2000) and Werber et al. (2009) describe waterborne outbreaks of viral gastroenteritis associated the consumption of sewage contaminated drinking water due to defective waste and municipal water systems. The South African water quality guideline for these viruses are <1 count/100 ml (SANS 241, 2006).

2.3.4 Faecal coliforms

Faecal coliform bacteria are naturally present among the intestinal flora of humans and other warm blooded animals. *Escherichia coli* forms part of the faecal coliform group of bacteria and is generally used as an indicator of microbiological water quality and faecal contamination of water (Ishii and Sadowsky, 2008). These organisms have the ability to grow at elevated temperatures and enter the environment as faecal waste products (Rompre et al., 2002). *Escherichia coli* bacteria are commonly associated with diarrheal disease and thus far, six strains have been identified (Prescott et al., 2005). These strains include the enterotoxigenic *E. coli* (ETEC), the enteropathegenic *E. coli* (EPEC), the enteroinvasive *E. coli* (EIEC), the enterohemorrhagic *E. coli* (EHEC), the enteroaggregative *E. coli* (EAEC) and the diffusely adhering *E. coli* (DAEC) (Prescott et al., 2005). These bacteria are transmitted via the faecal-oral route by contaminated or poorly treated drinking water supplies (McFeters et al., 1986; Rompré et al., 2002). Contamination may also be caused by a

cross connection between contaminated water into the potable water supply (Clark et al., 1996) or regrowth in the distribution system (LeChevallier, 1990).

The ETEC strains attach to the epithelial cells and produce either a heat-stable or a heat-labile enterotoxin. The toxins then stimulate an increase in the production of cGMP and cAMP respectively, which in turn causes the hypersecretion of electrolytes into the intestinal lumen thus causing a watery diarrhoea (Bennett, 1998; Prescott et al., 2005). EHEC strains produce the Shiga-like toxin, which destroys the intestinal epithelium causing hemorrhagic colitis and bloody diarrhoea. This toxin has also been associated with haemolytic uremic syndrome. The EHEC serotype *E. coli* 0157:H7 has caused many outbreaks of waterborne disease. Studies by Swerdlow et al. (1992) and Hrudey et al. (2003) report waterborne diseases associated with drinking unchlorinated municipal water contaminated with *E. coli* 0157:H7 and drinking faecally contaminated well water due to a defective water treatment system after flooding. The EPEC strains are known to be the leading cause of watery diarrhoea in infants in developing countries (Trabulsi et al., 2002). The South African water quality guideline for *E. coli* and faecal coliforms are <1 count/100 ml (SANS 241, 2006).

2.3.5 Cholera

Cholera is frequently associated with inadequate sanitation and poor hygiene practices (Ashbolt, 2004). Cholera is caused by the gram-negative *Vibrio cholera* bacterium and is frequently described as a water borne disease due to its association with water (Sack et al., 2004). Infection is usually acquired through the ingestion of water contaminated with faecal waste from already infected carriers. This disease is characterised by watery diarrhoea (a rice-water appearance) which rapidly leads to severe dehydration (Prescott et al., 2005). The

bacterium attaches to the epithelial cells of the small intestine where they produce an enterotoxin, choleragen (Prescott et al., 2005). The toxin is composed of an enzymatic A-subunit and a receptor binding B-subunit. Upon B-subunit binding to the epithelial cells, the A-subunit enters the epithelial cells causing the release of cAMP. This results in interferences with the absorption of sodium and causes chloride and water secretion (Sack et al., 2004; Prescott et al., 2005).

2.4 Pesticide contamination in drinking water

Pesticides are a group of natural or artificially synthesized substances that are deliberately used to control pests and improve agricultural production (Ormad et al., 2008). The pollution of source water by pesticides can occur through point or diffuse sources (Holvoet et al., 2007). Pesticides may contaminate drinking water sources through agricultural and urban run-off, direct application to control insects and vegetation, domestic usage and leaching from pesticide wastes (Ikehata and Eldin, 2005). The different classes of pesticides found to contaminate both surface and ground water include the organochlorine pesticides, the organophosphorus pesticides and the triazines.

Organochlorine pesticides are persistent organic pollutants in surface water (Zhou et al., 2006). These pesticides have the ability to resist biodegradation and can therefore be recycled through the food chain, biomagnify and ultimately affect humans via drinking water and fish consumption (Golfinopoulos et al., 2003; Turgut, 2003; Sankararamakrishnan et al., 2005; Zhou et al., 2006). Currently their use has been prohibited in many countries due to persistence, bioaccumulation and toxic impact on human health (Turgut, 2003). The latter is attributed to the lipophilic nature of these compounds, which enables them to penetrate cell

membranes with relative ease (Turgut, 2003). However, South Africa still uses the organochlorine insecticide DDT for malaria control (Dalvie et al., 2004; Bouwman et al., 2006).

Organophosphorus (OP) pesticides are degraded quite rapidly in the environment. They are currently used as substitutes for organochlorine pesticides (Ballesteros and Parrado, 2004) but have been found to be toxic in vertebrates (Chambers et al., 2001). Acute toxicity of OPs occurs through the inhibition of acetylcholinesterase which alters the cholinergic signalling pathway by disrupting the breakdown of neurotransmitter acetylcholine (Pope et al., 2005). The excessive acetylcholine accumulates in the synaptic cleft, which ultimately causes neuromuscular paralysis (Seeley et al., 2003; Pope et al., 2005).

The pesticide atrazine, belonging to the chemical group triazines, is frequently detected in both surface and drinking water (Rodriguez-Mozaz et al., 2004; Hua et al., 2006). The triazines are herbicides that are used to control weeds (Nagaraju and Huang, 2007). They are characterised by their mobility and solubility in water and strong adsorption onto soil (Shen and Lee, 2003). These herbicides and their degradation products are known for their toxic endocrine disruption effects (Nagaraju and Huang, 2007). Studies have shown that atrazine inhibits androgen-mediated development whilst at the same time producing estrogen-like effects in exposed individuals (Eldridge and Wetzel, 1999; Matsushita et al., 2006). It has also been reported that exposure to atrazine modulates aromatase activity (Benachour et al., 2007; Fan et al., 2007), reduces semen quality and fertility in men (Swan et al., 2003) as well as increases the incidence of breast cancer in women (Kettles et al., 1997). The cut off level for atrazine in drinking water as set by the US Environmental Protection Agency is 0.003 mg/L (USEPA, 2003). This contaminant is not yet regulated in South African drinking water.

2.5 Inorganic chemical contaminants in drinking water

Inorganic pollutants constitute a large proportion of chemical contaminants in drinking water. They are present as a consequence of natural processes as well as anthropogenic activities. Copper, arsenic, nitrate, lead and cadmium are some of the most important inorganic constituents in drinking water. These chemicals have the ability to induce a variety of adverse effects in humans.

2.5.1 Copper

Copper (Cu) is an important trace element for the human body and is widely dispersed throughout the environment (Sidhu, 1995). This element is found in minimal amounts in an array of cells and tissues (Turnlund, 1998). Copper acts as a cofactor for both structural and catalytic properties of many enzymes including cytochrome c oxidase, tyrosinase and p-hydroxyphenyl pyruvate hydrolase (Uauy et al., 1998). These enzyme systems are vital in biological processes required for growth, development and maintenance. The common sources for contamination in drinking water are corrosion of household plumbing systems and erosion of natural deposits. Refer to Table 2.1 for the South African water quality guidelines for copper. The adverse health effects identified by Knobelach et al. (1998) with regard to drinking water contaminated with copper include acute gastrointestinal disturbances such as abdominal pain, nausea, vomiting, diarrhoea, dizziness and headaches. Chronic health effects include liver or kidney damage.

2.5.2 Arsenic

Arsenic (As), a heavy metal, is widely distributed in the earth's crust and is the 20th most abundant natural element (Duker et al., 2005). It occurs in both inorganic and organic forms in the environment. Arsenic is considered a human carcinogen and is associated with skin, lung, kidney and bladder cancer (ATSDR, 2003). Human exposure to arsenic in drinking water occurs orally but there have been reported cases of dermal exposure by bathing in contaminated water. Chronic arsenic toxicity presents as pigmentation changes on the trunk and extremeties as well as keratosis on the palms and soles (Kakkar and Jaffery, 2005). Arsenic contamination of drinking water is through leaching from natural geological sources (Ratnaike, 2003). Refer to Table 1.1 for the South African water quality guidelines for arsenic.

Arsenic is also known as an immunotoxicant, exhibiting its effects on a variety of immune cells and responses. Some of the effects include suppression of delayed hypersensitivity reactions, the modulation of co-receptor expression and release of lymphokines, reducing mitogen activated T cell proliferation and increasing free intracellular Ca^{2+} (Galicia et al., 2003; Goytia-Acevedo et al., 2003). It was also reported that low concentrations of inorganic arsenic decreased chemotactic migration and adhesion of splenic macrophages (Bishayi and Sengupta, 2003).

2.5.3 Nitrate

Nitrate (NO_3) is ubiquitous in the environment (Sadeq et al., 2008). The presence of nitrate in drinking water comes from both natural and man-made sources such as soil leaching, waste

waters and agricultural and urban run-off (Fields, 2004; Gatseva and Argirova, 2008). Refer to Table 1.1 for the South African water quality guidelines for nitrate. Toxic effects to humans occur when nitrate is endogenously reduced to nitrite leading to the formation of methemoglobin, a derivative of haemoglobin. Methemoglobin does not bind oxygen because the ferrous iron in haemoglobin is oxidised to the ferric form resulting in a condition known as methemoglobinemia. This condition, 'baby blue syndrome', affects infants younger than 6 months and is characterised by a bluish skin colour and chocolate coloured blood (Knobeloch et al., 2000; Fewtrell, 2004; Sadeq et al., 2008).

2.5.4 Lead

Lead (Pb), a naturally occurring element, is a blush gray heavy metal with considerable importance as an environmental pollutant (ATSDR, 2005). This metal has been widely used by humans for thousands of years and its toxic effects have been acknowledged for several centuries (Hu et al., 2010). The lead source in drinking water is more often than not lead-bearing plumbing materials. Lead release into drinking water is a direct result of corrosion from lead soldered joints connected with copper lines in household plumbing (Subramaniam and Cooner, 1991; Isaac et al., 1997; Korshin et al., 2000). Toxic levels of lead can cause pathophysiological changes to several organ systems such as renal, central nervous, hematopoietic, reproductive and immune systems (Goyer, 1986).

Studies by Heo et al. (1996, 1998) reported lead treatment *in vitro* and *in vivo* enhanced the development of Th 2 cells whilst at the same time inhibiting that of Th 1 cells. This is evidenced by an increase in the production of Th 2 cytokine interleukin-4 and a decrease in the production of Th 1 cytokine interferon gamma. It has also been reported that lead down-

regulates the cell mediated immune response in mice, decreasing resistance to intracellular pathogens (Kishikawa et al., 1997). In infants and young children, lead toxicity could lead to impaired cognitive function and behaviour disorders (Jarup, 2003). Refer to Table 1.1 for the South African water quality guidelines for lead.

2.5.5 Cadmium

Cadmium (Cd) is an important industrial metal (Kaličanin, 2009) and environmental pollutant (Castro-González and Méndez-Armenta, 2008). Small amounts of cadmium enter the environment from natural weathering of mineral deposits however, most is released by anthropogenic activities such as mining, smelting operations and the application of phosphate fertilizers (Leffel et al., 2003; Kaličanin, 2009). Cadmium contaminates drinking water sources through industrial emissions, agricultural runoff and soil leaching (Leffel et al., 2003). Cadmium is known to be toxic in humans at very low levels (Kaličanin, 2009). Human exposure can result from consumption of cadmium contaminated food and drinking water. Upon exposure, cadium is transported by the blood and distributed to the liver and kidney. These organs are the principal sites of long-term storage in the body (Goyer and Clarsksom, 2001). cadmium has a long biological half-life (Castro-González and Méndez-Armenta, 2008) and chronic exposure may result in immunotoxicity, renal, pulmonary, hepatic, skeletal, and reproductive adverse effects as well as various types of cancers (Kaličanin, 2009). Refer to Table 1.1 for the South African water quality guidelines for cadmium.

2.6 Disinfection by-product contamination in drinking water

Disinfectants react with naturally occurring organic matter or inorganic substances during the production of drinking water to form chemical compounds known as disinfection by-products (DBPs) (Richardson et al., 2007; Legay et al., 2010). Amongst other DBPs, the trihalomethanes and haloacetic acids have been found in the highest concentrations in treated drinking water (Richardson, 2003).

The trihalomethanes (THM) were the first class of disinfection by-products to be identified in drinking water (Rook, 1974). THM include four species namely, chloroform, bromoform, bromodichloromethane and chlorodibromomethane (Richardson, 2007). The South African limit for total THM in drinking water is <200 µg/L. These compounds are a group of volatile organic compounds formed when chlorine reacts with humic and fulvic acids. Chronic health effects in humans include adverse birth outcomes as well as bladder and colon cancer (King and Marrett, 1996; King et al., 2000; Wright et al., 2003; Wright et al., 2004).

Haloacetic acids (HAA), a major class of disinfection by-products, are widespread in drinking waters. The five common HAA compounds include bromoacetic acid, dibromoacetic acid, chloroacetic acid, dichloroacetic acid and trichloroacetic acid (Richardson, 2007). Currently there is no South African limit for HAA. However, the US Environmental Protection Agency regulates these HAA compounds by a cut off level of 60 µg/L in drinking water (USEPA, 2003). The chemistry of HAA indicates that they are non volatile, highly hydrophilic, ionic compounds (Urbansky, 2000). They are usually formed at high concentrations with the disinfectant chlorine. The human health concern is attributed to

their widespread distribution in drinking water and potential carcinogenic properties (Graves et al., 2002; Malliarou et al., 2005; Scott et al., 2005).

2.7 Endocrine disrupters

In the last 40 years, much attention has been focused on a group of chemicals that have the potential to alter the endocrine system of wildlife and humans (Mendes, 2002). These chemicals are collectively termed the endocrine disrupter chemicals (EDCs). The US Environmental Protection Agency (1997) has defined 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". Various types of natural and synthetic chemical compounds have been recognised as potential EDCs.

Mechanisms in which EDCs can interfere with endocrine function include:

- Mimicking the effect of endogenous hormones by binding and activating hormone receptors;
- Antagonizing the effect of endogenous hormones by binding and inhibiting hormone receptors;
- Disrupting the pattern of synthesis and metabolism of endogenous hormones;
- Disrupting the synthesis of hormone receptors (Mendes, 2002; Soto et al., 2006).

A variety of adverse health effects associated with EDCs have been reported in recent years (Sonnenschein and Soto, 1998; Mendes, 2002; Kim et al., 2007) for both humans and wildlife. For the most part, EDCs affect reproductive development and function due to its

regulation by an array of hormonal signalling pathways (Mills and Chichester, 2005). However, literature has shown that these compounds also have the ability to modulate the immune system (Mendes, 2002). The activation of immune and immune accessory cells directs cytokine production. These cytokines have the ability to stimulate or inhibit the production and secretion of hormones from the hypothalamus pituitary adrenal axis and the hypothalamus pituitary ovarian axis. In turn, cytokine synthesis and secretion can be affected by adrenal and ovarian hormones creating a negative feedback effect (Chryssikopoulos, 1997).

Mammalian hormonal systems that need to be assessed for the impact of EDCs include the hypothalamic-pituitary-gonadal axis, the hypothalamic-pituitary-adrenal axis, hypothalamic-pituitary-thyroid axis and the immune system.

2.7.1 Hypothalamic-pituitary-gonadal (HPG) axis

The HPG axis regulates the reproductive processes in males and females. This axis involves the gonadotropin releasing hormone (GnRH) neurons in the hypothalamus, gonadotropes in the anterior pituitary gland and the somatic cells of the gonads (theca and granulosa cells in the ovary, Leydig and Sertoli cells in the testes). The decapeptide GnRH is synthesized and secreted in brief pulses from the terminals of GnRH neurons into the hypothalamohypophysial portal system (Clarke and Cummins, 1982; Kimura and Funabashi, 1998; Terasawa, 1998). This portal system delivers GnRH to the gonadotropic cells of the anterior pituitary gland. In response to GnRH stimulation, the anterior pituitary synthesizes and secretes gonadotropins, leutinizing hormone (LH) and follicle stimulating hormone (FSH).

