

**AN ASSESSMENT OF WATER QUALITY AND
ENDOCRINE DISRUPTION ACTIVITIES IN THE
EERSTE/KUILS RIVER CATCHMENT SYSTEM,
WESTERN CAPE, SOUTH AFRICA.**

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature

Date

Abstract

Water quality analysis forms the basis in assessing and monitoring catchments. As urban development continuously increase, pollution sources increase in either point source (wastewater treatment works, industrial effluents) and/or non-point source origin (storm water discharge, domestic pollutants), accumulating pollutants in the environment. It was only recently discovered that certain pollutants have subtle disrupting effects on the endocrine system resulting in health related problems associated with the reproductive system and thyroid system (growth and development) of animals and potentially humans. Natural water resource management proves to include limited biological assays measuring endpoints for cytotoxicity, inflammatory activity and endocrine disruption. The broad objective of this study was therefore to include several bioassays, not normally used in municipal (City of Cape Town) monitoring programmes, along with water quality data collected by the City of Cape Town. The Eerste/Kuils River catchment system, Western Cape, under the auspices of the City of Cape Town was chosen, and although this catchment does not contribute to drinking water resources, is subjected to a range of anthropogenic influences (industrial effluents, household wastewater, agricultural runoff). Within the short time-frame available for this study (six months) two months, July (following a dry summer and autumn season) and October (following a wet winter and early spring season) were selected for water quality monitoring. Spatial variation (with relevance to specific point and non-point contamination) among sampling sites were also obtained by choosing several (n=10) along the catchment. Specific aim of the study therefore included: Firstly (Chapter 2), the use of *in vitro* bioassays, lactate-dehydrogenase assay (LDH) for cytotoxic activity, pro-inflammatory hormone Interleukin-6 (IL-6) secretion by human blood cells and a specific *Salmonella* ELISA for faecal contamination, in conjunction with routine chemical and biological (mostly microbiological) monitoring activities. The study indicated significant variation among sites in all microbiological measures as well in IL-6 secretion and *Salmonella* presence. Between months, variations were also evident in certain variables. Secondly (Chapter 3), two bioassays using the yolk precursor protein, vitellogenin (Vtg) as endpoint was implemented in a) an *in vitro* *Xenopus laevis* liver slice assay (five day exposure) and b) an *in vivo* Zebrafish (*Danio rerio*) bioassay (seven day exposure) assessing estrogenic activity in the Eerste/Kuils River

catchment. Although estrogen spiked positive control water samples stimulated Vtg production *in vitro* as well as *in vivo*, no dramatic estrogenic activity was measured at any of the selected sites. Thirdly (Chapter 4), a bioassay using the thyroid controlled metamorphosis in *Xenopus laevis* tadpoles to assess effects on the thyroid hormonal system was implemented. Thyroid stimulatory activity, compared with a negative control sample, was measured at two sites along the catchment. Although the practical implementation of the tadpole semi-static exposure protocol (water replacement) proved to be labour intensive, all the added bioassays proved to be valuable tools to add valuable information regarding water quality. It is clear that more research related to anthropogenic influences along the Eerste/Kuils River catchment system are needed, specifically in monitoring monthly variations to better understand annual variation in several of the endpoints studied.

Uittreksel

Waterkwaliteit vorm die basis vir die evaluering en monitering van opvangsgebiede. Voortdurende stedelike ontwikkeling gee aanleiding tot 'n toename in die voorkoms van besoedelstowwe in die natuurlike omgewing deur gelokaliseerde (punt) bronne (rioolwerke/industriële uitvloeisel) en/of nie gelokaliseerde (nie punt) bronne (vloed uitlaat/huishoudelike uitvloeiels) van besoedeling. Dit het onlangs aan die lig gekom dat van hierdie chemiese besoedelstowwe subtiel die endokriene sisteem versteur en so aanleiding gee tot gesondheidsprobleme in terme van die voortplantings sisteem en tiroïed sisteem (groei en ontwikkeling) by diere en moontlik ook die mens. Daar is beperkte gebruik van biologiese toetse wat inligting verskaf oor sitotoksiteit, inflammatoriese aktiwiteit en endokriene versteuring. Die doel van hierdie studie was dus om van hierdie biologiese toetse, wat normaalweg nie deel uitmaak van die roetine munisipale (Stad van Kaapstad) opvangsgebied monitering nie, gebruik te maak. Die Eerste-, Kuilsrivier, Wes Kaap, onder beheer van Stad Kaapstad is gekies en alhoewel die opvangsgebied nie water bydra tot drinkwaterbronne nie, word die opvangsgebied beïnvloed deur verskeie mensgemaakte bronne van besoedeling (afloop vanuit omliggende landbougebiede). Binne die kort tydsraamwerk van die projek (ses maande) is besluit om twee maande, Julie (volg 'n droë somer en herfs seisoen) en Oktober (volg 'n nat winter en vroeë lente seisoen) vir water kwaliteit monitering te kies. Ruimtelike variasie langs die loop van die opvangsgebied is ingesluit deur moniteringspunte (n=10), met in ag name van die potensiële besoedelingsbronne. Spesifieke doelwitte van die projek sluit in: Eerstens (Hoofstuk 2), om die *in vitro* biotoetse, laktaat hidrolise (LDH) vir sitotoksiteit, pro-inflammatoriese hormoon Interleukin-6 (IL-6) vir inflammatoriese aktiwiteit, vrygestel deur menslike bloedselle en 'n *Salmonella* ELISA vir ontlasting besoedeling saam met bestaande chemiese en biologiese (hoofsaaklik mikrobiologiese) veranderlikes te gebruik. Die studie het getoon dat beduidende variasie in alle mikrobiologiese toetse asook IL-6 vrystelling en *Salmonella* voorkoms bestaan het tussen versamelpunte. Maandelikse variasie in sekere van die veranderlikes het ook voorgekom. Tweedens (Hoofstuk 3), is twee biotoetse wat die dooiervoorloperproteïene, vitellogeen (Vtg) as eindpunt gebruik geïmplimenteer in a) 'n *in vitro* *Xenopus laevis* lewersnit biotoets (vyf dag blootstelling) en b) 'n *in vivo* Zebra vis (*Danio rerio*) biotoets (sewe dag blootstelling) om estrogenisiteit in die

Eerste-, Kuilsrivier opvangsgebied te evalueer. Alhoewel, die estrogeen behandelde positiewe kontrole water monsters Vtg produksie veroorsaak het in beide die *in vitro* lewer-kulture en *in vivo* vistsoets, is geen dramatiesse estrogeeniese aktiwiteit by enige van die moniteringspunte gevind nie. Derdens (Hoofstuk 4), is 'n biotoets wat die tiroïedbeheerde metamorfose in *Xenopus laevis* paddavis se gebruik om effekte op die tiroïedsisteem te evalueer. Die differensiële stimulerings (versnelling), in vergelyking met 'n negatiewe kontrole watermonster, van die tiroïed sisteem is by twee moniteringspunte in die opvangsgebied waargeneem. Alhoewel die praktiese implementering van die paddavis semi-stadiese (water word gereeld vervang) biotoets arbeidsintensief is, het alle toekomstige biotoetse waardevolle toekomstige inligting oor water kwaliteit verskaf wat 'n belangrike bydrae tot ingeligte bestuursbesluite kan verleen. Dit is duidelik dat meer navorsing in verband met die menslike invloed langs die Eerste-, Kuilsrivier opvangsgebied nodig is, veral met maandelikse monitering vir seisoenale veranderinge.

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ACRONYMNS

ANOVA	One-way Analysis of Variance
DWAF	Department of Water Affairs and Forestry
EDCs	Endocrine disrupting compounds
EDSP	Endocrine Disrupter Screening Programme
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
ELISA	Enzyme-linked Immunosorbent Assay
IL-6	Interleukin-6
LDH	Lactatedehydrogenase
NAEBP	National Aquatic Ecosystem Biomonitoring Programme
NCMP	National Chemical Monitoring Programme
NEMP	National Eutrophication Monitoring Programme
NF	Nieuwkoop and Faber
NMMP	National Microbial Monitoring Programme
NTMP	National Toxicological Monitoring Programme
NWA	National Water Act
OECD	Organisation for Economic Development
PTU	6-n-propyl-2-thiouracil
RHP	River Health Programme
SA	South Africa
STW	Sewage Treatment Works
T₄	Thyroxine
TH	Thyroid hormone
UK	United Kingdom
US	United States
US EPA	United States Environmental Protection Agency
USA	United States of America
Vtg	Vitellogenin
WRC	Water Research Commission
WWTW	Wastewater Treatment Works
XEMA	<i>Xenopus</i> Metamorphosis Assay

CHAPTER 1

Background

Changes in the health and fecundity of wildlife populations and humans, caused by the disruption of the hormonal system through chemical compounds found in the aquatic environment, has become a growing concern over the past 10 years (Colborn *et al.*, 1996). It has been suggested that these changes relate to the endocrine-altering action of various chemical contaminants, originating from sewage release, industrial effluent discharge and agricultural runoff into rivers and streams (Archand-Hoy and Benson, 1998; Kime, 1998).

Freshwater ecosystems have suffered the most intense human related intervention of all ecosystems over the past 100 years of human history on both national and global scale (Collares-Pereira *et al.*, 2002). According to Jewitt (2002), humans rely on renewable freshwater for drinking, irrigation of crops, industrial use, production of fish and waterfowl, transportation, recreation and waste disposal. Renewable freshwater, however, only amounts to 0.26% of the total global water quantity available for utilisation. It is, therefore not surprising that projections are that sub-Saharan African countries will become physically water scarce in 2050 (Seckler *et al.*, 1998). With the human population relying almost entirely on rivers and aquifers for water, it is therefore of no surprise that the management of the quality of these resource have received top priority throughout the global community. Water quality management is not a new concept and has taken on the policy of pollution control for rivers and water resources. As rivers act as drains to the surrounding landscapes, catchments become the sinks for accumulating materials, severely degrading the environment (Dallas and Day, 2004). A significant proportion of wastes is also generated from urban areas (private homes), as either solid or liquid wastes. Leaching from dumping sites, effluents from sewage treatment works and runoff from agricultural areas direct many of the chemical compounds into waterways (Kime, 1998).

The toxicity of accumulated environmental chemicals in aquatic organisms is a major concern in the evaluation of chemical hazards, health risks as well as ecological risks for wildlife and humans. Chemical analyses in combination with toxicity tests may link adverse effects and chemicals originating from environmental sources. In addition, integrating exposure and effects data with field surveys

ultimately contribute towards evaluation of ecological effects and establish cause/effect relationships between environmental chemicals and adverse ecological impacts (Sparling *et al.*, 2000). Toxicity of water bodies and sediments that are potentially contaminated by chemicals or pose health risks because of conditions that favour microbial health risks can be assessed by either a chemistry approach, toxicant approach or a microbial approach. The chemical approach mainly concerns chemical analyses and known water quality criteria (usually based on laboratory exposure experiments) to indirectly estimate toxicity. Ecotoxicity testing on the other hand, involves the measurement of a biological effect (e.g. mortality, growth, reproductive output) associated with the exposure to chemical compound or mixture of compounds. Ecotoxicity testing could provide data on the acute (immediate) and chronic (long-term) toxicity of the contaminated source to organisms. Ecotoxicity tests are mostly run in laboratories, with most tests being conducted under static (no water exchange), static-renewal (periodic water exchange) or flow-through (continual exchange) conditions. Alternatively, field (*in situ*) toxicity test can also be conducted.

Acute toxicity testing mostly measure lethal effects, but sub lethal effects can also be used. Acute toxicity testing usually are expressed as LC₅₀Ss and EC₅₀S for a relative short test period (e.g. 96hr) (Sparling *et al.*, 2000). Chronic testing on the other hand potentially detects both chronic lethal and sub lethal toxicity, mostly including endpoints regarding growth and reproduction. Therefore, toxicity data, field data and chemical analysis data may be integrated to get a better picture or understanding of adversely affected ecosystems or communities and provide more complete information for assessing effects caused by hazardous wastes (Sparling *et al.*, 2000).

The microbial approach mainly concerns poor sanitation (lack of) and faecal contamination (threat of) with associated waterborne diseases such as gastroenteritis, *salmonellosis*, dysentery, cholera, typhoid fever and hepatitis. Microbial testing mostly measures the level of pathogens (sources of waterborne diseases) present in water resources utilised for drinking, recreation and irrigation purposes (DWAF, 2002b). Microbial tests are mainly run in laboratories of each regional catchment area according to the guidelines set by DWAF and data is obtained from the supply and sanitation database for each of the regions (DWAF, 2002b).

Water quality and monitoring in South Africa

Freshwater supplies in South Africa (SA) are dependant mostly on seasonal rains to replenish fluctuating water levels (Cape Metropolitan Council, 2003). As one of the fastest growing third world countries in the southern hemisphere, with a population growth of around 1.32% (1999 estimate), SA is becoming a highly urbanised and industrialised third world country with water demand on resources estimated to have doubled by the year 2050 (Dallas and Day, 2004). The problem arises that SA's water resources is not only being utilised for rural drinking water supplies, food security and the maintenance of natural systems, but is constantly being degraded by industrial and sewage work effluents, storm water discharges and agricultural runoff (Cape Metropolitan Council, 2003; Dallas and Day, 2004; Davies and Day, 1993; Deksissa *et al.*, 2003; DWAF, 1996; Hayes, 2002; Johnson *et al.*, 2001; Kamara and Sally, 2003; Lange, 1998; Perret, 2002; Schulz *et al.*, 2001a; Van Wyk *et al.*, 2002).