In the testes, LH acts on Leydig cells and promotes the synthesis and secretion of testosterone. Testosterone has a negative feedback effect on the hypothalamus and the anterior pituitary to reduce GnRH, LH and FSH secretion. FSH acts on Sertoli cells and stimulates the secretion of inhibin B which has negative feedback effect on the pituitary gland by specifically inhibiting FSH secretion (Anawalt et al., 1996; McLachlan et al., 2002).

In the ovaries, the secretion of FSH increases before ovulation, stimulating the development of follicles and the secretion of estrogen. The estrogen causes proliferation of the endometrium and increase LH secretion by the hypothalamus. The result is therefore an LH surge prior to ovulation. This LH surge allows maturation of follicles and subsequent ovulation. The formation of a corpus luteum follows with the secretion of progesterone and some estrogen. A negative feedback loop exists on FSH and LH secretion from the pituitary gland (Seeley et al., 2003).

Adverse human health effects in males due to EDC's remain highly controversial (Foster et al., 2004). Speculated effects include changes in sperm quality and quantity as well as an increased incidence in prostate and testicular cancers (Mendes, 2002). Disruptions affecting females could result in secondary amenorrhea in women. This condition may result from tumours in the pituitary gland, which decreases LH and FSH levels or autoimmune diseases that target the ovary (Seeley et al., 2003). Breast cancer may also develop due to altered regulation of normal breast development (Helderman and Ellis, 2006).

2.7.1 Hypothalamic-pituitary-thyroid (HPT) axis

The HPT axis forms part of the endocrine system and is responsible for the regulation of metabolism in some tissues, and growth and maturation in others. The thyroid gland is one of the largest endocrine glands. It is an essential component for normal metabolic function (Seeley et al., 2003). Thyroid releasing hormone (TRH) is secreted from hypothalamic neurons into the blood. TRH enters the hypothalamohypophysial portal system and acts on thyrotropes in the anterior pituitary gland to regulate the synthesis and secretion of thyroid stimulating hormone (TSH) (WHO, 2002). TSH passes to the thyroid gland via the general circulation and stimulates synthesis and secretion of the thyroid hormones, triiodothyronine (T3) and tetraiodothyronine (T4). Circulating T3 and T4 have a negative feedback effect on the hypothalamus and anterior pituitary by inhibiting TRH and TSH. Therefore, as T3 and T4 levels increase, they inhibit TRH and TSH secretion and vice versa (WHO, 2002; Seeley et al., 2003).

Thyroid dysfunction due to autoimmune disease, drugs and diseases of the hypothalamus and pituitary gland can lead to reduced thyroid hormone levels resulting in hypothyroidism (Franklyn, 2005). Overstimulation of thyroid hormones results in hyperthyroidism, which could be caused by the autoimmune thyroid disease, Graves' disease (Ashton, 2005). A study by Ghisari and Bonefeld-Jorgensen (2005) investigated the effects of different EDCs (bisphenol A, iprodion, nonylphenol) on the function of thyroid hormone in rat pituitary GH3 cells. The results showed disrupted thyroid hormone activity by these EDCs and the authors concluded that these EDCs have the potential to increase the risk of thyroid hormone disruption and have adverse effects on cognitive function. Crisp et al. (1998) also observed hypothyroidism in rodents after exposure to PCB, TCDD and chlorinated pesticides.

2.7.2 Hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis regulates growth, reproduction and other biological functions (Maccari and Morley-Fletcher, 2007). It involves the hypothalamus, pituitary and adrenal glands. Under stressful conditions, the autonomic nervous system and HPA axis become activated. In response to stress the hypothalamic neurons release corticotropin-releasing hormone (CRH), which enters the hypothalamohypophysial portal system and binds to cells in the anterior pituitary gland. This stimulates the secretion of adrenocorticotropic hormone (ACTH). ACTH subsequently binds to membrane bound receptors on cells in the adrenal cortex and stimulation of cortisol secretion occurs. A negative feedback effect exists as cortisol inhibits CRH and ACTH secretion. A few major effects of cortisol include increased fat and protein breakdown as well as increased blood glucose levels (Seeley et al., 2003). Anti-inflammatory effects have also been noted (De Prada et al., 2007).

A study by Watson and Mackin (2006) suggested that hypothalamus-pituitary-adrenal disruption plays a role in the pathophysiology of mood disorders. According to Buckley and Schatzberg (2005), disruption of this axis affects sleep leading to sleep disorders such as sleep apnea and insomnia. Associations with chronic fatigue syndrome has also been suggested where cortisol levels in patients were found to be reduced due to prolonged hyperactivation of the axis caused by stress (Devanur and Kerr, 2006).

2.8 Conclusion

The quality of drinking water and potential associated health risks vary throughout the world. The variations in water quality and health risks lead to different treatment priorities and

drinking water quality management strategies. To be able to set guidelines for contaminants in drinking water, good quality research and data on levels of contaminants in water and their associated morbidity and mortality risk assessments are required. Overall, however, it is evident, that an adequate supply and maintenance of safe drinking water remain key requirements for public health.

Table 2.1: WHO, EU, SA and EPA guidelines for drinking water quality

Health Related Chemical Parameters					
Inorganic chemicals	**Unit**	**WHO max limit**	**EU max limit**	**SA max limit**	**USEPA max limit**
Aluminium	µg/ℓ	200	200	300	50-200
Ammonia	µg/ℓ	No guideline	500	1000	Not mentioned
Antimony	µg/ℓ	5	5	10	6
Arsenic	µg/ℓ	10	10	10	10
Bromate	µg/ℓ	Not mentioned	10	Not mentioned	10
Cadmium	µg/ℓ	3	5	5	5
Chloride	mg/ℓ	250	250	200	250
Chromium	µg/ℓ	50	50	100	100
Copper	mg/ℓ	2	2	1	1
Cyanide	µg/ℓ	70	50	50	200
Fluoride	mg/ℓ	1.5	1.5	1	4
Iron	µg/ℓ	300	200	200	300
Lead	µg/ℓ	10	10	20	15
Manganese	µg/ℓ	500	50	100	50
Mercury	µg/ℓ	1	1	1	2
Nickel	µg/ℓ	20	20	150	Not mentioned
Nitrate	mg/ℓ	50 (as total N)	50	10 (as total N)	10 (as total N)
Nitrite	mg/ℓ	50 (as total N)	0.5	10 (as total N)	10 (as total N)
Selenium	µg/ℓ	10	10	20	50
Sodium	mg/ℓ	200	200	200	Not mentioned
Sulphate	mg/ℓ	500	250	400	250
Zinc	mg/ℓ	3	Not mentioned	5	5
Microbiological					
E. coli		Not mentioned	<1/250 ml	<1/100 ml	<1/100 ml
Faecal Coliforms (Includes *Salmonella spp.*, *Shigella spp.*, *Vibrio cholera*)		Not mentioned	<1/100 ml	<1/100 ml	<1/100 ml
Reference		WHO, 1993	EU, 1998	SANS 241, 2006	USEPA, 2003

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CHAPTER 3: The immune system

3.1 Overview of the Immune system

The immune system is a tightly regulated, intricate organ system (Ladics, 2007) which plays a fundamental role in the maintenance of health (Krzystyniak et al., 1995). This system relies on the communication between different immune cell types, cell products, tissues and other organ systems for optimal efficiency (Ladics, 2007). The chief function of the immune system is to protect the host against infectious microbes such as bacteria, viruses, parasites and fungi that possess varied pathologic mechanisms (Chaplin, 2006). This system has also evolved to aid in the elimination of neoplastic cells and non-self components (Roitt et al., 1993).

The host defence mechanisms against infectious microbes vary, depending on the nature of the pathogen and the type of tissue targeted (Chaplin, 2003). The immune responses rely on detecting structural features of pathogens thereby marking them distinct from host cells. Thus, the ability of the host to differentiate between self and non-self components is crucial in the process of controlling or eliminating pathogens without causing too much damage to its own tissues (Chaplin, 2006).

Immunity can be categorized into two components based on speed and developmental origin of the response. These components are termed the innate (present at birth) and acquired (upon post-partum exposure to pathogen) responses (Pruett, 2003). The innate response employs phagocytic cells as well as cellular products and does not require prior sensitization to an antigen (Ag). Acquired immune responses however, requires prior exposure to an Ag and

includes both the cell mediated (T helper type 1, Th 1) and humoral (T helper type 2, Th 2) immune pathways (Ladics, 2007).

3.2 The innate immune system

Innate immunity is the first line of defence against an invading pathogen hence the host is provided with immediate defence. Different defence mechanisms are in place that allows this type of immunity to be effective. The skin and epithelial barriers of the host, when intact, is highly efficient at preventing entry of pathogens into the host. The mucociliary action removes inhaled or ingested particles (Chaplin, 2006). Gastric acid in the stomach, spermine and zinc in semen as well as lysozyme in sweat, tears, saliva and nasal secretions possess bactericidal properties that inactivates pathogens. Once the pathogen has penetrated the body, mechanisms including phagocytosis and the complement system come into play (Roitt, 1984).

The innate response is almost immediately after exposure to pathogens and may cause damage to normal tissue while fighting pathogens. It does not require prior pathogen sensitization, and a second exposure to the same pathogen does not become more vigorous therefore memory is not displayed (Delves and Roitt, 2000; Pruett, 2003; Ladics, 2007).

3.2.1 Phagocytosis

The response of phagocytes to the site of infection is the first step required to initiate the phagocytosis process. The circulating phagocytes such as neutrophils and macrophages then respond to a variety of signalling events generated at the site of infection. Some of the signals

triggers the activation of endothelial cells close to the site of infection to upregulate the expression of cell adhesion molecules such as E-selectin and intracellular adhesion molecule 1 (ICAM-1) (Tosi, 2005). These cell adhesion molecules then associate with specific components on the surface of phagocytic cells and subsequently causes the phagocytes to adhere to the endothelium. The production of chemoattractants such as C5a, N-formyl bacterial oligopeptides and leukotriene B_4 at the site of infection creates a chemotactic gradient for phagocyte migration into damaged tissue (Parkin and Cohen, 2001). Phagocytes then move along this gradient and leave the circulation by diapedesis, through spaces between the endothelial cells to the infection site (Parkin and Cohen, 2001).

Upon reaching the infection site, the phagocytes must recognise and phagocytose the invading microbe. Phagocytes have a variety of pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) which are highly conserved structures expressed on the surfaces of microorganisms (Kumar et al., 2009). An example of PRRs is the toll-like receptors (TLRs) which contribute to signal transduction. These receptors are homologues to the *Drosophila* receptor Toll and allow recognition of PAMPs such as lipopolysaccharide, from gram-negative bacteria, peptidoglycan, and lipoteichoic acid (Medzhitov et al., 1997). The activation of TLRs induces an appropriate cascade of effector adaptive responses. TLRs are cell surface transmembrane proteins and thus far, ten human TLRs have been identified. TLR4 recognizes lipopolysaccharide (LPS) of Gram-negative bacteria when in combination with CD 14 whereas TLR2 recognizes both lipoproteins and peptidoglycans of Gram-positive bacteria. TLR9 recognizes hypomethylated cytosine and guanine rich repeats (Falgarone et al., 2005; Kumar et al., 2009) in the bacterial cell wall.

The association of the PRRs and the PAMPs activates the cytoskeletal contractions of the phagocytic cells causing an invagination of the membrane and forming pseudopods around the microbe (Tosi, 2005). The pseudopods engulf the microbe and this membrane bound vesicle is known as a phagosome. The phagosome then fuses with the lysosomal compartments in the cells cytoplasm to form the phagolysosome. In this protected compartment, killing of the microbe can occur by either oxygen dependent or oxygen independent mechanisms (Parkin and Cohen, 2001). The oxygen dependant response depends on the reduction of oxygen by an NADPH oxidase to form toxic oxygen metabolites such as superoxide anion, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Parkin and Cohen, 2001; Tosi, 2005). The oxygen independent response utilizes toxic proteins and enzymes, which are found in primary and secondary granules of the lysosome, to kill the microbe (Tosi, 2005). The enzyme lysozyme, cleaves linkages in the peptidoglycan layer of bacterial cell walls. This leads to the disintegration of the cell wall. The bactericidal permeability increase protein permeabilizes the cell wall, allowing the microbial proteins and nutrients to leach out of the microbe thus killing the microbe (Tosi, 2005). The major basic protein changes the charge of the microbe thereby disrupting the microbial membrane leading to microbial death (Tosi, 2005).

The antigen presenting cells such as macrophages, dendritic cells and B lymphocytes utilizes phagocytosis to direct antigens to both MHC I and MHC II compartments which are required for the induction of acquired responses. Phagocytosis therefore serves as a bridge between innate and acquired immune responses (Greenberg and Grinstein, 2002).

3.2.2 The complement system

The complement system is an essential component of both the innate and acquired immune responses (Chaplin, 2003; Chaplin, 2006). The complement system is composed of at least 30 serum and cell surface proteins as well as three distinct activation pathways (Tosi, 2005). These protein molecules are mainly synthesised in the liver and released into the serum for immune surveillance (Tosi, 2005). Upon exposure to a foreign pathogen, activation of the complement cascade can occur via either the alternative pathway, the classical pathway or the mannan-binding lectin pathway (Parkin and Cohen, 2001; Beutler, 2004; Zipfel, 2009).

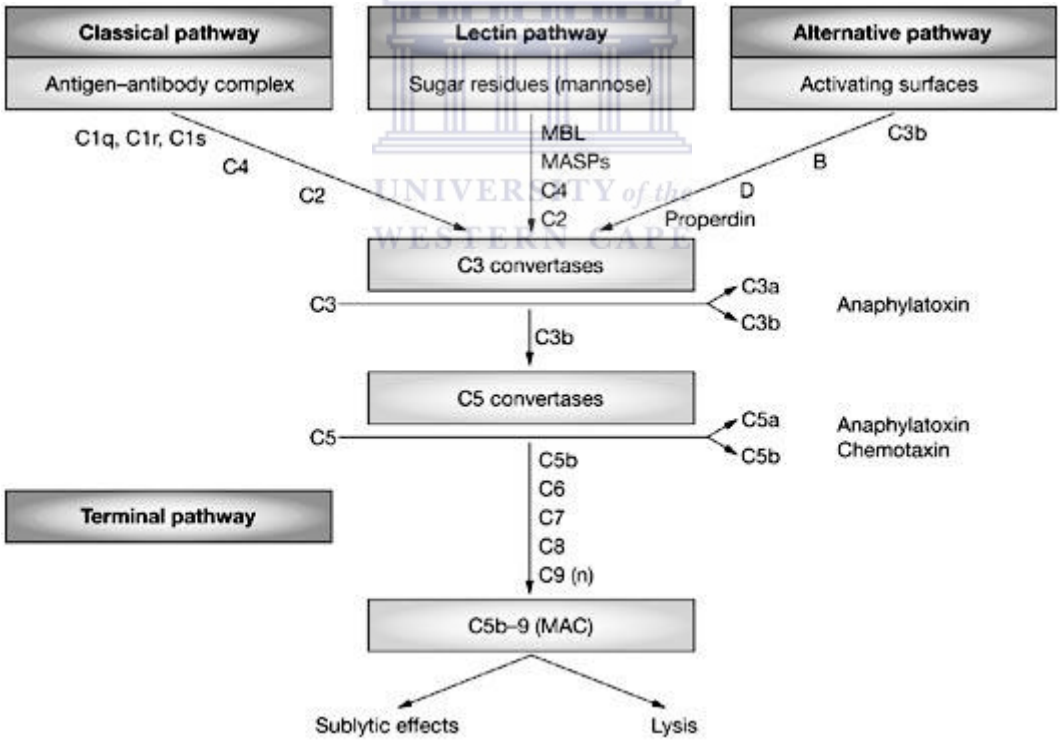

Figure 3.1: A simplified view of the complement system (Cook and Botto, 2006)

The C3 serum protein mediates the alternative pathway. This pathway is activated by the conformational change of C3 and subsequent binding to microbial surfaces (Zipfel, 2009). The activated C3 then binds complement factor B to form C3B. C3B then attracts factor D which cleaves C3B to form the intermediate C3-convertase C3Bb. This convertase cleaves C3 to form C3a, which is released into the serum, and C3b that binds to the microbial cell surface to activate the remainder of the cascade (Tosi, 2005). C3b once again binds complement factor B and undergoes proteolytic cleavage by factor D to form the C3-convertase C3bBb. This C3-convertase is stabilised by properdin, and is responsible for the C3 amplification loop, which allows for the rapid synthesis of C3a and C3b (Tosi, 2005). C3a is a anaphylatoxin that induces histamine release from degranulated mast cells, which creates edema and susequently recruits various phagocytic cells (Hugli, 1984). C3b acts as an opsonin, which serves as a recognition signal for phagocytosis (Fishelson, 1991). It also binds the C3-convertase C3bBb to form the C5-convertase C3bBbC3b. The C5-convertase cleaves C5 into C5a and C5b. C5a is a potent chemoattractant, which also acts as an anaphylatoxin. C5b binds to the microbial cell surface and assembles with the terminal complement proteins, C6, C7, C8 and C9 to form the membrane attack complex (MAC). MAC drills holes in the cell surface leading to osmotic cell lysis (Tosi, 2005; Zipfel, 2009).