The 1910 Irrigation Conservation Act saw the first change to then existing legislation, becoming the first nationally applied water law through the reduction of government's management of water to only that of irrigation related works. This was followed by the 1956 Water Act during the Post Industrial WWII period development, which changed the "ownership" state of water to "exclusive" use of water for conservation, household, urban, agricultural and industrial developments (Perret, 2002; Pollard, 2002). Democratic change in SA gave birth to a new National Water Act (Act 36 of 1998) (NWA), revising former regulations of water ownership to considering water as a common asset to all. The NWA states specific requirements in terms of the use, re-use and treatment of all water bodies through the assignment of the Department of Water Affairs and Forestry (DWAF) as custodian to the nation's water resource (Pollard, 2002). This has given rise to the establishment of Integrated Water Resources Management (IWRM) and Integrated Catchment Management (ICM) frameworks as tools to manage local water resources (Pollard, 2002, Hughes and Hannart, 2003).

Biomonitoring is an approach being relatively widely applied in SA, notably through the River Health Programme (RHP) (Brown, 2002) and generates information about sustainable impacts on the environment. Unfortunately, inadequate treatment of industrial and wastewater treatment work (WWTW) effluent, released unchecked, cripples SA by inflicting so-called poverty diseases associated predominantly with rural areas where large numbers of people collect their drinking water from local rivers (Levite & Sally, 2002). In the Western Cape alone, approximately 50% of

rivers have been urbanised and are being affected by effluents. The NWA specifically mandates the Minister to establish monitoring programmes to monitor, record, assess and disseminate information on the different water resources utilised in SA. Current monitoring programmes include the monitoring of chemical, microbial, eutrophication and aquatic ecosystem parameters through the structuring of national monitoring programmes under the auspices of DWAF (DWAF, 2002a, 2002b).

The National Chemical Monitoring Programme (NCMP) with the aim of providing information on the major inorganic chemical water quality constituents of surface waters across South Africa, to water resource managers, scientists, decision-makers and the public was established in 1998 (DWAF, 2002a). Water quality is assessed on the basis of fitness for use by the domestic and irrigated agriculture water use sectors and is presented in such a way to be useful in water resource management purposes for the 19 WMA's in SA (DWAF, 2002a). A water quality criterion is therefore provided for each of the constituents and is known as the Target Water Quality Range (TWQR). The range for chemical concentrations is set at safe levels for human consumption. NCMP does not, however, deal with the microbiological status of water resources.

With numerous dense settlements (both formal and informal), increasing urbanisation and other factors, South Africa's water resources have come under increasing threat of faecal contamination. The establishment of the National Microbial Monitoring Programme (NMMP) aims at providing information on the status and trends of the extent of faecal pollution, in terms of microbial quality of surface water resources, and to assess potential health risks to humans (DWAF, 2002b). The NMMP is implemented on a national scale with primary managerial responsibility resting with DWAF. TWQR has also been set for microbial activity and levels of pathogen activity within water resources. A shortcoming of the NMMP is that no provision is made for assessment of direct human inflammatory activity or cytotoxic effects caused by pathogens and toxins remaining in the water after the death of micro-organisms (Pool *et al.*, 2000).

Cytotoxic reaction/activity can be demonstrated by assessing damage caused to erythrocytes in the blood stream, with the resultant release of lactate dehydrogenase (LDH) which catalyses the conversion of pyruvic acid to lactic acid in the cell. In addition, the detection of inflammatory activity following *in vitro* exposures of whole blood cell cultures may be done. When exposed to contaminants, including microbes or toxicants release (dead or alive), human blood stimulates the secretion of pro-

inflammatory hormones, one of which is known as Interleukin-6 (IL-6) (Pool, 1999). Increased production of pro-inflammatory hormones by culture cells has been used as early warning biomarkers in water quality assessments (Pool *et al.*, 2003).

Salmonella is a pathogenic organism transferred via the faecal-oral pathway, mainly from contaminated waters and results in extreme cases of diarrhoea (mostly causing infant deaths). Water samples could be screened for *Salmonella* antigens by using polyclonal antibodies which are very specific for *Salmonella* H and O antigens. Moreover, *Salmonella* and IL-6 response have also shown a close correlation in levels of contamination and have been used successfully as indicators for water quality (Pool *et al.*, 2003).

The National Eutrophication Monitoring Programme (NEMP) was developed by the Water Research Commission to address negative ecological and economic impacts such as the deterioration of water quality, loss of biodiversity, recreation and human health impacts. SA's water resources exhibits high nutrient enrichment and eutrophication related problems creating a need for consistent monitoring (DWAf, 2002a). NEMP therefore aims to measure, assess and regularly report on the current trophic status, the nature of eutrophication problems and the potential trophic changes in SA's (WRC, 2000) surface waters.

National Aquatic Ecosystem Biomonitoring Programme (NAEBP) established a nation-wide programme (River Health Programme) focussing on using biological indicators in conjunction with traditional physical and chemical indicators, to assess and monitor the health of South Africa's freshwater ecosystems. The River Health Programme (RHP) aims to measure, assess and report on the ecological state of aquatic ecosystems, detect spatial and temporal trends, identify and report emerging problems and to serve as a source of information regarding aquatic ecosystems (DWAf, 1994).

Monitoring toxic effluents and hazardous wastes have proven to be more effective if the discharging effluents are monitored prior to their release into the environment (Murray *et al.*, 2003). In addition to the existing National monitoring programmes, DWAf was mandated by the National Water Act to develop a National Toxicants Monitoring Programme (NTMP) with the aim of measuring, assessing and reporting on the biological activity of toxic substances in SA watercourses (Murray *et al.*, 2003).

Endocrine disrupting contaminants: an emerging environmental problem.

Endocrine disruption (ED), as a potential non-lethal hazard/risk to wildlife and humans received increased attention since the publication of Rachel Carson's book "Silent Spring", published in 1962, followed by the publication of "Our Stolen Future" (Colborn *et al.*, 1996) and Cadbury's book, "Feminization in Nature", recently published in 1998. The basic hypothesis states: "Synthetic and some naturally occurring chemical substances in the environment are disrupting the normal functions of the endocrine system and its hormones in humans and wildlife (Juberg, 2000; SETAC, 2000). Exposure to these environmental chemicals has been linked to birth defects in the reproductive organs, reductions in sperm count and increased breast, prostate and testicular cancer risks (EHP, 1996). Endocrine disrupting compounds (EDCs) may potentially interfere with the synthesis, secretion, transport, binding action or elimination of the body's natural hormones responsible for the reproduction, development and behavioural maintenance. Although initial studies linked EDCs to interaction (agonistic or antagonistic) with the female hormone, estrogen, interaction with androgen, thyroid systems, immune and nervous systems increasingly received attention (Jimenez, 1997; Davies, 1998; Fairbrother, 2000; Baker, 2001; Guillette and Gunderson, 2001; Damstra *et al.*, 2002; Petrelli and Mantovani, 2002; Vasudevan *et al.*, 2002; Andersen *et al.*, 2003; Branchi *et al.*, 2003; Ishihara *et al.*, 2003; Brion *et al.*, 2004; Ho *et al.*, 2004).