The classical pathway is activated by antibodies IgG or IgM, which are bound to pathogenic cell surface antigens. This antibody-antigen complex makes a site available on the Fc portion of the antibody to bind to complement protein C1q to form a complex that then activates complement proteins C1r and C1s. The activated C1s then proteolytically cleaves C4 into C4a and C4b, and C2 into C2a and C2b to form C4b2a, the classic pathway C3-convertase. The C3-convertase will then rapidly synthesise C3b for the alternative pathway as a positive

feedback loop. C4a acts as an anaphylotoxin causing vascular dilation and permeability (Parkin and Cohen, 2001; Walport, 2001; Chaplin, 2003; Tosi, 2005).

The mannan-binding lectin pathway is triggered by the association of mannan-binding lectin (MBL) to mannose containing carbohydrates on microbial cell surfaces. MBL is a serum protein that belongs to the collectin family (Petersen et al., 2001; Tosi, 2005). This interaction subsequently leads to the activation of the MBL-associated serine proteases 1 and 2 (MASP-1 and MASP-2). MBL and the MASPs have structural and functional similarities to the activated C1 serum protein in the classical pathway and can therefore activate and cleave C4 and C2 to form the C3-convertase C4b2a (Chaplin, 2003). The C3-convertase once again generates C3b for the alternative pathway thus increasing phagocytosis (Tosi, 2005).

3.3 The acquired immune system

The acquired immune response displays specificity as well as a memory response after a second exposure to the initiating pathogen (Pruett, 2003). The acquired immune response requires interactions between antigen presenting cells (APC), T-helper cells (Th 1 and Th 2), B lymphocytes, T cytotoxic cells (Tc) as well as several cytokines for efficient pathogen defences to occur (Pruett, 2003). Acquired immunity is divided into two branches namely, humoral and cell mediated responses.

3.3.1 Cells of the acquired immune system

B and T lymphocytes are derived from stem cells within the red bone marrow. Pre-T cells migrate through the blood to the thymus where they are processed into functional T

lymphocytes. Pre-B cells remain in the bone marrow for the duration of their development into functional B lymphocytes. Both cell types re-enter the blood and circulates to secondary lymphatic organs and tissues (diffuse lymphatic tissue, tonsils, lymph nodes and the spleen) where they communicate with other cells to elicit an immune response (Parkin and Cohen, 2001; Seeley et al., 2003).

The expression of antigen specific receptors on both B and T lymphocytes are specific features of acquired immunity. The antigen specific receptors are produced in both cell types during early developmental processing (Parkin and Cohen, 2001). T-helper and T cytotoxic lymphocytes can be differentiated from progenitor T cells by the expression of T cell antigen receptor-2 and T cell antigen receptor-1 as well as the appearance of CD4 and CD8 molecules on the surface (Roitt, 1989). The function of these receptors is to identify antigens bound to major histocompatibility complex (MHC) molecules. The MHC gene complex can be separated into two genes, MHC class I and MHC class II. T lymphocytes with CD8 molecules recognize MHC I-antigen bound molecules and T lymphocytes with CD4 molecules recognize MHC II-antigen bound molecules (Roitt, 1989).

3.3.2 Humoral immunity

The central function of humoral immunity is the production of antigen specific antibodies (Ab), which are predominantly immunoglobulin G (IgG), by B lymphocytes. In early development, IgD immunoglobulins are inserted into the surface of the B cells and act as antigen specific receptors (Roitt, 1989). Antibodies are able to neutralise toxic substances from bacteria, prevent microbial attachment to mucosal surfaces, activates the complement

pathway, opsonises bacteria to enhance phagocytosis and sensitizes cells infected with intracellular pathogens for antibody dependant cytotoxic killing (Pruett, 2003).

The antigen presenting cells phagocytose the antigenic proteins from extracellular pathogens that have been degraded/killed during phagocytosis (Chaplin, 2003). The internalised antigenic proteins are then proteolytically cleaved into peptide fragments by enzymes within an endosome (Chaplin, 2003). Meanwhile, the endoplasmic reticulum releases MHC class II molecules bearing an invariant chain to the endosome where MHC II can interact with the peptide fragment. Before peptides can bind, the invariant chain is cleaved by proteases and the peptide fragment takes its place. The MHC II-peptide complex is then delivered to the cell surface for antigen presentation to CD 4 T-helper cells (Chaplin, 2003). The MHC II-peptide complex stimulates precursor Th 0 lymphocytes to differentiate into T-helper 2 cells (Th2) (Parkin and Cohen, 2001).

The humoral immune response is mediated by the Th2 cells and is characterized by the production of Th2 cytokines interleukin 4 (IL-4), interleukin 5 (IL-5) and interleukin 10 (IL-10) (Ladics, 2007). In turn, these cytokines aid B lymphocyte proliferation and differentiation into antibody-secreting plasma cells (Ladics, 2007). These antibodies are produced several days after antigen exposure and released into the general circulation (Ladics, 2007).

3.3.3 Cell mediated immunity

Intracellular pathogens such as viruses and mycobacteria replicate within the host cell and synthesise proteins within the cytoplasm. The newly synthesised pathogen proteins are digested by the proteosome into antigenic peptide fragments. The peptides are then

transported into the endoplasmic reticulum by the TAP transporter and attach to the antigenic groove of the MHC class I molecule that associates with the β_2-microglobulin subunit. The MHC I-peptide complex then detaches from the TAP transporter and is delivered to the cell surface for antigen presentation to CD 8 cytotoxic T cells and CD 4 T-helper cells (Chaplin, 2003).

The cell mediated immune response is mediated by the T-helper 1 (Th1) cells and is characterized by the production of cytokines interleukin 2 (IL-2), tumour necrosis factor beta (TNF-β) and interferon gamma (IFN-γ). The Th1 response is required for the production of cytotoxic T lymphocytes and provides immunity against intracellular pathogens such as viruses, certain bacteria and protozoa and cancers (Xie et al., 2008).

3.3.4 Th1/Th2 Immune balance

The differentiation between the T-helper 1 and T-helper 2 cell types is dependent on a group precursor T cells termed ‘naïve T cells’. The process of differentiation into either of the two cell types is called Th polarization. Before differentiation can take place, ‘naïve T cells’ enter a pre-activation stage (Th 0) (Kidd, 2003). The process of Th polarization is dependent on the time period of antigenic exposure as well as the surrounding cytokine environment (Langenkamp et al., 2000; Storni et al., 2005). This combination allows the Th1 and Th2 cells to regulate one another. A cell mediated immunity feedback loop exists as IFN-γ stimulates Th0 to differentiate into Th1 whilst inhibiting Th2 differentiation (Parkin and Cohen, 2001). Cell mediated immunity is regulated by the Th1 response and is responsible for defense against intracellular pathogens and fight cancerous cells (Kidd, 2003). A humoral immunity feedback loop also exists as IL-4 activates Th0 to differentiate into Th2 whilst suppressing

Th1 differentiation (Parkin and Cohen, 2001). Humoral immunity is mediated by the Th2 response, which increases antibody production and offers protection against extracellular pathogens such as bacteria, fungi and yeasts (Kidd, 2003). A negative aspect to these feedback loops is over reactivity, which could lead to disease. Th1 hyperactivity can cause organ specific autoimmunity such as rheumatoid arthritis and multiple sclerosis. Th2 hyperactivity is associated with allergic disease (Singh, 1999).

3.3.5 Cytokines

Cytokines are soluble protein molecules that promote cell to cell communication in order to regulate and maintain immune function (Kidd, 2003). They form an essential component in determining the nature of the immune response and what effector mechanism should be chosen (Godoy-Ramirez et al., 2004). Cytokines may act as autocrine, paracrine or endocrine messengers (Parkin and Cohen, 2001). Cytokines includes the interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs) and transforming growth factors (TGFs) (Roitt et al., 1993; Kidd, 2003). Interleukins and interferons involved in the acquired immune response are briefly discussed below.

Interleukin-6 (IL-6) is a major proinflammatory cytokine and is important in protection against pathogens during infection (Dienz and Rincon, 2008). This cytokine is produced by antigen presenting cells (APCs) such as dendritic cells, macrophages and B cells (Kamimura et al., 2003). A response to external stimuli such as IL-1, TNF α and platelet derived growth factor induces IL-6 production (Kamimura et al., 2003). The IL-6 family includes IL-6, IL-11, oncostatin M, leukemia inhibitory factor and ciliary neurotrophic factor. IL-6 exerts its action by connecting to plasma membrane receptor complexes which contains the signal

transducing receptor chain glycoprotein 130 (Heinrich et al., 2003). This leads to the Jak/Stat (Janus kinase/signal transducer and activator of transcription) pathway and the MAPK (mitogen-activated protein kinase) cascade (Heinrich et al., 2003).

Interleukin-10 (IL-10) was initially named cytokine-synthesis inhibitory factor because of its ability to inhibit the production of cytokines IL-2, IFN-γ and TNF α by murine Th 1 cells (Fiorentino et al., 1989). In humans, IL-10 inhibits the activation of and cytokine production by Th 1 cells as well as their proliferation and chemotaxis (Mocellin et al., 2004). IL-10 inhibits Th 1 and natural killer (NK) cell cytokine production by way of inhibiting accessory cell function. Studies have also shown that IL-10 inhibits a wide range of activated macrophage/monocyte functions, amongst others, NO production; monokine synthesis, class II MHC expression and co-stimulatory molecule expression such as IL-12 and CD80/CD86 (Bogden et al., 1991; de Waal et al., 1991; Ding et al., 1993; Mocellin et al., 2004).

The cytokine interferon-gamma (IFN-γ) is the principal mediator of innate and acquired immune responses. It is synthesised by macrophages, natural killer cells, CD8 T cells and CD4 T-helper cells. It is mainly produced by activated $CD4^+$ or $CD8^+$ T cells and natural killer cells (Muhl and Pfeilschifter, 2003). IFN-γ signals activity via the Jak/Stat pathway. Once the IFN-γ molecule and its receptor interact, the recruitment and activation of Jak, and then Stat 1 occurs. The activated Stat 1 then translocates to the nucleus and binds to a defined DNA sequence to initiate transcription. Interferon regulating factors IRF-1, IRF-2 and IRF-9 also play a role in the signalling pathway (Billiau and Matthys, 2009). IFN-γ plays a significant role in the control of intracellular infection and tumour development. It enhances antigen presentation through the MHC-I and MHC-II pathways and regulates the cell cycle, growth and apoptosis of various cell types (Boehm et al., 1997; Schroder et al., 2004).

3.4 Disruption of normal immune function

The immune system, among others, is an important target organ for chemical induced toxicity. The term immunotoxicity describes the adverse effects of chemicals agents, therapeutic drugs or xenobiotic agents on the immune system (Snodin, 2004). The effects that follow can modulate immune activity leading to a reduced ability of the host to fight infectious diseases and cancers and/or facilitate hypersensitivity and autoimmunity (De Jong and Van Loveren, 2007).

Immunosuppression directly affects the function of the immune organs or cells and manifests as a decrease in lymphoid organ weight and/or a decrease in the number of immune cells. The result may be a reduction in immune function displayed as a reduced resistance against infection (De Jong and Van Loveren, 2007). An example depicting the effects of immunosuppression can be seen in opportunistic infections in HIV patients and cytomegalus infections in patients who have had organ transplants (Bower et al., 2006; Tan and Goh, 2006; Tang et al., 2006).

Cyclosporin A is an immunosuppressive drug used extensively to decrease the risk of organ rejection after an organ transplants. Cyclosporin A acts on T lymphocytes specifically (Carfi' et al., 2007). It causes a reduction and deactivation of T cells which results in an impairment of proliferative responses of T cells, thus decreasing the synthesis of IFN-γ, IL-1, and IL-2. It has been shown to induce various cancers and increase organ transplant patient's susceptibility to infections.

Another example is the use of the drug verapamil, a calcium channel blocker. Verapamil is used in the treatment of hypertension and cardiovascular arrhythmias. A study by Chow and Jusko (2004) showed that this substance has toxic effects on the immune system by inhibiting mitogen induced T cell proliferation (Carfi' et al., 2007).

Additionally, Tributyltin chloride (TBTC) a toxic organotin compound is used as plastic stabilizers, agricultural pesticides and antifouling agents in paint (Snoeij et al., 1987). Literature has shown that TBTC inhibits the proliferation of immature thymocytes at low doses and causes a depletion of thymocytes by apoptosis at higher doses (Raffray and Cohen, 1993; Genneri et al., 1997).

In contrast, immunostimulation occurs when there is a direct stimulation on the immune system. Immunostimulation is coupled with an increase in the activity of immune organs or cells which may lead to enhanced or excessive responses. The result is an exacerbated immune response against foreign agents or a decreased capability of the immune system to respond to external stimuli (De Jong and Van Loveren, 2007).

A hypersensitivity reaction results from an increase in specific responses to various xenobiotics leading to hyper-immune responses causing tissue damage. Allergic disease to the xenobiotic may be a consequent effect (De Jong and Van Loveren 2007). The Coombs and Gell classification system categorizes hypersensitivity reactions into four classes (Coombs and Gell, 1975). Hypersensitivity reactions I, II and III are antibody mediated whereas T lymphocytes and macrophages mediate the hypersensitivity type IV response (Putman et al., 2003). The most frequent types of immunotoxic reactions are hypersensitivity type I and type IV. Hypersensitivity type I or anaphylactic reaction is IgE mediated and has

an impact on mediators released from basophils and mast cells. Type IV (delayed hypersensitivity reaction) responses affect stimulated T lymphocytes which generate cytokines as well as other mediators. This may result in cellular and tissue damage (Anderson and Langone, 1999). Hypersensitivity type IV reactions appear as skin eruptions on the body (Van Wijk and Nierkens, 2006). This type of reaction also manifests as allergic contact dermatitis due to drug and environmental exposure (Putman et al., 2003).

Autoimmunity occurs from the interaction between xenobiotics and tissue components. This leads to responses to auto antigens which are initiated by enhanced immune reactivity, immune stimulation or reactions to altered self antigens. The induction of antinuclear antibodies may also occur (De Jong and Van Loveren 2007). Chemicals also have the ability to alter self antigens. The immune system would therefore detect these components as foreign resulting in autoimmunity disorders (Van Loveren et al., 1995).

3.5 Conclusion

The immune system is structurally and functionally complex. Literature indicates that environmental contaminants can result in a variety of adverse effects on several organs and tissues of the immune system. These effects are known to increase the incidence of infectious disease and cancers as well as exacerbate allergic responses and autoimmune diseases. Exposure to environmental contaminates can occur through various occupational environments or via consumption of contaminated food or water. Chronic exposure to contaminants in drinking water can alter the homeostasis of the immune system thus resulting in immune dysfunction and the development of disease in humans and animals.

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CHAPTER 4: The chemical and steroid hormone analysis of raw and drinking water from two drinking water treatment plants in Gauteng.

4.1 Abstract

Steroid hormones are constantly excreted into the environment by human and animal sources. These hormones are known as endocrine disrupting compounds (EDCs) because they have the potential to interfere with normal endocrine functioning. Surface water contamination by EDCs can cause adverse effects on human and wildlife reproductive systems as well as markedly influence their immune systems. It is therefore essential to determine if drinking water could be contaminated by these EDCs when produced from polluted surface waters. The aim of this study was to determine the chemical quality, estrone (E1) and testosterone (T) levels in raw and drinking water samples. The water samples were collected from two drinking water treatment plants (M-plant and Bar-plant) during spring, summer, autumn and winter. The chemical quality was determined by Rand Waters Analytical Services using South African certified protocols. Samples were also subjected to C18 solid phase extraction and E1 and T levels were determined using commercially available ELISA kits. The concentration of calcium, hardness, magnesium, potassium, sodium, sulphur, chloride, nitrate and sulphate was higher in the Bar-plant than in the M-plant. The Bar-plant is downstream from the M-plant and may therefore be impacted on by human activities. The levels of testosterone in raw and drinking water from the M-plant ranged from 1.2 - 3.9 ng/l and 2.6 - 4.9 ng/l respectively, for all the seasons. The levels of testosterone in raw and drinking water from the Bar-plant ranged from 1.7 - 7.3 ng/l and 0.2 - 1.5 ng/l respectively, for all the seasons. The ranges for estrone in raw and drinking water from the M-plant were 0.1 - 3 ng/l and 1.1 - 2.5 ng/l respectively. The ranges for estrone in raw and drinking water from the

Bar-plant were 4.5 - 12.2 ng/l and 0.6 - 1.6 ng/l respectively. Significant differences were observed between raw and drinking water from each plant. The levels of T in drinking water from the M-plant was significantly higher than the raw water ($P<0.050$). This was probably due to the biotransformation of steroid conjugates to free hormone during the treatment process. The levels of T in raw water from the Bar-plant was significantly higher than the drinking water. This shows that the treatment processes utilised in the plant were effective in removing these steroid hormones. No significant difference was observed in E1 levels from the M-plant. The E1 levels in raw water from the Bar-plant was significantly higher than the drinking water. This study confirms the presence of endocrine disrupters in water resources and shows the efficiency of the treatment processes in removing them.

4.2 Introduction

Water is an important natural resource and life-sustaining drink to both humans and animals (Bates, 2000; Van Leeuwen, 2000). The provision of high quality water is therefore essential for safeguarding the health and well-being of the human population (Van Leeuwen, 2000). Changes in water quality can occur through natural and anthropogenic inputs from point and non-point sources along the length of the river. However, it is observed that anthropogenic activities significantly influence these changes (Rand Water, 2005). The growth in urban settlements, industries and agriculture, produce nutrient concentrates, toxic chemical compounds and natural hormones that enter the environment (Calderon, 2000; Van Leeuwen, 2000). Due to their persistence in the environment, these chemical contaminants may pollute ground and surface water, which are the main resources for drinking water (Eichhorn et al., 2001). In addition, the inadequate removal of contaminants by treatment processes and the consequent exposure of contaminated drinking water to humans may result in a variety of acute and chronic health effects. A group of contaminants causing much concern in the water industry are the steroid hormones.

Steroid hormones are produced by both humans and animals and are constantly excreted into the environment (Drewes and Shore, 2001; Shore and Shemesh, 2003). They are a group of biologically active compounds that are structurally related due to a common precursor, cholesterol (Lintelmann et al., 2003). The steroid hormones include estrogens, androgens, progestogens, glucocorticoids, and mineralcorticoids (Raven and Johnson, 1999). These natural steroids are synthesized and secreted by the adrenal cortex, testis, ovary and placenta. Estrogens (estradiol, estrone, estriol) are female hormones responsible for the development and maintenance of reproductive tissues and secondary sex characteristics in females

(Martini, 2006). Androgens (testosterone, dihydrotestosterone) are male hormones responsible for the development and maintenance of male reproductive organs, sperm production and secondary male sex characteristics (Seeley et al., 2003; Martini, 2006). It also plays a major role in tissue regeneration of bone and muscle (Ying et al., 2002; Lintelmann et al., 2003). Progestogen (progesterone) helps to regulate the changes that occur during the menstrual cycle. Glucocorticoids (cortisol) are synthesised by the adrenal glands in response to stressors such as emotional upheaval, exercise, illness or starvation (Ying et al., 2002).

Steroid hormones may enter the environment through sewage discharge or animal waste disposal (Ying et al., 2002). These hormones, which are chemically stable, enter the environment in the free form or as conjugates (Ternes et al., 1999). The steroid conjugates have the ability to biotransform back to the free form under appropriate environmental conditions (Panter et al., 1999).

Steroid hormones have been detected in sewage effluent and surface waters (Desbrow et al., 1998; Belfroid et al., 1999; Baronti et al., 2000; Kuch and Ballschmiter, 2001; Kolpin et al., 2002; Yamamoto et al., 2006; Chen et al., 2007; Velicu and Suri, 2009). The pollution of water by these natural steroids is a major concern because they may interfere with the normal functioning of the endocrine system, thus affecting reproduction and developmental aspects in wildlife and humans (Jobling et al., 1998; Lintelmann et al., 2003). These hormones have been shown not only to modulate the endocrine system but also the immune system resulting in an alteration of development, homeostasis, and behaviour (Mendes, 2002). Currently, estrogens are the most reviewed endocrine disruptors (Jobling et al., 1998; Folmar et al., 2002).

Estrogens are known to modulate immune functions via the presence of estrogen receptors (ER) on immune cells (Gredel et al., 2008). These receptors are found in macrophages, monocytes, $CD4^+$ T cells, B cells and dendritic cells (Kogiso et al., 2006). Natural estrogens induce immunotoxic effects in immune organs such as the thymus and the spleen. In the thymus, estrogen induces thymic atrophy by triggering apoptosis (Okasha et al., 2001). This negative effect suppresses the proliferation and differentiation of T lymphocytes. Estrogens enhance the humoral immune response by increasing immunoglobulin production by B lymphocytes (Lang, 2004). This enhancement is suggested to play a role in the pathogenesis of the autoimmune disease, systemic lupus erythematosus (Kanda and Tamaki, 1999). Literature shows that testosterone inhibits the production of immunoglobulins IgG and IgM in human peripheral blood mononuclear cells, suggesting suppression of the humoral immune response (Kanda et al., 1996).

Very few studies have been done to assess the effectivity of drinking water treatment processes in removing steroid hormones. The aim of this study was to determine the chemical quality and levels of steroid hormones in the raw and drinking water from two drinking water treatment plants.

4.3 Materials and Methods

4.3.1 Collection of water samples

Water samples were collected in September (Spring), February (Summer), May (Autumn) and August (Winter). Water was collected in 2 x 1000 ml sterile plastic bottles (chemistry) and 1 x 1000 ml sterile glass bottle (hormone assays). Immediately after collection, 20 ml of nitric acid was added to one plastic bottle to prevent oxidation of the heavy metals. The plastic bottles were transported at 4 ºC to Rand Water's Scientific Division for chemical analysis using South African certified protocols. Extractions were done within 24 hours of collection. Water samples were stored at 4 °C until extraction.

4.3.2 Analytical techniques used for the chemical analysis of water samples

The inorganic compounds Al, B, Cd, Ca, Cr, Co, Cu, Fe, Pb, Mg, Mn, Mo, Ni, P, K, Si, Na, S, V and Zn were measured by using Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-OES; Spectro CCD, South Africa). The chloride, nitrate and sulphate ions were determined by ion chromatography (Metrohm 761 Compact IC; Swiss Lab, South Africa). Silica and ammonia were analysed via an Aquakem automated colorimetric analysis system (Aquakem 250 automated colorimetric analyser; Anatech). Cyanide was determined by using the ion selective electrode method (Cyanide selective electrode; Swiss Lab, South Africa).

4.3.3 Detection of steroid hormones in water

4.3.3.1 Solid phase extraction (SPE) of water samples

Water samples were extracted on C18 SPE columns (Anatech) using our in-house extraction procedure. In brief: C18 columns were pre-washed with 4 ml of solvent mixture (40 % hexane, 45 % methanol and 15 % 2-propanol), followed by another wash with 4 ml of ethanol. The column was then washed with one column volume of reverse osmosis water after which the water sample was applied onto the column. Distilled water was used as a control. The column was then air-dried. The bound hydrophobic substances were eluted with 2 ml solvent mixture. The eluate was dried under air and reconstituted in dimethyl sulfoxide (DMSO) to give a final volume 1/1000 times that of the original sample volume. The samples were stored at –20 °C.

4.3.3.2 Estrone ELISA

Concentrated (1000 x) water extracts were diluted 1/100 using 0.1 % (w/v) bovine serum albumin (BSA) in 0.9 % NaCl. The diluted (10 x concentrated) extracts were assayed directly on the estrone ELISA kits (IBL, Germany) using the manufacturer's instruction manual. In brief: microtitre plate strips pre-coated with polyclonal anti-Estrone were removed from the strip holder and firmly fixed in the ELISA plate. All assays were done in duplicate. The samples and standards were transferred to the wells (25 µl/well), followed by the addition of working conjugate solution (estrone conjugated to horse radish peroxidase) (100 µl/well). The contents of the wells were mixed by tapping the plate. The ELISA plate was then incubated for 1 hour at room temperature, followed by washing the plate four times with

wash buffer (300 µl/well). Tetramethylbenzidine (TMB) substrate was dispensed at 150 µl/well after which the plate was incubated for 15 minutes at room temperature. The reaction was stopped by the addition of 50 µl stop solution (2M H_2SO_4) to each well. The optical density (OD) was measured at 450 nm using a plate reader (Mulitskan Ex, Thermo Electron Corporation). A standard curve was drawn using the OD readings obtained for the standards and the concentrations for the samples were read off this curve. The reader can refer to Swart and Pool (2007) for validation papers for steroid ELISAs.

4.3.3.3 Testosterone ELISA

Concentrated (1000 x) water extracts were diluted 1/100 using 0.1 % (w/v) bovine serum albumin (BSA) in 0.9 % NaCl. The diluted (10 x concentrated) extracts were assayed directly on the testosterone ELISA kits (IBL, Germany) using the manufacturer's instruction manual. In brief: microtitre plate strips pre-coated with mouse monoclonal anti-Testosterone were removed from the strip holder and firmly fixed in the ELISA plate. The samples and standards were transferred to the wells (25 µl/well), followed by the addition of working conjugate solution (testosterone conjugated to horse radish peroxidase) (100 µl/well). The contents of the wells were mixed by tapping the plate. The ELISA plate was then incubated for 1 hour at room temperature, followed by washing the plate four times with wash buffer (300 µl/well). TMB substrate was dispensed at 150 µl/well after which the plate was incubated for 15 minutes at room temperature. The reaction was stopped by the addition of 50 µl stop solution (2M H_2SO_4) to each well. The optical density (OD) was measured at 450 nm using a plate reader (Mulitskan Ex, Thermo Electron Corporation). A standard curve was drawn using the OD readings obtained for the standards and the concentrations for the samples were read off this curve.

4.3.4 Statistical analysis

All data is presented as mean ± standard deviation (SD). Statistical significance was determined by one way analysis of variance (ANOVA). Differences were considered statistically significant at $P<0.050$.

4.4 Results

4.4.1 The physical and chemical analysis of water

The main physical and inorganic chemical properties of the raw and drinking water samples are presented in Table 4.1, 4.2, 4.3, and 4.4 for spring, summer, autumn and winter respectively. Higher values for calcium, hardness, magnesium, potassium, sodium, sulphur, chloride, nitrate and sulphate were identified in the Bar-plant in comparison to the M-plant. The calcium levels detected in the M and Bar-plants ranged from 15-19 mg/l and 27-74 mg/l respectively. The hardness of the samples collected from the M-plant were in the range of 69-86 mg/ml $CaCO_3$ and 115-295 mg/ml $CaCO_3$ in the Bar-plant. Sodium levels in the M and Bar-plant ranged from 8-12 mg/l and l22-74 mg/l respectively. The sulphur levels in the M and Bar-plants were found in the ranges of 4.8-6.8 mg/l and 19-65 mg/l respectively. Chloride ions were found in the range of 6.8-10 mg/l and 27-100 mg/l in the M and Bar-plant respectively. The range for nitrate ions in the M and Bar-plants were <0.10-0.34 mg/l and 1.1-3.6 mg/l respectively, whereas sulphate ion ranged between 15-18 and 48-130 mg/l respectively.

Table 4.1: The physical and inorganic chemical properties in raw and drinking water samples from the Bar and M treatment plants during spring.

SPRING						
Inorganic Quality variable	**Unit**	**Reporting Limit**	**M Raw**	**M Drink**	**Bar Raw**	**Bar Drink**
Aluminium	mg/l	<0.010	0.04	0.01	<0.010	0.09
Boron	mg/l	<0.010	0.01	0.01	0.11	0.19
Cadmium	ug/l	<2.5	<2.5	<2.5	<2.5	<2.5
Calcium	mg/l	<0.400	17	19	69	74
Chromium	mg/l	<0.010	<0.010	<0.010	<0.010	<0.010
Cobalt	mg/l	<0.015	<0.015	<0.015	<0.015	<0.015
Copper	mg/l	<0.010	<0.010	0.02	0.01	0.01
Hardness	mg/l CaCO3		79	86	285	295
Iron	mg/l	<0.006	0.04	<0.006	<0.006	0.15
Lead	ug/l	<8.00	<8.00	<8.00	<8.00	<8.00
Magnesium	mg/l	<0.040	8.6	9.2	28	27
Manganese	mg/l	<0.003	<0.003	<0.003	0.01	0.06
Molybdenum	mg/l	<0.010	<0.010	<0.010	<0.010	<0.010
Nickel	mg/l	<0.015	<0.015	<0.015	0.02	<0.015
Phosphorus	mg/l	<0.0410	0.3	0.29	0.79	0.37
Potassium	mg/l	<0.310	2.2	2.4	14	14
Silicon	mg/l	<0.065	3.8	3.9	0.54	1.2
Sodium	mg/l	<1.500	10	12	68	74
Sulphur	mg/l	<0.260	6.2	6.8	65	62
Total Silica	mg/l	<0.14	8.1	8.3	1.1	2.6
Vanadium	mg/l	<0.030	<0.030	<0.030	<0.030	<0.030
Zinc	mg/l	<0.008	<0.008	<0.008	<0.008	0.01
Ammonia	mg/l		0.08	0.1	0.18	0.14
Chloride	mg/l		7.8	10	61	100
Cyanide	mg/l		<0.03	<0.03	<0.03	<0.03
Nitrate	mg/l		0.34	0.27	2	2.4
Sulphate	mg/l		-	-	-	-

- Not determined

Indicates higher values for the Bar-plant than for the M-plant

Table 4.2: The physical and inorganic chemical properties in raw and drinking water samples from the Bar and M treatment plants during summer.

SUMMER						
Inorganic Quality variable	**Unit**	**Reporting Limit**	**M-Raw**	**M-Drink**	**Bar-Raw**	**Bar-Drink**
Aluminium	mg/l	<0.010	0.43	<0.010	0.53	<0.010
Boron	mg/l	<0.010	0.01	0.01	0.06	0.12
Cadmium	ug/l	<2.5	<2.5	<2.5	<2.5	<2.5
Calcium	mg/l	<0.400	15	16	27	42
Chromium	mg/l	<0.010	<0.010	<0.010	<0.010	<0.010
Cobalt	mg/l	<0.015	<0.015	<0.015	<0.015	<0.015
Copper	mg/l	<0.010	0.01	0.01	0.01	0.01
Hardness	mg/l CaCO3		69	70	115	145
Iron	mg/l	<0.006	0.25	<0.006	0.32	0.01
Lead	ug/l	<8.00	<8.00	<8.00	<8.00	<8.00
Magnesium	mg/l	<0.040	7.5	7.4	11	9.7
Manganese	mg/l	<0.003	<0.003	<0.003	0.01	<0.003
Molybdenum	mg/l	<0.010	<0.010	<0.010	0.01	0.01
Nickel	mg/l	<0.015	<0.015	<0.015	<0.015	<0.015
Phosphorus	mg/l	<0.0410	<0.0410	<0.0410	0.36	<0.0410
Potassium	mg/l	<0.310	1.7	1.6	6	6.2
Silicon	mg/l	<0.065	3.5	2.6	6.4	4.7
Sodium	mg/l	<1.500	9.3	9.4	22	38
Sulphur	mg/l	<0.260	4.9	4.8	21	19
Total Silica	mg/l	<0.14	7.4	5.7	14	10
Vanadium	mg/l	<0.030	<0.030	<0.030	<0.030	<0.030
Zinc	mg/l	<0.008	0.01	<0.008	0.01	0.01
Ammonia	mg/l		<0.243	<0.243	0.31	<0.243
Chloride	mg/l		6.8	8.3	27	72
Cyanide	mg/l		-	-	-	-
Nitrate	mg/l		0.12	0.15	1.4	1.1
Sulphate	mg/l		15	15	64	56

- Not determined

Indicates higher values for the Bar-plant than for the M-plant

Table 4.3: The physical and inorganic chemical properties in raw and drinking water samples from the Bar and M treatment plants during autumn.