Modulation (disruption) of the reproductive hormones, Estrogens and Androgens

Estrogenic activity, in particular, has been associated with wastewater treatment works (WWTWs) as point source polluters in the environment (Harries *et al.*, 1997; Allen *et al.*, 1999; Harries *et al.*, 1999; Haung and Sedlak, 2001; Kirk *et al.*, 2002). Reproductive impairments were documented in several wildlife species, including fish (Kime, 1998), amphibians (Harries *et al.*, 1999), alligators (Guillette, 2000), birds (Guillette and Gunderson, 2001) and mammals. Studies of the Water Works Department in England indicated the presence of substances such as nonaphemol, octaphenol and ethynyl estradiol (the major component of birth control pills) in surface waters of rivers and streams (Guillette, 2000). EDC research in Europe (Kirk *et al.*, 2002) and the United States (Haung and Sedlak, 2001) focused on the release of industrial effluent into surrounding rivers and streams. Europe has since established various working groups such as the Environment Working Group on Endocrine Disruption and European Union Scientific Committee of Toxicity,

Ecotoxicity and Environment (CSTEE) to solely support continued research and actions against EDCs (Baker, 2001). This has led to the establishment of the UK Water Industry Research Limited (2002), reviewing the current state of knowledge on the removal and degradation of EDs in WWTWs. Concern surrounded not only xeno-estrogens or natural estrogens, but also the bioaccumulation of alkyl phenol ethoxylates (APOEs), phthalate esters, bisphenol A and their associated biodegradable products such as nonylphenol and octaphenol, all of which act as strong estrogen mimics when released into the environment.

Various studies have monitored estrogen activities, more specifically in their mimicking actions in the replacement of sex steroids (Arnold *et al.*, 1996; Archand-Hoy and Benson, 1998; Kime, 1998; Rodgers-Gray *et al.*, 2000; Guillette and Gunderson, 2001; Hurter *et al.*, 2002; Mosconi *et al.*, 2002). In the early 1970's, hermaphroditic freshwater roach (*Rutilus rutilus*) was identified with effects of estrogen mimics causing altered male to female sex ratios, gonad abnormalities, reproductive dysfunction, precocious sexual development and feminisation of the animal (Sumpter and Jobling, 1995; Archand-Hoy and Benson, 1998; Jobling *et al.*, 1998; Kime *et al.*, 1999; Kloas *et al.*, 1999). Further studies indicated declines in various wildlife populations e.g. American alligators in Lake Apopka in the USA (Guillette, 2000) stimulating subsequent studies on the release of effluents into local rivers (Harries *et al.*, 1997; Allen *et al.*, 1999; Harries *et al.*, 1999; Haung and Sedlak, 2001; Kirk *et al.*, 2002). Following various whole organism exposure studies, bioassays was employed as an alternative for the measuring of estrogenic activities through both *in vivo* and *in vitro* biomarker models (Hurter *et al.*, 2002). Although the link to several reproductive related diseases in humans remains controversial, epidemiological data suggest that endocrine disruption in exposed humans may be a real health threat (Foster *et al.*, 2004; Toft *et al.*, 2004).

Biomonitoring relates to the techniques used to measure the effect of environmental change (environmental chemicals) on species present. This may be measured in terms of numbers present, or diversity, or more often by indicator species whose disappearance or disturbance provides an early warning signal (Tribe, 2004). EDC activity in water resources or effluents are commonly screened for estrogen activity through the use of various *in vivo* or *in vitro* exposure bioassays, including the ligand receptor binding assay, cell culture techniques and measurements of estrogen mediated protein production (Takatsuki and Yamaguchi, 2001). This has led to the research and validation of several endocrine related endpoints, including the estrogen

controlled yolk precursor, vitellogenin (Vtg) (Jones *et al.*, 2000), as well as several estrogen receptor binding and reporter gene assays (Jobling *et al.*, 1998). The problem, however, rests in the choice of test to be employed, the validity of the test and guidelines to follow during detection methods (Baker, 2001).

Vitellogenin production is a natural process under multi hormonal control, predominantly 17 β -estradiol, and is active during seasonal reproduction cycles of female fish (Sumpter and Jobling, 1995). During peak levels of 17 β -estradiol concentrations, Vtg is synthesised by the liver and released into the bloodstream. Circulating vitellogenin (Vtg) is absorbed by the ovaries and stored in growing oocytes as egg-yolk for developing embryos (Allen *et al.*, 1999). Although the Vtg synthesis pathways exist in male, vitellogenin levels are normally undetectable low because of low circulating estrogen levels in males (Rose *et al.*, 2002; Fenske *et al.*, 2001; Sherry *et al.*, 1999). Because of this phenomenon, males represent an ideal exposure model for estrogenicity. The presence of Vtg in the blood plasma of fish, male or female, can be detected by measuring indirect changes in the plasma associated with the high circulating Vtg levels, for example, increased lipoproteins or alkaline-labile phosphorous (Hurter *et al.*, 2002). Because Vtg is a lipoprotein complex, circulating plasma concentrations could also be determined through homologous immunoassays such as enzyme-linked immunosorbent assays (ELISA) (Fenske *et al.*, 2001).

Standardized protocols for estrogenicity screening by utilizing small cultured fish species, including Zebrafish (*Danio rerio*), Medaka (*Oryzias latipes*) and Fathead minnows (*Pimephales promelas*) as model systems have been validated by the USA-EPA, OECD and Japan Environmental Agency (US EPA, 1996). Fish, as experimental models, proves advantageous in its similarity of the endocrine system to that of mammals and the ability to be used in full life cycle exposures and multigenerational studies (Tamatsuki and Yamaguchi, 2001). The presence of environmental estrogens can also be detected through the use of *in vitro* bioassays. In short, the assay is based on the production of Vtg by the liver slices of (*in vivo*) estrogen exposed *Xenopus* (African clawed frog). Liver slice bioassays have proved to be a valuable high-throughput *in vitro* assay for detecting estrogen activity in water samples (Hurter *et al.*, 2002).

In addition to estrogenic screening, several studies have showed that environmental contaminants interfere with androgen dependant processes, for example, effluents from pulp and paper mill industries, include unknown substances

that act as androgen receptor agonists (Durhan *et al.*, 2002). These effluent exposures have been linked to masculinization of females in fish populations. Fungicides, for example vinclozolin and pesticides, *p,p'*-1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (*p,p'*-DDE), have been found to demasculinize mature male fish (Damstra *et al.*, 2002; Boudreau *et al.*, 2005). Apart from androgen receptor binding assays and reporter gene assays few functional androgen endpoints have been used in screening programmes to assess disruption of the androgen system.