AUTUMN						
Inorganic Quality variable	**Unit**	**Reporting Limit**	**M Raw**	**M Drink**	**Bar Raw**	**Bar Drink**
Aluminium	mg/l	<0.010	0.1	0.02	0.02	<0.010
Boron	mg/l	<0.010	0.01	0.01	0.09	0.07
Cadmium	ug/l	<2.5	<2.5	<2.5	<2.5	<2.5
Calcium	mg/l	<0.400	17	17	47	55
Chromium	mg/l	<0.010	<0.010	<0.010	<0.010	<0.010
Cobalt	mg/l	<0.015	<0.015	<0.015	<0.015	<0.015
Copper	mg/l	<0.010	0.02	0.01	<0.010	0.01
Hardness	mg/l CaCO3		72	72	195	210
Iron	mg/l	<0.006	0.07	0.02	0.01	0.03
Lead	ug/l	<8.00	<8.00	<8.00	<8.00	<8.00
Magnesium	mg/l	<0.040	7.3	7.3	19	18
Manganese	mg/l	<0.003	0.01	<0.003	0.01	<0.003
Molybdenum	mg/l	<0.010	<0.010	<0.010	<0.010	<0.010
Nickel	mg/l	<0.015	<0.015	<0.015	0.02	<0.015
Phosphorus	mg/l	<0.0410	<0.0410	<0.0410	0.24	<0.0410
Potassium	mg/l	<0.310	2.4	2.4	9.6	9.8
Silicon	mg/l	<0.065	3.4	3.2	4.8	4.1
Sodium	mg/l	<1.500	8	8.1	48	52
Sulphur	mg/l	<0.260	6.2	6.3	38	38
Total Silica	mg/l	<0.14	7.3	6.8	10	8.8
Vanadium	mg/l	<0.030	<0.030	<0.030	<0.030	<0.030
Zinc	mg/l	<0.008	0.1	0.1	0.12	0.21
Ammonia	mg/l		<0.243	<0.243	<0.243	<0.243
Chloride	mg/l		6.9	9	60	84
Cyanide	mg/l		-	-	-	-
Nitrate	mg/l		<0.10	0.17	3	2.6
Sulphate	mg/l		15	16	130	125

- Not determined

Indicates higher values for the Bar-plant than for the M-plant

Table 4.4: The physical and inorganic chemical properties in raw and drinking water samples from the Bar and M treatment plants during winter.

WINTER						
Inorganic Quality variable	**Unit**	**Reporting Limit**	**M Raw**	**M Drink**	**Bar Raw**	**Bar Drink**
Aluminium	mg/l	<0.010	0.67	<0.010	<0.011	<0.012
Boron	mg/l	<0.010	0.01	0.01	0.07	0.08
Cadmium	mg/l	<2.5	<2.5	<2.5	<2.5	<2.5
Calcium	mg/l	<0.400	16	16	52	55
Chromium	mg/l	<0.010	<0.010	<0.010	<0.010	<0.010
Cobalt	mg/l	<0.015	<0.015	<0.015	<0.015	<0.015
Copper	mg/l	<0.010	0.01	0.01	0.01	0.01
Hardness	mg/l CaCO3		71	71	215	220
Iron	mg/l	<0.006	0.39	<0.006	0.01	0.03
Lead	mg/l	<8.00	<8.00	<8.00	<8.00	<8.00
Magnesium	mg/l	<0.040	7.4	7.5	20	20
Manganese	mg/l	<0.003	<0.003	<0.003	0.01	<0.003
Molybdenum	mg/l	<0.010	<0.010	0.01	0.01	0.02
Nickel	mg/l	<0.015	<0.015	<0.015	<0.015	<0.015
Phosphorus	mg/l	<0.0410	<0.0410	<0.0410	0.38	<0.0410
Potassium	mg/l	<0.310	3	2.9	12	12
Silicon	mg/l	<0.065	4.4	3	0.68	1.4
Sodium	mg/l	<1.500	8.6	8.7	55	61
Sulphur	mg/l	<0.260	4.9	4.9	46	46
Total Silica	mg/l	<0.14	9.5	6.5	1.4	3.1
Vanadium	mg/l	<0.030	<0.030	<0.030	<0.030	<0.030
Zinc	mg/l	<0.008	<0.008	0.008	<0.008	0.04
Ammonia	mg/l		<0.243	<0.243	<0.243	<0.243
Chloride	mg/l		6.9	9.3	52	71
Cyanide	mg/l		<0.03	<0.03	<0.03	<0.03
Nitrate	mg/l		0.2	0.19	3	3.6
Sulphate	mg/l		18	18	48	49

- Not determined

Indicates higher values for the Bar-plant than for the M-plant

4.4.2 The validation of steroid hormone assays

4.4.2.1 Testosterone ELISA

Figure 4.1 illustrates the standard curve for the testosterone ELISA. The testosterone ELISA has a detection range between 200 and 16 000 pg/ml. A polynomial relationship is displayed between absorbance and testosterone concentration (R^2=0.997). Sensitivity (minimum detection limit) of the assay was 83 pg/ml.

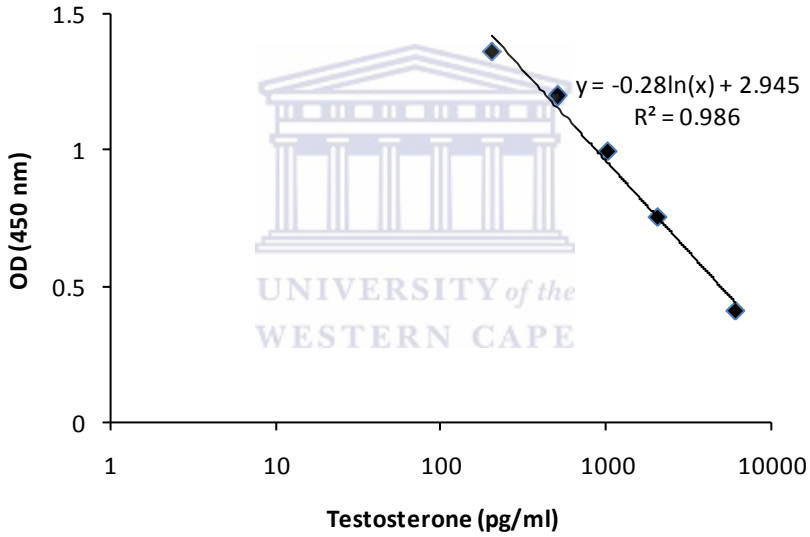

Figure 4.1: Standard curve for the testosterone ELISA to determine hormone concentration in raw and drinking water. The curve was based on concentrated samples.

4.4.2.2 Estrone ELISA

Figure 4.2 illustrates the standard curve for the estrone ELISA. The estrone ELISA has a detection range between 15 and 2 000 pg/ml. Dilutions in the standard curve display a logarithmic relationship with regard to the estrone concentrations in raw and drinking water. A good inverse relationship is displayed between absorbance and estrone concentration (R^2=0.996). Sensitivity (minimum detection limit) of the assay was <6.3 pg/ml.

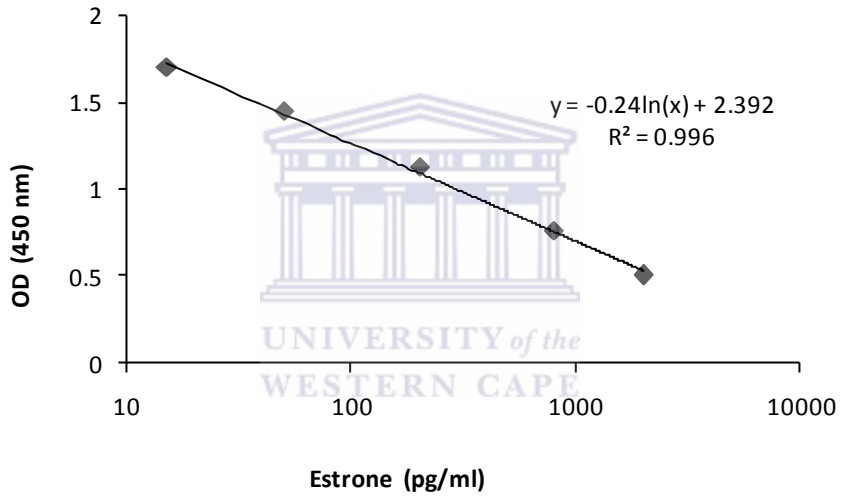

Figure 4.2: Standard curve for the estrone ELISA to determine hormone concentration in raw and drinking water. The curve was based on concentrated samples.

4.4.3 The detection of testosterone in raw and drinking water from the M and Bar plants

Figure 4.3 illustrates testosterone concentration in raw and drinking water from both the M and Bar-plants. In the M-plant, testosterone was detected in raw water at concentrations in the range 1.2 to 3.9 ng/l and in drinking water in the range of 2.6 to 4.9 ng/l for all the seasons. In addition, the concentrations of testosterone were significantly higher in the drinking water during spring (P=0.005) and winter (P=0.036) compared to the raw water. In the Bar-plant, testosterone concentrations detected in raw and drinking water ranged from 1.7 to 7.3 ng/l and 0.2 to 1.5 ng/l respectively, for all the seasons. There were significantly higher concentrations of testosterone detected in the raw water during spring (P=0.039), summer (P=0.031), autumn (P<0.001) and winter (P=0.005) compared to the drinking water. The result shows that the treatment processes in the Bar-plant is efficient in removing these androgens from raw water thus providing safe drinking water to consumers.

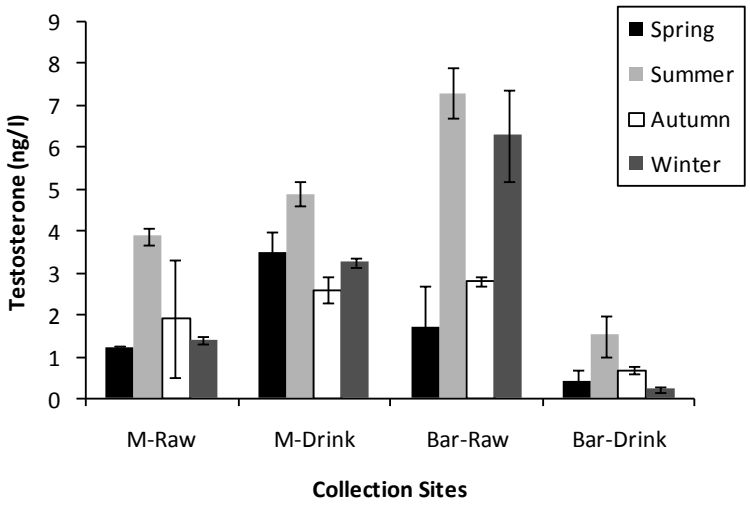

Figure 4.3: The analysis of testosterone levels in raw and drinking water from two drinking water treatment plants. Bars = standard deviation.

4.4.4 The detection of estrone in raw and drinking water from the M and Bar-plants

Figure 4.4 illustrates estrone levels in raw and drinking water from the M and Bar-plants. In the M-plant, the concentration of estrone detected in raw and drinking water ranged from 0.1 to 3 ng/l and 1.1 to 2.5 ng/l respectively, for all the seasons. However, there was no significant difference between the raw and the drinking water during all the seasons. In the Bar-plant, the ranges for estrone concentrations in the raw and drinking water were 4.5 to 12.2 ng/l and 0.6 to 1.6 ng/l, respectively. The raw water has significantly higher concentrations of estrone during spring ($P<0.001$), summer ($P=0.008$), autumn ($P=0.002$) and winter ($P=0.007$) in comparison with the drinking water. This result shows that the treatment processes in the Bar-plant is efficient in removing these estrogens from raw water thus providing safe drinking water to consumers.

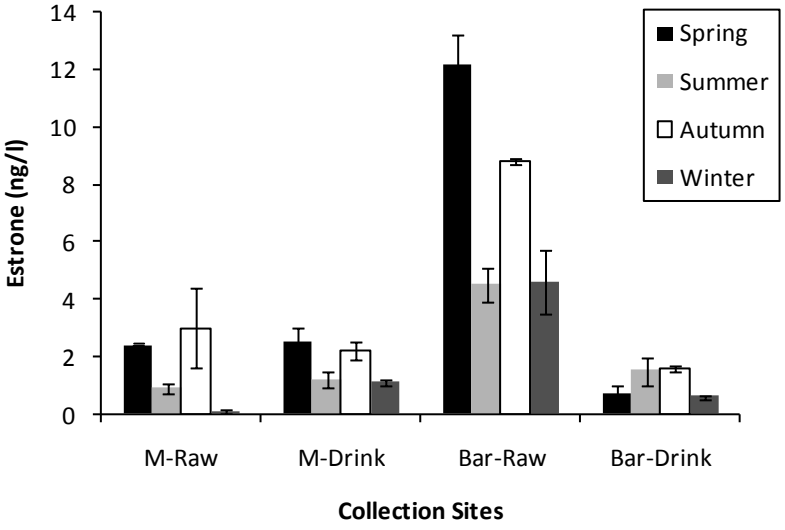

Figure 4.4: The analysis of estrone levels in raw and treated water from two drinking water treatment plants. Bars = standard deviation.

4.5 Discussion

The chemical analysis reveals that all values fall within the World Health Organisation and South African National Standard 241 guidelines for drinking water (WHO, 1993; SANS 241, 2006). Table 4.1, 4.2, 4.3, and 4.4 shows that the levels of hardness, chloride, sulphate, nitrate, sulphur, calcium, magnesium, potassium and sodium are higher for the Bar-plant than for the M-plant. Both plants are located on the same river catchment system. The Bar-plant however, is situated downstream from the M-plant and the increased levels in the above parameters can potentially be attributed to human activity such as mining, wastewater treatment, industrial activities and general urbanisation.

The analysis of estrone and testosterone levels showed a significant difference between the raw and drinking water from both the M and Bar-plants. The raw water from the Bar-plant contains high (>5 ng/l) testosterone levels during summer and winter and high (>5 ng/l) estrone levels during spring and autumn. The concentration of 5 ng/l estrogen was the lowest observed effect concentration (LOEC) that induced endocrine disruption in fish (Kitamura et al., 2009). Steroid hormones can contaminate surface water through discharges from wastewater treatment plants and the runoff from soil after the application of wastewater effluent for irrigation (Desbrow et al., 1998; Ternes et al., 1999), runoff from agricultural fields where animal manure and biosolids are used as fertilizers, and runoff from agricultural feeding operations (Finlay-Moore et al., 2000; Lee et al., 2007). This could offer an explanation for the increased steroid hormone levels in the raw water from both plants. In the Bar-plant, the drinking water however had significantly lower testosterone and estrone levels (<5 ng/l) after treatment. The result shows that the water treatment processes in the Bar-plant

are highly effective in removing these estrogens and androgens from raw water. This can be attributed to the inclusion of GAC as part of the treatment process.

The testosterone levels in the drinking water from the M-plant were found to be higher than the raw water for all the seasons. However, these levels were always below 5 ng/l. Natural steroid hormones are excreted by humans and animals in the urine and faeces as glucoronates or sulphate conjugates. A possible explanation for these elevated levels is that these conjugated forms can be deconjugated back to free hormones during the treatment process and under appropriate environmental conditions (Lintelmann et al., 2003; Rodriguez-Mozaz et al., 2004). In addition, the M-plant does not utilise the granular activated carbon (GAC) adsorption process as opposed to the Bar-plant. Studies have shown that adsorption processes remove steroid hormones to a higher degree than chlorination processes (Westerhoff et al., 2005; Bodzek and Dudziak, 2006). This may indicate that the additional GAC adsorption process in the treatment process increase the effectiveness of plants to remove potential steroids from raw water.

Literature searches done by us found no studies on the effect of UV disinfection on steroid hormone degradation and removal from water or the seasonal variation of steroid hormones in water.

4.6 Conclusion

In conclusion, the raw and drinking water from the two treatment plants have been analysed and compared in terms of the chemical quality and the levels of steroid hormones present. The efficiency of the treatment plants in removing chemicals and steroid hormones were also

assessed. There is currently no guidelines available in South Africa for steroid hormone contamination in drinking water. The results shows high levels of testosterone and estrone in the raw water. Significant levels of these steroid hormones can potentially affect the endocrine system causing endocrine disruption in both wildlife and humans. It is for this reason that treatment processes such as GAC must be considered as part of the treatment if the raw water has high steroid hormone levels. Routine monitoring programmes needs to be set up to screen water resources. Future *in vivo* studies should be done to establish the biological effects of these steroid hormones. One should keep in mind that the steroids studied in this study does not represent the total estrogenicity and androgenicity since chemicals that mimic these hormone were not assessed. Estrogenic activity may be underestimated.

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CHAPTER 5: The immunotoxicity of raw and drinking water from two drinking water treatment plants in Gauteng.

5.1 Abstract

The immune system is a tightly regulated, intricate organ system (Ladics, 2007) which plays a fundamental role in the maintenance of health (Krzystyniak et al., 1995). This system relies on the communication between different immune cell types, cell products, tissues and other organ systems for optimal efficiency (Ladics, 2007). Literature searches have shown that very few studies have been done on the immunomodulatory effects of water pollutants. The aim of this study was therefore to assess the impacts of raw and drinking water from two drinking water treatment plants on specific immune pathway biomarkers using an *in vitro* whole blood culture (WBC) assay. Water samples were collected during spring, summer, autumn and winter from two drinking water treatment plants located on a catchment system in the Vaal river. Whole blood cultures were incubated with the raw and drinking water samples in the presence and absence of stimuli (LPS and PHA). The culture supernatants were then screened for specific immune pathway biomarkers. Cytotoxicity was determined using an LDH assay. The Enzyme Linked Immunosorbant Assays (ELISA's) was used to assess cytokine synthesis. The specific biomarkers used in this study were IL-6 (inflammatory activity), IL-10 (humoral immunity) and IFN-γ (cell mediated immunity). The raw and drinking water samples were not cytotoxic. The addition of drinking water samples from both the M and Bar-plants to unstimulated cultures, had no significant effect on IL-6, IL-10 and IFN-γ secretion for all the seasons compared to the negative control cultures. The addition of raw water from the Bar-plant to unstimulated cultures induced higher IL-6 secretion during autumn and winter, higher IL-10 secretion during winter and had no effect on IFN-γ secretion

compared to the negative control. The addition of raw water from the M-plant to unstimulated WBC had no significant effect on IL-6, IL-10 and IFN-γ secretion. The addition of raw water from both the M and Bar-plants to stimulated WBC induced higher IL-6 secretion during spring and winter compared to the positive control. Additionally, the raw water from the Bar-plant inhibited IL-6 secretion during summer in comparison to the positive control. The addition of raw and drinking water from both plants to stimulated WBC induced higher IL-10 than the control cultures during spring, autumn and winter. The addition of raw water to stimulated WBC induced higher IFN-γ secretion during all the seasons compared to the control. The immunomodulatory effects may, in fact, be due to the presence or absence of microbes and breakdown products pre and post treatment.

5.2 Introduction

The immune system is a tightly regulated, intricate organ system (Ladics, 2007) which plays a fundamental role in the maintenance of health (Krzystyniak et al., 1995). This system relies on the communication between different immune cell types, cell products, tissues and other organ systems for optimal efficiency (Ladics, 2007). Cytokines are soluble protein molecules secreted by immune cells to promote cell to cell communication (Kidd, 2003). With the aid of other molecular components, cytokines play an essential role in the induction and regulation of the innate and acquired immune responses thus maintaining homeostasis (Godoy-Ramirez et al., 2004). Exposure to pollutants via drinking water can potentially cause chronic health effects, which in turn can modulate the functions of cytokines and other mediators of the immune system, thus resulting in immune dysfunction and the development of disease in humans and animals.

The innate immune response can be defined as natural immunity (Pruett, 2003). This immune pathway is present at birth. It provides the first line of defense against pathogenic microorganisms (Hogenkamp et al., 2008). It is mediated by phagocytic cells such as macrophages, neutrophils and dendritic cells and a number of cytokines (Falgarone et al., 2005). The innate immune response is triggered upon pathogen recognition by pattern recognition receptors (PRR) on the surface of phagocytic cells. These PRRs recognise molecular carbohydrate patterns expressed on the surfaces of invading microbes (Medzhitov and Janeway, 1997). This results in the induction of a pro-inflammatory cascade of cell signalling to defend against the invading microbe (Dalpke et al., 2008).

Interleukin 6 (IL-6) plays a significant role in mediating the innate immune response (Akira et al., 1990). IL-6 initiates inflammatory events via the activation of T lymphocytes, differentiation of B lymphocytes and the induction of acute phase protein synthesis by hepatocytes (Jones et al., 2001). It is classified as both a proinflammatory and anti-inflammatory cytokine (Steensberg et al., 2003; Dienz and Rincon, 2008). This cytokine is produced by antigen presenting cells (APCs) such as dendritic cells, macrophages and B cells (Kamimura et al., 2003). A response to external stimuli such as IL-1, TNF α and platelet derived growth factor induces IL-6 production (Heinrich et al., 2003).

The acquired immune responses develop upon post partum exposure to a pathogen and displays immunological memory. This response is mediated by interactions between antigen presenting cells, T-helper cells (Th 1 and Th 2), B lymphocytes, T cytotoxic cells (Tc) as well as several cytokines for efficient pathogen defences to occur (Pruett, 2003). Acquired immunity is divided into two branches namely, humoral and cell mediated immune pathways (Ladics, 2007).

The humoral immune response is mediated by the Th2 cells and is characterized by the production of Th2 cytokines interleukin 4 (IL-4), interleukin 5 (IL-5) and interleukin 10 (IL-10) (Ladics, 2007). In turn, these cytokines aid B lymphocyte proliferation and differentiation into antibody-secreting plasma cells (Ladics, 2007). The Th2 response provides immunity against extracellular pathogens such as bacteria, fungi and yeast (Twigg, 2005). Interleukin-10 (IL-10) is produced by B and T cells, monocytes and macrophages (Moore et al., 2001; Pestka et al., 2004). IL-10 was initially named cytokine-synthesis inhibitory factor because of its ability to inhibit the production of cytokines IL-2, IFN-γ and TNF-α by murine Th 1 cells (Fiorentino et al., 1989). In humans, IL-10 inhibits Th 1 cell

activation and cytokine production, as well as their proliferation and chemotaxis (Mocellin et al., 2004). IL-10 inhibits Th 1 and natural killer (NK) cell cytokine production by way of inhibiting accessory cell function. Studies have also shown that IL-10 inhibits a wide range of activated macrophage and/or monocyte functions, amongst others, NO production, monokine synthesis, class II MHC expression and co-stimulatory molecule expression such as IL-12 and CD80/CD86 (Bogden et al., 1991; de Waal et al., 1991; Ding et al., 1993; Mocellin et al., 2004).

The cell mediated immune response is mediated by the T-helper 1 (Th1) cells and is characterized by the production of cytokines such as interleukin 2 (IL-2), tumor necrosis factor beta (TNF-β) and interferon gamma (IFN-γ) (Xie et al., 2008). The Th 1 response is required for the induction of cytotoxic activity by T cells, macrophages and natural killer (NK) cells and provides immunity against intracellular pathogens such as viruses, certain bacteria, protozoa and tumor development (Pietrzak et al., 2008; Xie et al., 2008). The cytokine interferon-gamma (IFN-γ) is the principal mediator of innate and acquired immune responses. It is mainly produced by Th1 $CD4^+$ T cells, $CD8^+$ T cells and natural killer cells (Muhl and Pfeilschifter, 2003). IFN-γ activates monocytes and macrophages and promotes antibody isotype switching by B cells which binds complement and promotes phagocytosis (Sinigaglia et al., 2000). It enhances antigen presentation through the MHC-I and MHC-II pathways and regulates the cell cycle, growth and apoptosis of various cell types (Boehm et al., 1997; Schroder et al., 2004). IFN-γ also regulates immune function by inhibiting Th2 $CD4^+$ T cell development (Chen and Liu, 2009).

The aim of this study is to evaluate the effectiveness of water treatment processes of two drinking water plants on the Vaal River, Gauteng to remove immunotoxins. The one plant

gets raw water from a relatively “clean” site while the other plant receives raw water that may be contaminated due to human activity. The specific aims of this study are to determine the inflammatory potential of the raw and treated water from both plants and determine the effects of raw and treated water from both plants on the humoral and cell mediated pathways of the immune system.

5.3 Materials and Methods

5.3.1 Collection of water samples

Water samples were collected in 1 x 1000 ml sterile glass bottles. Samples were collected in September (Spring), February (Summer), May (Autumn) and August (Winter). Water samples were stored at 4 °C and processed within 24 hours of collection. Distilled water was used as a control. Neat water samples were stored at -20 °C.

5.3.2 Blood collection

Human blood was obtained from healthy male volunteers. Blood samples were collected directly into 4 ml sodium citrate vacuette tubes (Greiner Bio-One GmbH, Kremsmünster, Austria) via venous puncture. The blood was stored at ambient temperature and processed within 4 hours after collection under sterile conditions in a laminar flow cabinet. Informed consent was obtained from each individual.

5.3.3 Whole blood culture for inflammatory activity

Whole blood cultures were performed using unstimulated and stimulated diluted blood. Unstimulated blood consisted of 2 ml blood and 18 ml RPMI-1640 (Roswell Park Memorial Institute 1640) (Sigma-Aldrich, St. Louis, MO, U.S.A.). For stimulated blood, 5 µl of a 1 mg/ml lipopolysaccharide (LPS) (Sigma-Aldrich, USA) solution was added to 2 ml of blood and 18 ml of RPMI-1640 medium. Samples and a distilled water control were added at 10 µl/well to 96 well cell culture plates (Nalge Nunc International, Thermo Fisher Scientific,

NY, USA). Stimulated or unstimulated blood were then added at 200 µl/well. Samples and controls were run in duplicate and incubated overnight at 37 °C in a 5 % CO_2 incubator (Thermo Scientific, Heraeus, HERA cell CO_2 incubator, U.S.A.). After the overnight incubation, supernatants were removed and analysed for cytokine secretion and cytotoxicity.

5.3.4 Whole blood culture for T helper cytokine synthesis

Whole blood cultures were performed using unstimulated and stimulated diluted blood. Unstimulated blood consisted of 2 ml blood and 18 ml RPMI-1640. For stimulated blood, 125 µl of a 1.6 mg/ml phytohemagglutinin (PHA) solution in RPMI-1640 was added to 2 ml of blood and 17.875 ml RPMI-1640 medium. Samples and a distilled water control were added at 10 µl/well to 96 well cell culture plates (Nalge Nunc International, Thermo Fisher Scientific, NY, USA). Stimulated or unstimulated blood was then added at 200 µl/well. Samples and controls were run in duplicate and incubated for 48 hours at 37 °C in a 5 % CO_2 incubator. After the incubation period, supernatants were removed and analysed for cytokine secretion.

5.3.5 Cytotoxicity assay

Lactate dehydrogenase (LDH) release into culture medium was used as a biomarker for cytotoxicity. A commercially available chromogenic LDH assay kit (Cytotoxicity Detection Kit (LDH), Roche Applied Science, Germany) was used to analyse the samples. For the 100 % standard, a control blood sample received 2.5 µl of a 10 % sodium dodecyl sulphate (SDS) solution to 250 µl of whole blood in medium. The cells lysed immediately and the lysate was used as the 100 % LDH standard. Dilutions of this sample were used to construct a standard

curve. Samples and dilutions of the standard were added to the plate at 10 µl/well. LDH kit substrate was added to all wells at 50 µl/well. The plate was read immediately at 592 nm and thereafter at 10 minute intervals. Between readings, the plate was incubated at room temperature in the dark.

5.3.6 Cytokine ELISA's

The cytokines IL-6, IL-10 and IFN-γ in culture supernatants were analysed using commercially available ELISA kits (Human IL-6, IL-10 and IFN-γ ELISA Ready-SET-Go!, eBioscience, Inc., San Diego, U.S.A.). All the reagents required for the ELISA were supplied with the kit. Assays were performed according to the manufacturer's instructions. In brief: 96 well ELISA plates (Nunc-Immuno plate, Maxisorp) were coated with 50 µl/well capture antibody diluted appropriately in coating buffer and incubated overnight at 4 °C. The wells were aspirated and washed 5 times with wash buffer (PBS, Tween-20 and distilled water). Non-specific binding sites were blocked with 100 µl/well assay diluent and incubated for 1 hour at room temperature. The wells were aspirated and washed 5 times with wash buffer. The standards were diluted appropriately in assay diluent and 2-fold serial dilutions of recombinant human cytokine standard (1 µg/ml) was performed. Cell free supernatants were added at 50 µl/well in duplicate to the 96 well plate. The plate was sealed and incubated at room temperature for 2 hours. The wells were aspirated and washed 5 times with wash buffer. Biotinylated detection antibody was added at 50 µl/well and incubated at room temperature for 1 hour. The wells were aspirated and washed 5 times with wash buffer. Bound cytokine was detected by adding 50 µl/well enzyme-conjugate (Avidin-horse radish peroxidase). The plate was incubated for 30 minutes. The wells were aspirated and washed 7 times with wash buffer. Tetramethylbenzidine (TMB) substrate was added at 50 µl/well and

incubated for approximately 15 minutes. The reaction was stopped with 50 µl/well 2M sulphuric acid (H_2SO_4) and the absorbance values read at 450 nm on an ELISA plate reader. (Thermo Electron Corporation, Multiskan, EX, Taiwan).

5.3.8 Statistical analysis

All data is presented as mean ± standard deviation (STD). Statistical significance was determined by one way analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.050$.

5.4 Results

5.4.1 The effect of water on cellular toxicity

The LDH assay was used as biomarker for cytotoxicity. The standard curve was used to determine the percentage toxicity in unstimulated and stimulated WBC induced by raw and drinking water. Figure 5.1 illustrates the standard curve for the LDH assay. A good correlation is observed between absorbance values and percentage toxicity ($R^2 = 0.997$).

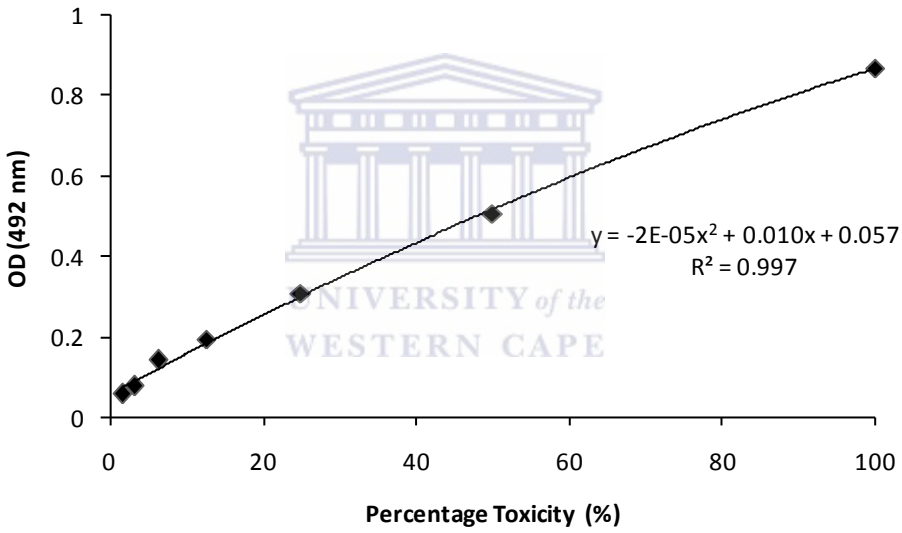

Figure 5.1: Standard curve for percentage toxicity versus corrected OD.

After assessing the cytotoxicity of raw and drinking water samples, data showed that the percentage toxicity of all samples were between 0-1 % different from the control indicating no significant toxicity in any of the samples (Figure 5.2).