Modulation of Thyroid hormone system

The majority of endocrine disruption research has been found to focus on anti- and estrogenic effects, anti- and androgenic effects and steroidogenesis, with less research on the disruption of the thyroid system (Colborn, 2002), despite the increasing evidence of adverse thyroid gland development and abnormal levels of circulating thyroid hormones (THs) in various animal systems. Colborn (2002) suggests that TH is critical for the development of the brain, intelligence and behaviour and it is therefore essential to develop screens to detect synthetic chemicals interfering with the thyroid system as well. The interest in the thyroid endocrine axis stems from several studies suggesting that disruption may occur because of interactions with synthetic chemicals, including pesticides, agricultural, medical and industrial chemicals (Brucker-Davis, 1998; Christian & Trenton, 2003; Jahnke *et al.*, 2004). Xenobiotics may affect thyroid function by altering any of the life-history components of thyroid hormones (Christian and Trenton, 2003). Potential effects of EDCs on the thyroid system involve the circulating levels of thyroid hormones (THs), T₃ and T₄ (Kloas, 2002) as EDCs mimic TH and bind to plasma proteins responsible for the distribution of endogenous hormones (Brucker-Davis, 1998). Some EDCs mimic TH through binding to thyroid hormone receptors (TRs), for example, tetrachlorodibenzo-*p*-dioxin (TCDD) acting as a TH agonist (Kloas, 2002). Due to the widespread regulatory involvement of the thyroids, thyroid function should be included when considering the potential risk of environmental chemicals (Christian and Trenton, 2003). A great deal of attention has focused on the role of THs in amphibian metamorphosis (Colborn, 2002; Hayes 1997c).

Amphibian metamorphosis is controlled by thyroid hormones (Shi, 2000) and has been identified as an excellent model system to investigate the potential modulatory effects of environmental chemicals on the thyroid system (Kloas, 2002; Touart, 2002). The continuous exposure of environmental contaminants during the

lifecycle and the easy susceptibility to pollutant accumulation (Gutleb *et al.*, 1999) are the main reasons for the study of TH during amphibian metamorphosis. TH plays an essential role in embryonic development and growth in vertebrate species, as well as in the process of amphibian metamorphosis. Metamorphosis is a period of substantial morphological change consisting of phases such as resorption or regression of tissues, remodelling of organ systems and development of tissues (Touart, 2002). The local African clawed frog, *Xenopus laevis*, represents one of few anuran species that have been well studied in this regard and whose life history and laboratory breeding characteristics are well known (Kloas, 2002; Kloas *et al.*, 2002; Mosconi *et al.*, 2002; Shi, 2000; Touart, 2002; Van Wyk *et al.*, 2003), providing the essential background for future studies.

Screening programmes for EDC activity

Internationally, the US Environmental Protection Agency (US EPA) was authorized to develop screening and testing programmes, utilizing a battery of tests to determine whether chemicals interact with the endocrine system of wildlife and therefore potentially also with the human endocrine system. This authorization followed on the 1996 amendments of the Safe Drinking Water Act within which the US EPA needed to screen drinking water for substances that may have potential endocrine disruption activity. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) were formed in 1996, making recommendations to the US EPA on the development of screening and testing programs. The Endocrine Disruptor Screening Programme incorporated recommendations in 1998 made by EDSTAC, based on the screening of potential environmental contaminants for endocrine modulating activities (agonistic and antagonistic) of estrogen, androgen and thyroid hormone systems (US EPA, 1996). Selected screens are currently being validated and standardized, in collaboration with the Organization for Economic Development (OECD) (based in Paris, France), to facilitate general use.

EDSTAC recommended a tiered screening and testing approach to refine assessment screenings towards early applicable and effective priority systems: Tier 1 screening focuses on assessment and priority settings of chemicals for which there is insufficient scientific data. Bioassays recommended include three *in vitro* assays and five *in vivo* assays. The *in vitro* assays included estrogen and androgen receptor binding (transcriptional activation assays) and a steroidogenesis assay using minced testes from the mammalian system. The five *in vivo* assays (US EPA, 1996) include a

rodent 3-day uterotrophic assay, a rodent 20-day pubertal female assay for effects on thyroidal function, a male rodent 5-7 day Hershberger assay, a frog metamorphosis assay for thyroid effects and a fish gonad recrudescence or vitellogenin production assay (Sherry *et al.*, 1999; Smeets *et al.*, 1999). Tier 2 testing is designed to characterize more defined responses and therefore includes endpoints that will give decisive evidence whether or not the tested chemicals may be endocrine disruptors. Tier 2 tests include several wildlife species and are therefore longer duration tests. The battery of tests suggested include a two-generation mammalian reproductive toxicity assay, an avian reproductive toxicity assay, a fish life cycle toxicity assay, an invertebrate life cycle toxicity assay and an amphibian development and reproductive assay.

Although no EDC screening programme exists for South Africa, the inclusion of selected bioassays in the envisaged National Toxicology Programme (Murray *et al.*, 2003) will be a first attempt. However, only Tier 1 screens will be used, including *in vitro* reporter gene assays (estrogen and androgen receptors) and a *Xenopus* liver slice assay (Hurter *et al.*, 2002). The zebrafish Vtg bioassay will represent the only *in vivo* screen. Initially, no screening for environmental effects on the thyroid system will be included (Murray *et al.*, 2003; Van Wyk, *pers comm.*). However, despite this state of affairs, the inclusion of relevant bioassays investigating specific functional endpoints to ensure a better understanding of the complete ecotoxicological profile remains an important need. This is especially true for endpoints associated with cytotoxic and inflammatory responses adding value to human health related biomarkers. In light of the international developments, the lack of assessing environmental effects on the functionality of the thyroid hormonal system is a great shortcoming.

Western Cape Rivers

Screening and testing for EDC activity in South African waters, has taken place on a very limited scale, mostly as research funded by the Water Research Commission of South Africa. In the Western Cape, because of the extensive use of agrichemicals (pesticides, herbicides and fungicides) in the intensely farmed areas (London *et al.*, 2000; Dalvie *et al.*, 2004) environmental assessment for estrogenic activity in selected rivers were conducted. The African clawed frog, *Xenopus laevis*, was used as model system, utilising the Vtg response as biomarker for estrogenicity (Hurter *et al.*, 2002; Van Wyk *et al.*, 2002). Male *Xenopus* frogs were also exposed *in situ* in three

different agricultural areas (Van Wyk *et al.*, 2002). Although estrogenicity was shown at exposure sites, data show that estrogenicity in the water occurred on a limited scale. Using the *in vitro* *Xenopus* liver Vtg bioassay, Hurter *et al* (2002) suggested that estrogenicity occur more widespread including in drinking water samples. In another study, Hayes (2002) used the *Xenopus* liver assay to screen three Western Cape Rivers, including Palmiet, Lourens and Hout Bay rivers. Although no estrogenicity could be detected in the point samples, Vtg in male tilapia fish collected from the Lourens River was reported.

The catchment of the Eerste/Kuils River is located within a well established but expanding urban area, which greatly affects the water quality and quantity within the catchment system. Urbanization has resulted in the hardening of soil surfaces, increasing runoff rates and causing a decrease in overall water quality (Cape Metropolitan Council, 2003). The Kuils River has been impacted by the increase of storm water runoff and discharges of sewage effluent, increasing river flow but deteriorating overall water quality. Flowing continuously during the winter months, stagnant during the summer season, the river has changed from a seasonal to perennial flow regime, consisting of treated sewage effluent from Scotsdene, Bellville and Zandvliet sewage works. The Eerste River in turn receives effluent from the Stellenbosch STW and discharges, along with effluent of the Macassar sewage works, directly into the Eerste River estuary. The Eerste/Kuils River Catchment System falls under the management of the City of Cape Town and is sampled on a regular basis to set water quality standards. This system could therefore be used as a model system to study the integral effects of a diverse range of potential pollution sources (household, industrial, sewage and agricultural), known to be potentially sources of toxic and endocrine disrupting activities in polluted water bodies. Although, this catchment system does not contribute to the drinking water resource it allows for the assessment and the practicalities associated with the incorporation of additional bioassays to assess inflammatory responses and activity of certain EDCs.

Objectives of this study

The objective of this study was therefore to use available data and locally developed bioassays to test specific hypotheses set for the Eerste/Kuils River Catchment System namely:

- 1) that routinely measured water quality parameters are within the standards set, spatially and temporally throughout the system (Chapter 2),
- 2) that effluents discharging into the system do not affect the general cytotoxicity and inflammatory activity of the water (Chapter 2),
- 3) that effluents discharging into the system do not result in estrogenic activity setting (Chapter 3),
- 4) that effluents discharging into the system do not affect the functioning of the thyroid hormonal system (Chapter 4).

Each chapter was written in manuscript format to be submitted for publication, therefore, sections in the Introductions, Material and Methods and Discussions will be repetitive.

CHAPTER 2

An Assessment of the water quality in the Eerste/Kuils River Catchment System, Western Cape*

2.1 Introduction

Social and economic development of the human population has globally placed extreme pressures on the fresh water resource sector, particularly in its demand for high water quality and quantity standards. International response to this increasing demand saw the implementation of various regulations, monitoring and assessment programs to sustain and determine levels of water quality standards. Leaders on this front included the European Environmental Commission (EEC), World Health Organisation (WHO) and in particular the US Environmental Protection Agency (EPA), emphasizing and implementing the policing of monitoring drinking water strategies and priorities. This is a considerable task considering the continuous population growth and increasing water demands through agricultural, industrial and urbanization activities on a mere 0.26% renewable freshwater resource (Jewitt, 2002).

Water demand trends have proven high for developing countries due to the agricultural and urbanization activities, exceeding global trends of 2.4% per annum increase (Clarke, 1993). Several policies and acts (Safe Drinking Water Act of 1974 (USA), The Constitution (SA), Bill of rights, Water Act of 1965, Water Services Act 1997, Water and Property Rights, White Paper on Water Supply and Sanitation (1994)) has established rights for the protection of water sources (DWAF, 1996) setting criteria and guidelines for water quality standard requirements (EEC, 1980; SABS, 1984; US EPA, 1996; WHO, 1984 and 1993).

Monitoring programs became the essential tool in maintaining standard water quality levels safe for human consumption, recreational and domestic use. On a national level, in various countries, programmes included toxicity, chemical and/or microbial testing of water resources assessing, recording, monitoring and disseminating information. For example, the United States (US) implemented the Biomonitoring of Environmental Status and Trends (BEST), Environmental Monitoring and Assessment Programme (EMAP) and the National Water-Quality Assessment Programme (NAWQA) to monitoring toxicant pollution. Canada

* Submitted to Water SA, 2004

implemented the Canadian Environmental Protection Act (CEPA), the Fisheries Act, the Pest Control Product Act and the Transport Act (Transportation of Dangerous Goods Regulation) to monitoring long and short-term effects of chemicals. The Asian-Pacific region focused on organochlorines with the Netherlands monitoring chemical effects in local water resources (Murray *et al.*, 2003).

Although many of these monitoring programs are still in either scoping or design phases, they have proved essential in establishing water quality indicators. Water health indicators include pathogens, trace elements, nitrates and/or sediments and general water quality parameters such as dissolved oxygen concentrations, temperature and/or pH (DWAF, 1996).

Water resources in South Africa have in recent years been subjected to threats of pollution, mainly due to urbanisation and the establishment of rural settlements with poor sanitary services. Democratic changes in SA gave rise to the acceptance of the National Water Act No 36 of 1998 and the Water Services Act 108 of 1997 (Stein and Niklaas, 2002), revising former regulations of water ownership. National Government was issued with the responsibility of implementing the values and purposes establishing the Department of Water Affairs and Forestry (DWAF) in 1996 as custodian for SA's water resources. DWAF developed the South African Water Quality Guidelines (1996) as management tool to maintain the fitness of national water resources. The maintenance and regulation of existing water resources was redistributed to regional governments and local authorities (municipalities and local councils) in an attempt to assist government and local municipalities with water management decisions. Currently various national monitoring programmes (NMP) are at different implementation and development stages (Murray *et al.*, 2003) and include programmes such as the National River Health Programme (NRHP), National Eutrophication Monitoring Programme (NEMP), National Chemical Monitoring Programme (NCMP) and the National Microbial Water Quality Monitoring Programme (NMMP). NMP's also included the monitoring of inorganic compounds and radioactivity within the environment and have recently included the final draft for the implementation of a National Toxicants Monitoring Programme (NTMP) in South Africa (Murray *et al.*, 2003).

Under the auspices of the old and revised South African Water Acts (Act 54 of 1956 and Act 36 of 1998), national government armed itself with knowledge on the sustainable use of water resources within the country. Following international example in sanitation and water, water resource managers focused their attention on

the improvement of water provision to rural settlements (Goldblatt, 1999). This stimulated various assessment studies on rivers (Douglas, 2001; Faniran *et al.*, 2001; Coetzee *et al.*, 2002), catchments (Fatoki *et al.*, 2001; Deksissa *et al.*, 2003) and urbanised regions (Lange, 1998; Naicker *et al.*, 2003). Although the State governs major responsibility on South African water resources through the stewardship of DWAF, it has become essential for local authorities to initiate “closer to home” studies to successfully implement the use of water quality management tools, as part of the decision making process.