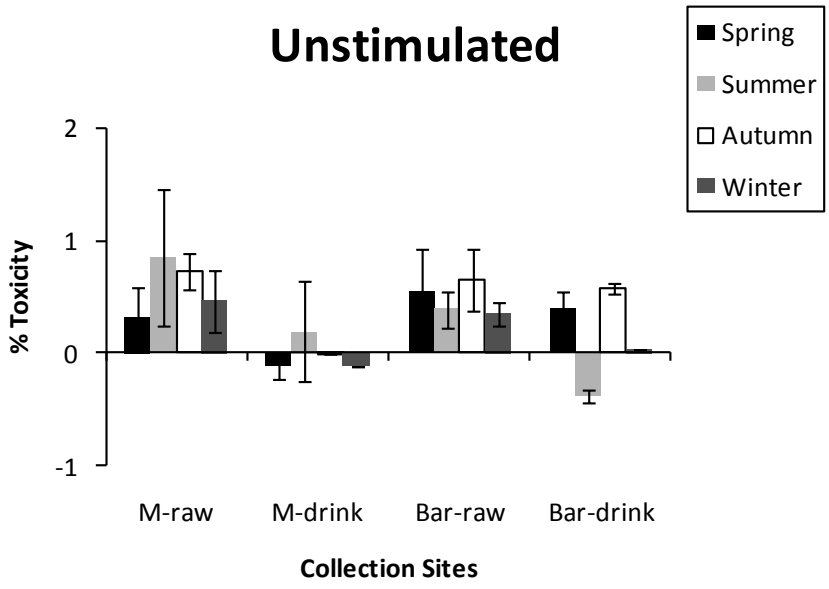

Figure 5.2: The cytotoxicity of raw and drinking water samples in unstimulated whole blood cultures. Data expressed as percentage toxicity.

5.4.2 Validation of the whole blood culture assay to monitor cytokine synthesis

5.4.2.1 Interleukin 6 (IL-6)

IL-6 was used as a biomarker for inflammatory activity. A standard curve was used to calculate the cytokine concentration in unstimulated and stimulated whole blood cultures. Figure 5.3 illustrates the standard curve for the IL-6 ELISA. A second order polynomial relationship is displayed between absorbance and IL-6 concentrations ($R^2 = 0.997$). The IL-6 ELISA has a detection range between 31 and 2 000 pg/ml.

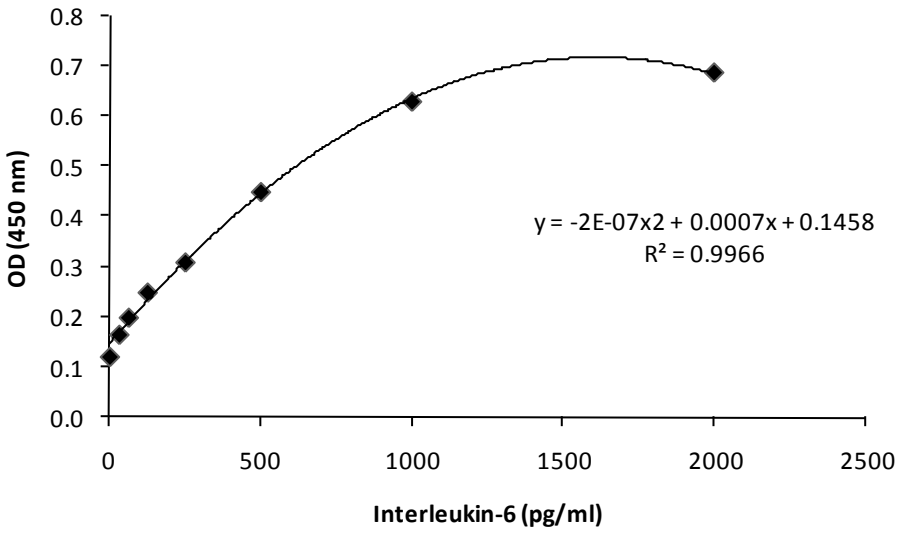

Figure 5.3: Standard curve for IL-6 ELISA assay. The standard curve shows that there is a good correlation (R^2=0.997) between absorbance and IL-6 concentration.

5.4.2.2 Interleukin 10 (IL-10)

IL-10 was used as a biomarker for humoral immunity. A standard curve was used to calculate the cytokine concentration in unstimulated and stimulated whole blood cultures. Figure 5.4 illustrates the standard curve for the IL-10 ELISA. A linear relationship is displayed between absorbance and IL-10 concentration (R^2 = 0.990). The IL-10 ELISA has a detection range between 21 and 1 333 pg/ml.

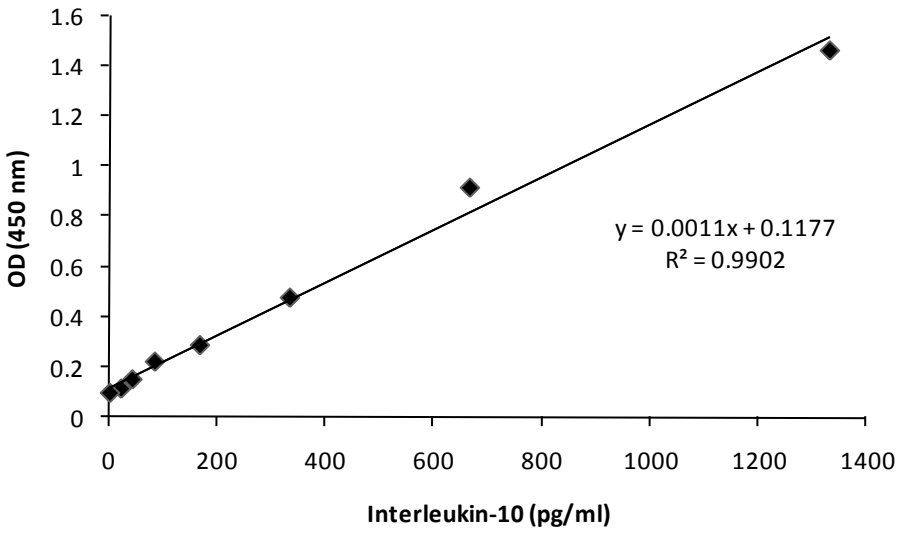

Figure 5.4: Standard curve for IL-10 ELISA assay. The standard curve shows that there is a good correlation (R^2 = 0.990) between absorbance and IL-10 concentration.

5.4.2.3 Interferon gamma (IFN-γ)

IFN-γ was used as a biomarker for cell mediated immunity. A standard curve was used to calculate the cytokine concentration in unstimulated and stimulated whole blood cultures. Figure 5.5 illustrates the standard curve for the IFN-γ ELISA. A linear relationship is displayed between absorbance and IFN-γ concentration (R^2= 0.999). The IFN-γ ELISA has a detection range between 63 and 4 000 pg/ml.

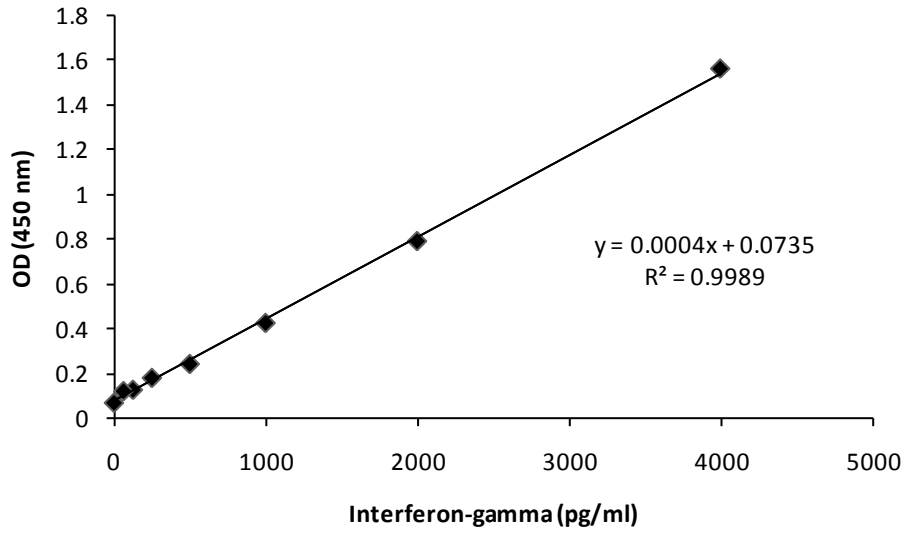

Figure 5.5: Standard curve for IFN-γ ELISA assay. The standard curve shows that there is a good correlation (R^2 = 0.999) between absorbance and IFN-γ concentration.

5.4.2.4 Mitogen stimulation

To assay unstimulated and stimulated cytokine production, whole blood was cultured in medium in the presence/absence of a stimulant. The specific stimulant depends on the cell population and immune pathway of interest. LPS, a component of the cell membrane of gram-negative bacteria, is a well characterized stimulus of pro-inflammatory leukocyte responses such as IL-6 release from monocytes and macrophages. In contrast PHA, a mitogenic lectin, is predominantly a stimulant of T lymphocyte cell proliferation and is therefore used for the cytokine specific cell mediated (Th1) and humoral (Th2) immune pathways. Table 5.1 illustrates that in the stimulated cultures, a mitogen dependant increase in cytokine IL-6, IL-10 and IFN-γ concentrations can be seen in comparison to unstimulated cultures.

Table 5.1: The effect of the absence/presence of mitogen LPS or PHA on whole blood cultures exposed to a distilled water control. The data is presented as the mean ± standard deviation (SD).

Cytokine	Unstimulated	Stimulated
IL-6 (LPS)	26.408 ± 4.585	613.841 ± 27.106
IL-10 (PHA)	18.455 ± 17.999	115.273 ± 18.642
IFN-γ (PHA)	191.250 ± 10.607	825 ± 68.943

5.4.3 The immunotoxicity of raw and drinking water from the M-plant

Figure 5.6 depicts a bar graph for IL-6 concentrations of unstimulated and stimulated whole blood cultures (WBC) exposed to raw and drinking water. In unstimulated WBC, both the raw and drinking water did not induce IL-6 levels significantly different to the control. This indicates that the samples do not induce an inflammatory response. In stimulated WBC, the raw water induced significantly higher IL-6 levels during spring ($P<0.001$) and winter ($P<0.001$) compared to the control. There was no significant difference between IL-6 levels induced by the drinking water during all the seasons compared to the control. However, the drinking water induced significantly lower IL-6 secretion during spring ($P=0.002$) and winter ($P<0.001$) compared to the raw water.

Figure 5.7 depicts a bar graph for IL-10 concentrations of unstimulated and stimulated WBC exposed to raw and drinking water. Addition of raw and drinking water to unstimulated WBC did not induce IL-10 levels significantly different to the control. The drinking water induced very low levels of IL-10 for all the seasons. Addition of raw water to stimulated WBC

resulted in a significantly higher IL-10 secretion during spring (P<0.001), autumn (P=0.028) and winter (P=0.02) compared to the control. The addition of drinking water to stimulated WBC did not induce IL-10 levels significantly different to the control. At the same time, drinking water induced significantly lower IL-10 secretion during spring (P<0.001) compared to the raw water.

Figure 5.8 depicts a bar graph for IFN-γ concentrations of unstimulated and stimulated WBC exposed to raw and drinking water. Addition of raw and drinking water to unstimulated WBC did not induce IFN-γ levels significantly different to the control. However, the addition of raw water to stimulated WBC induced significantly higher IFN-γ production during summer (P<0.001) and winter (P<0.001) compared to the control. Furthermore, drinking water induced significantly lower IFN-γ production during summer (P<0.001) and winter (P<0.001) compared to the raw water.

5.4.4 The immunotoxicity of raw and drinking water from the Bar-plant

Figure 5.6 depicts a bar graph for IL-6 concentrations of unstimulated and stimulated whole blood cultures (WBC) exposed to raw and drinking water. In unstimulated WBC, the raw water induced significantly higher IL-6 secretion during autumn (P=0.005) and winter (P<0.001) compared to the control. The drinking water however, did not induce significant IL-6 secretion for all the seasons compared to the control. This indicates that the samples do not induce an inflammatory response. In stimulated WBC, the raw water induced significantly higher IL-6 secretion during spring (P<0.001) and winter (P=0.022) in comparison to the control. However, raw water induced a significant decrease in IL-6 secretion during summer (P=0.015) compared to the control. The drinking water shows the

same trend with significantly lower IL-6 secretion during summer (P=0.029) compared to the control.

Figure 5.7 depicts a bar graph for IL-10 concentrations of unstimulated and stimulated WBC exposed to raw and drinking water. In unstimulated WBC, the raw water induced significantly higher IL-10 secretion during winter (P=0.009) compared to the control. The drinking water induced low levels of IL-10 for all the seasons. In stimulated WBC, the raw water induced significantly higher IL-10 secretion during spring (P<0.001) and winter (P<0.001) compared to the control. The bar graph also shows that drinking water induced significantly higher IL-10 during spring (P<0.001) compared to the control. Furthermore, drinking water induced significantly lower IL-10 during winter (P<0.001) compared to the raw water.

Figure 5.8 depicts a bar graph for IFN-γ concentrations of unstimulated and stimulated WBC exposed to raw and drinking water. In unstimulated WBC, both the raw and drinking water did not induce significant IFN-γ secretion compared to the control. However, in stimulated WBC, the raw water induced significantly higher IFN-γ secretion during spring (P<0.001), summer (P<0.001), autumn (P<0.001) and winter (P<0.001) compared to the control. The drinking water induced significantly higher IFN-γ cytokine secretion during spring (P=0.005) and winter (P<0.001) compared to the control. At the same time, drinking water induced significantly lower IFN-γ secretion during autumn (P<0.001) and winter (P<0.001) compared to the raw water.

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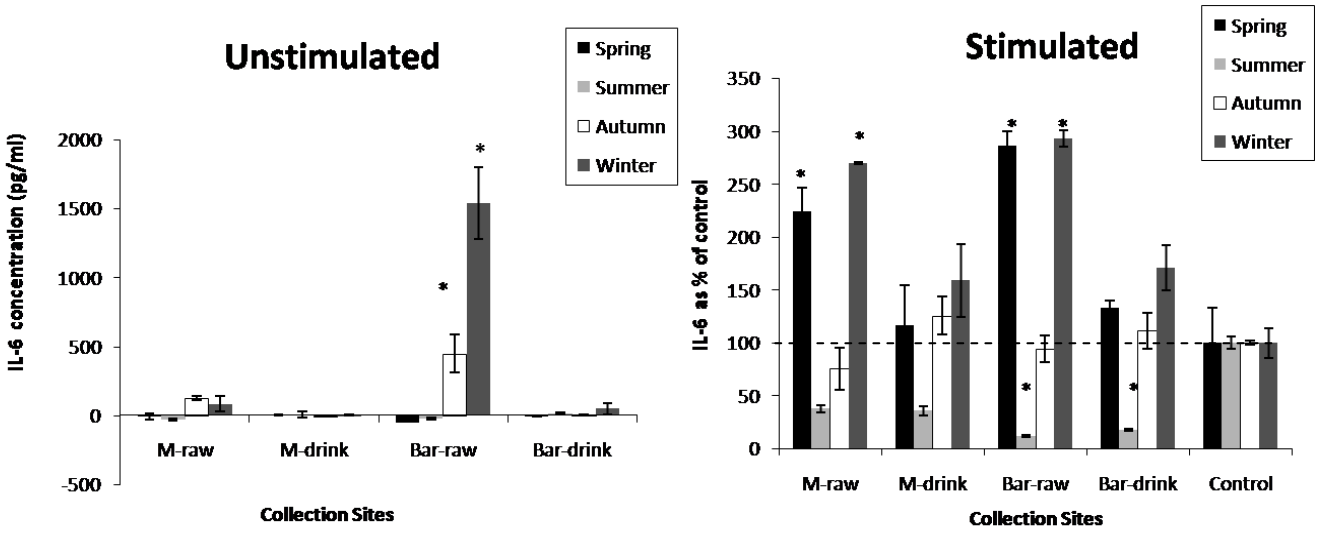

Figure 5.6: IL-6 production of human whole blood cultures exposed to raw and drinking water. (A) Data presented as pg/ml in the absence of a stimulus. (B) Data presented as percentage of control in the presence of a stimulus (LPS). * Statistical significance (P<0.050) compared to the control. Bars = standard deviation.