The City of Cape Town, located in the Western Cape, monitor and control various catchment systems under their authority. The assessment task relating to the various catchments is executed through the Scientific Services Department, directorate Water Services. Through the process of monthly water samples, endpoints are assessed through seasonal variances, long term impacts, pollution sources and domestic and recreational effects. The Water Quality Target Range (WQTR) for human health and the natural environment forms the basis on which water quality monitoring for the City of Cape Town exists. Catchments in the City of Cape Town include the Salt, Diep, Lourens, Houtbay, Zeekoei and Noordhoek catchment areas, of which the Eerste/Kuils catchment have stimulated interest due to four sewage treatment works located along the banks of the river. The quality of the water in the Eerste/Kuils River System proves problematic as factors such as physical pollution, canalization due to urbanization, loss of habitat and hydrological physical alterations to the river increased dramatically over the past 5 years (Cape Metropolitan Council, 2003).

Within the strategic support governed by monitoring systems, two main aspects; chemical/analytical extensions and bioassays, best define the structure of monitoring tools assisting with management decisions. The City of Cape Town is responsible for the full spectrum of providing a potable water and sewerage service to all the people in the greater Cape Town (City of Cape Town, 2005). The spectrum includes all aspects of bulk water supply to the City of Cape Town, Drakenstein and Stellenbosch, water distribution within the City as well as the collection, treatment and safe disposal of the effluent. Monitoring these various aspects, the City of Cape Town conducts chemical analyses which include aspects such as physical, chemical, microscopic and bacteriological methods of water examination. Data collected, in the format of suspended or dissolved constituents of water, describe water quality in terms of temperature, colour, concentration (nutrients or chemicals) or percentage

water body coverage (DWAF, 1996). Data obtained from the City of Cape Town will be used as both a comparative and supportive tool in addition to potential more sensitive, rapid, robust and reliable detection methods (bioassays).

Bioassays have become an essential tool in the ecotoxicological evaluation of environmental contaminants. Bioassays have been defined as “measurements of the concentration or potency of a substance by its effect on living cells, tissues or processes” (Kime, 1998) and mostly conducted *in vitro* as opposed to whole (*in vivo*) organism exposures. Advantages of *in vitro* bioassays include the successful repeatability, simplicity, cost and time factors as they prove to be cheaper, rapid and more robust and capable of measuring integrated agonistic or antagonistic as well as synergistic effects (Martin-Diaz *et al.*, 2004; Schirmer *et al.*, 2004). The success of bioassays lies within their effective endpoint analysis, identification abilities, compound concentration measurements, rapid sampling testing and consistency with the assessment of toxicity (Martin-Diaz *et al.*, 2004).

For the present study, in addition to the normal water quality data collected by authorities, for example the City of Cape Town, specific bioassays were included, lactate dehydrogenase (LDH) for cytotoxic activity (Diamantino *et al.*, 2000), Interleukin-6 (IL-6) for inflammatory activity and a specific *Salmonella* bioassay, as sensitive early detection methods to screen for the presence of biotoxins. Inflammatory activity caused by Inflammatory activity caused by microbial or biotoxin activity has been identified through the *in vitro* exposure of human whole blood cell cultures and measuring of the response through the secretion of pro-inflammatory hormones (immune response), including Interleukin-6 (IL-6) (Pool, 1999). Natural responses to pathogenic invasions include fever, the production of antibodies and macrophage endocytosis.

Salmonella, a bacterial pathogen, is transferred via the faecal-oral pathway and is used as indicator for faecal pollution of water resources by warm-blooded animals. Gastrointestinal diseases such as salmonellosis, cholera and typhoid fever are infectious diseases linked to the ingestion of contaminated waters (DWAF, 1996). *Salmonella* is detected through the use of specific polyclonal antibodies (Kim and Slauch, 1999; Olsen *et al.*, 2003; Španová *et al.*, 2003; Pool *et al.*, 2003a). In a study by Pool *et al* (2003a), *Salmonella* contamination in water resources closely correlated with changes in the immune response by human leucocytes (IL-6).

The broad objective of this study was to utilize the bioassays for cytotoxicity, inflammatory response and presence of *Salmonella* in conjunction with the physical

water quality parameters (chemical and microbiological parameters) in the Eerste/Kuils River catchment. It is clear from the information available for the anthropogenic activities along the Eerste/Kuils River catchments that potentially considerable variation could be expected in water quality as well as microbiological contaminations (RHP, 2001; RHP, 2003). The specific aims of this study include: 1) to establish if the water quality parameters of the Eerste/Kuils River Catchment System falls within SA water quality guidelines as safe for human use, 2) to assess potential biotoxin activity, including cytotoxic, inflammatory and *Salmonella* activities in the system, 3) to assess the relationship of the measured physical variables and the biological endpoints in relation to the anthropogenic activities along the catchments (spatial variation) as well as between two selected collecting months (selected within the time frame allowed for the study).

2.2 Materials and Methods

2.2.1 Study area

Situated within the Western Cape, the Eerste/Kuils River Catchment System is located amongst the Northern suburbs of Cape Town. Along with 10 other major catchments, the Eerste/Kuils River Catchment falls under the auspices of the City of Cape Town, Western Cape (South Africa) (Figure 2.1). Four major sewage works, Macassar, Zandvliet, Scotsdene and Stellenbosch release treated effluents into the river. Locations of these sewage works have been indicated in Figure 2.2.

Physical-chemical measurements and biological endpoints are collected on a monthly basis by the Water Services Department of the City of Cape Town. Data points along the river have been allocated according to possible influences and effluent discharging points and water samples collected at these points.