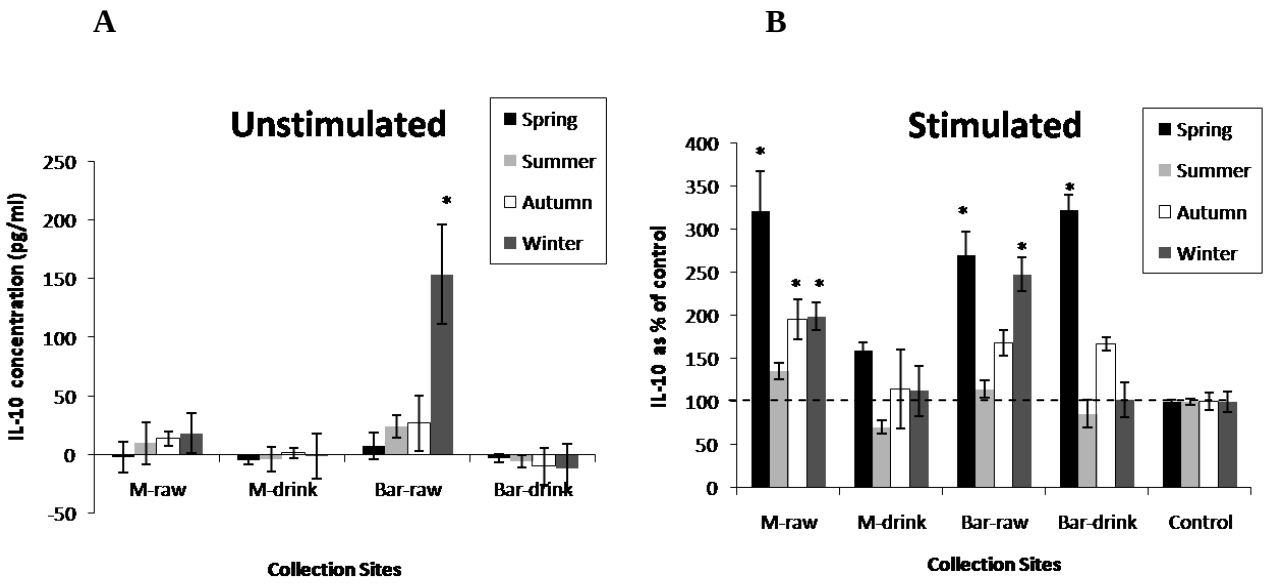

Figure 5.7: IL-10 production of human whole blood cultures exposed to raw and drinking water. (A) Data presented as pg/ml in the absence of a stimulus. (B) Data presented as percentage of control in the presence of a stimulus (PHA). * Statistical significance ($P<0.050$) compared to the control. Bars = standard deviation.

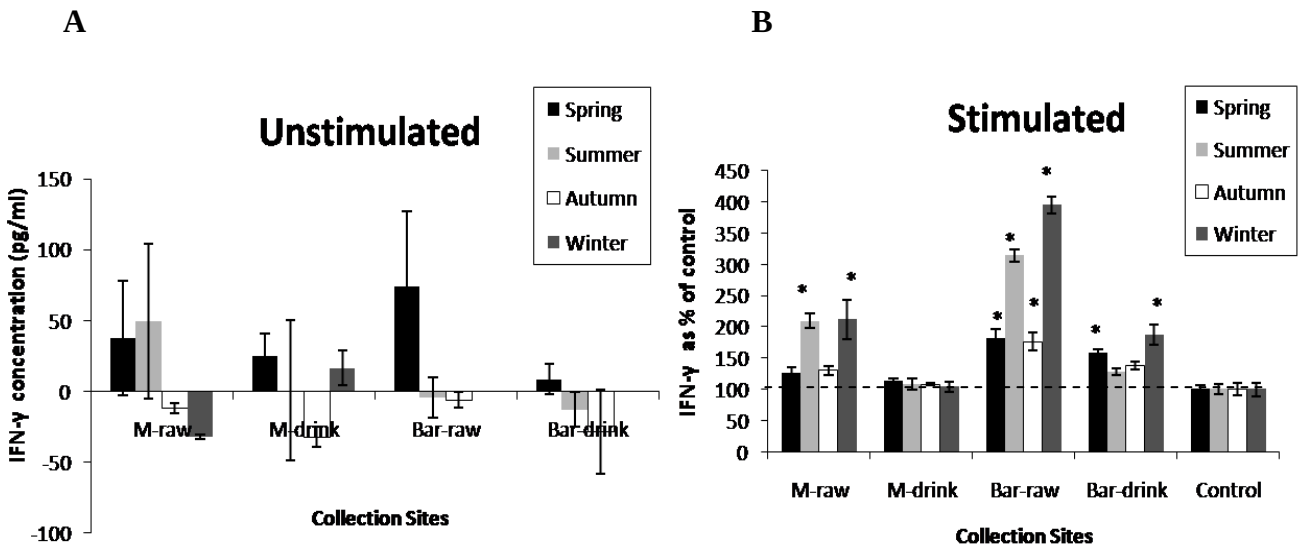

Figure 5.8: IFN-γ production of human whole blood cultures exposed to raw and drinking water. (A) Data presented as pg/ml in the absence of a stimulus. (B) Data presented as percentage of control in the presence of a stimulus (PHA). * Statistical significance ($P<0.050$) compared to the control. Bars = standard deviation.

5.5 Discussion

Raw and drinking water samples showed no cytotoxic effects on whole blood cultures. The LDH assay is a sensitive assay that determines cytotoxicity, therefore the results obtained from this assay does not exclude the effects on specific cellular pathways. Studies have shown that the absence of cytotoxicity does not necessarily indicate that the samples have no effect on the biochemical processes of the cell (Ganey et al., 1993). Therefore, it was decided to further analyse the raw and drinking water samples for their effects on the immune system using specific biomarkers for immunotoxicity.

The pleiotropic cytokine, IL-6 was used as a biomarker to determine the inflammatory activity of the raw and drinking water from both treatment plants. IL-6 production is relatively low in homeostatic conditions, but can be upregulated rapidly in response to viruses, bacteria or chronic inflammation (Vaisman et al., 2003; Naugler and Karin, 2007). The results from this study indicate that in unstimulated whole blood cultures, the drinking water samples from both plants did not induce inflammatory activity during all the seasons. However, the raw water from the Bar-plant induced high inflammatory activity in autumn and winter respectively. This may be linked to an upregulation of monocytes or macrophages (Krakauer, 1998) in response to microbial pathogens or breakdown products present in the raw water. Antibodies have the ability to neutralise microbial toxins, activate complement and opsonise bacteria for phagocytosis (Parkin and Cohen, 2001). This result was to be expected because the Bar-plant receives raw water that is contaminated due to human activity. In addition, Gauteng experiences its rainy season in summer rather than winter. Due to this factor, substances inducing inflammatory activity are more concentrated when there is no rain and thus low river flow.

In stimulated cultures, the raw water from both the M-plant and the Bar-plant induced high inflammatory activity during spring and winter. This may potentially lead to hypersensitivity reactions such as allergies. Hypersensitivity is an exacerbated immune response resulting in tissue damage (Putman et al., 2003). However, both the raw and drinking water from the Bar-plant inhibits inflammatory activity during summer. A possible explanation could be that Gauteng experiences its rainfall during summer and therefore contaminants present in the raw water are washed away during river flow. Upon treatment of the raw water in the two plants, the level of inflammatory activity decreased significantly for all the seasons to give drinking water that induced low levels of IL-6 upon exposure to whole blood cultures.

IL-10 is produced by macrophages, B cells and T cells and in the current study it is as used as a biomarker for humoral immunity. IL-10 directs Th0 differentiation to T-helper 2 cells which stimulate antibody production by B lymphocytes (Mocellin et al., 2004). This allows the host to effectively fight off extracellular pathogens such as bacteria, fungi and yeast (Twigg, 2005). In this study the drinking water collected from both the Bar and M-plants induced low IL-10 secretion for all the seasons in unstimulated whole blood cultures. This indicates that there was no extracellular pathogens or their breakdown products present at high enough levels in the drinking water to activate the humoral immune response. The raw water from the Bar-plant however, induced high IL-10 secretion during winter in unstimulated Whole blood cultures. This indicates that the raw water is contaminated with extracellular pathogens such as bacteria and helminths, which activates the immune system to mount an effective humoral response. In addition, Gauteng experiences a dry winter therefore the immunostimulatory substances present are more concentrated in the surface water due to low river flow.

In stimulated cultures, the raw from both the M and Bar-plants induce higher IL-10 than the control cultures during spring, autumn and winter indicating overstimulation of the humoral response. This increased humoral response can lead to a more effective defense against extracellular pathogens. However, overstimulation of humoral immunity can also cause hypersensitivity reactions. The drinking water from the Bar-plant induced higher IL-10 during spring than both the control cultures and the raw water. It is known that during the treatment process, chemicals may be added to water to alter properties of the finished water (Bates, 2000). An increase in calcium and calcium carbonate was noted in drinking water from the Bar-plant during spring, which could possibly have been added during the treatment process. Calcium is an essential second messenger that controls the activation of lymphocytes in the presence of a pathogen. It also plays a crucial part in T lymphocyte proliferation (Berridge et al., 1999; Berridge et al., 2000). Kinases play a crucial role in regulating immune system responses and are calcium dependent (Parkin and Cohen, 2001; Racioppi and Means, 2008). The increase in calcium and calcium carbonate allowed optimal IL-10 induction by drinking water therefore mounting an effective humoral response. After treatment of the raw water in the two plants, the level of IL-10 decreased significantly to give drinking water that induced lower levels of IL-10 upon exposure to whole blood cultures.

No significant effect on IFN-γ production was observed for unstimulated WBC exposed to raw and drinking water from the M-plant and the Bar-plant. These values were near to the lower detection limit of the ELISA kit. In the stimulated cultures however, hypersensitivity can be seen in raw water from both plants as IFN-γ levels exceed the control. This occurs during all four seasons. The stimulated cultures also show that the drinking water from the Bar-plant induced IFN-γ levels during spring and winter but at a lower level compared to raw water. These concentrations exceed the control. This could possibly be associated with the

increase in calcium, and its direct effect on calcium-dependant kinases which regulates an optimal cell-mediated immune response (Racioppi and Means, 2008). Increased IFN-γ directs Th0 differentiation to Th1 cells, indicating upregulation of cell mediated response. The cell mediated responses combat intracellular pathogens such as viruses and mycobacteria (Janeway et al., 2005) which may be present in the raw water from both plants. Upon treatment of the raw water in the two plants, the level of IFN-γ decreased significantly to give drinking water that induced lower levels of IFN-γ upon exposure to whole blood cultures.

5.6 Conclusion

In conclusion, the raw and drinking water from the two treatment plants have been analysed and compared in terms of their effects on specific immune pathway biomarkers, and the efficiency of their respective treatment plants in removing immunotoxins to give drinking water that is safe for human consumption. The results of this study show that raw water from the Bar-plant initiates inflammatory responses, humoral and cell mediated immunity. The immunomodulatory effects observed is a response to immunotoxin contamination in the water resources. Due to the potential adverse effects on immune function, it is important to set up routine monitoring programmes to screen water resources intended for domestic, agricultural, and recreational use for immunotoxins. The implementation of the assays used in this study will allow early detection of immunotoxin contamination and potentially avert these immune defects, which could have major health and economic implications for the country. This study forms part of a national programme that investigates the efficiency of drinking water treatment plants to remove potentially harmful substances present in raw water to give drinking water that is safe for human consumption.

5.7 References

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CHAPTER 6: Summary

6.1 Conclusion

Water is an important natural resource and life-sustaining drink to both humans and animals. The provision of a safe water supply is therefore essential for safeguarding the health and well-being of all. However, there is a great concern regarding the pollution of this natural resource by pathogenic microorganisms, toxic chemical compounds and natural hormones. Pollutants have the ability to affect various physiological systems and target the general health of humans and wildlife. Certain water pollutants are also known for their immunotoxic properties and ability to modulate specific immune pathways.

Cytokines are soluble intracellular factors that promote cell to cell communication in order to regulate and maintain immune function. Cytokines play a key role in defending the body against intracellular and extracellular pathogen invasion, certain cancers as well as toxic compounds. IL-6 mediates innate immunity by initiating inflammatory events in response to an infectious challenge. Humoral immunity is mediated by T-helper 2 cells and is characterised by the production of IL-10. This cytokine drives the proliferation of B cells into antibody-secreting plasma cells and provides defense against bacteria and parasites. Cell mediated immunity is mediated by T-helper 1 cells and is characterised by the production of IFN-γ. This cytokine induces the cytotoxic activity of natural killer cells and provides defense against viruses and *Mycobacterium tuberculosis*. Whilst these cytokines assist in defending the host against pathogenic and chemical insults, over production or suppression of these cytokines may lead to pathophysiological effects. Therefore, these cytokines are used as biomarkers for immunotoxicity testing.

Raw and drinking water samples from two drinking water treatment plants have been assessed in terms of chemical quality, steroid hormone activity, effects on specific immune pathways and the effectivity of treatment processes utilised by the plants.

The Bar-plant depicted higher levels of inorganic chemicals calcium, chloride, hardness, magnesium, nitrate, potassium, sodium, sulphate and sulphur than the M-plant. However, all the values fall within the South African National Standard and World Health Organisation guidelines for drinking water. The Bar-plant is located downstream from the M-plant on the same catchment system. Therefore, the increased levels in the above parameters can be attributed to mining, wastewater treatment, industrial activities and general urbanisation.

High testosterone levels (>5 ng/l) were detected in raw water from the Bar-plant during summer and winter and high estrone levels (>5 ng/l) during spring and autumn. Steroid hormones can contaminate water resources through wastewater discharges and run off from agricultural activities. The treatment processes, especially the GAC adsorption, used by this plant were highly effective in removing these steroid hormones (< 5ng/l) from raw water thus providing safe drinking water for human consumption. On the other hand, higher testosterone levels were detected in drinking water from the M-plant compared to raw water during all four seasons. However, these testosterone levels detected in the drinking water from the M-plant were always below 5 ng/l. Steroid hormones are excreted into the environment as conjugates by both animals and humans. These conjugates have the ability to biotransform back to free hormone during treatment processes. The higher testosterone levels may reflect the free hormones. The estrone levels showed no significant difference between the raw and the drinking water from the M-plant.

Raw and drinking water from both plants were not cytotoxic to human whole blood cells. This study shows that the drinking water from both the M and Bar-plants did not induce significant IL-6, IL-10 and IFN-γ secretion for all the seasons in unstimulated cultures. This indicates that the treatment processes employed were effective in removing contaminants, which could activate the inflammatory response as well as the humoral and cell-mediated immune pathways. The raw water from the Bar-plant induced higher IL-6 during autumn and winter, higher IL-10 during winter and had no effect on IFN-γ secretion for all four seasons. These responses indicate the presence of microbial pathogens in the raw water. The raw water from the M-plant had no significant effect on IL-6, IL-10 and IFN-γ secretion in unstimulated cultures.

Raw from both the M and Bar-plants induced high IL-6 secretion during spring and winter in stimulated cultures. This indicates an overstimulation of the inflammatory response, which can develop into a hypersensitivity reaction. Both the raw and drinking water suppress the inflammatory response during summer. Due to seasonal rains, the contaminants present in the raw water are washed away during river flow. The data also shows that the plants were effective in removing contaminants which induce inflammation. The IL-10 data shows that both the raw and drinking water induce IL-10 secretion during spring, autumn and winter. This indicates overstimulation of the humoral immune response and the possibility of developing a hypersensitivity reaction. Once again the treatment processes from both plants were effective in removing contaminants which could activate the humoral immune response. The raw water from both plants induces high IFN-γ secretion for all the seasons. This indicates the upregulation of the cell mediated responses which combat intracellular pathogens. This data reflects the efficiency of both treatment plants in removing contaminants which can activate inflammatory, humoral and cell mediated responses.

6.2 Future recommendations

All water resources intended for domestic, agricultural and recreational use should be routinely monitored and screened for immunotoxins. If high responses are detected, the removal efficiency should be investigated and then possibly be monitored. The implementation of the following assays can help prevent adverse health effects associated with the exposure to water pollutants and immunotoxins:

- Steroid hormone ELISA's which can successfully quantitate the levels of testosterone and estrone in water.
- Whole blood cultures which are sensitive in detecting immunotoxins.

One also has to keep in mind that these results reflect *in vitro* immunomodulatory effects and further *in vivo* studies are required to characterise the nature of the responses.