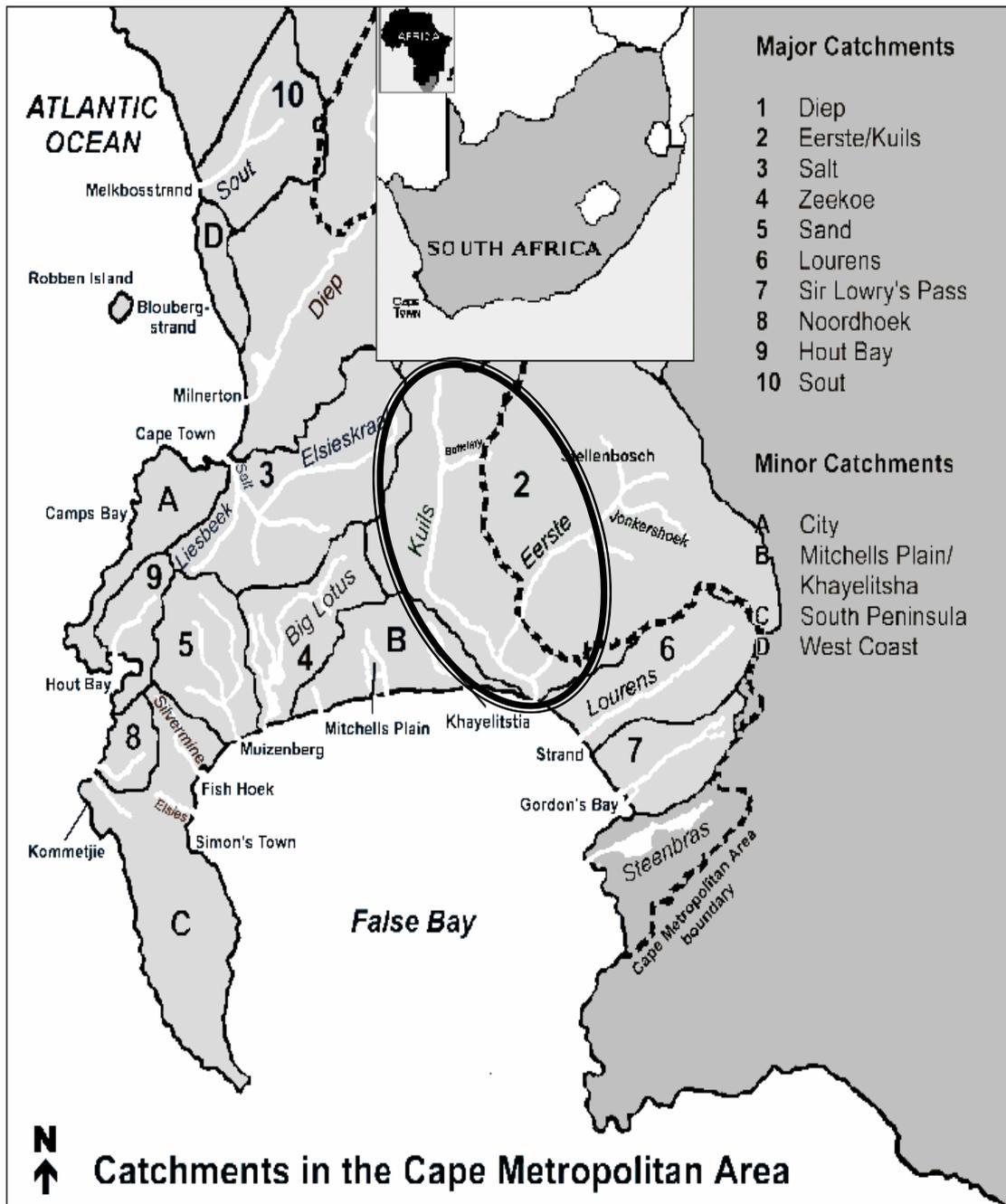


Figure 2.1: Major and Minor catchment areas under the local authority of the City of Cape Town, Cape Town. Catchment monitoring and quality assessments are obtained through the services of the Water Services Department of the City of Cape Town.

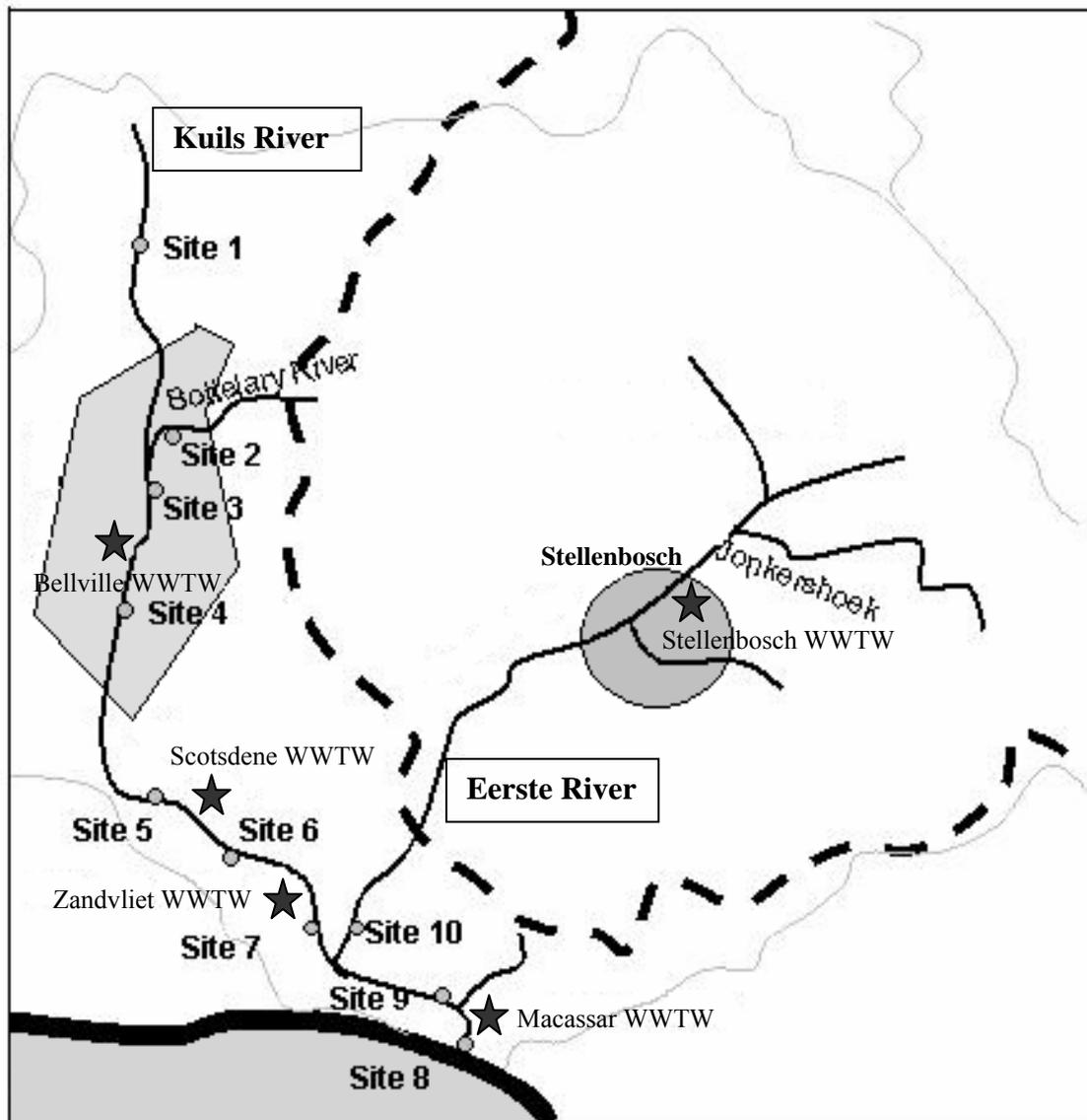


Figure 2.2: Sampling sites 1-10 located along the reaches of the Eerste/Kuils River Catchment System (Table 1), Western Cape, for sampling periods July and October 2003. Location allocated according to the impacts along the reaches, including the Bellville, Zandvliet, Macassar, Scotsdene and Stellenbosch wastewater treatment works (WWTWs) (effluent discharge points).

The City of Cape Town receives 80% of its annual rainfall in the winter months (May – August) (Figure 2.3). Sampling periods for the study were selected within the time-frame available for the project. July 2003 (winter) following a reasonable dry summer and autumn (<30mm total monthly rainfall; Figure 2.3) and October 2003 (late spring) following a period of high rainfall (between 60 and 100 mm total monthly rainfall).

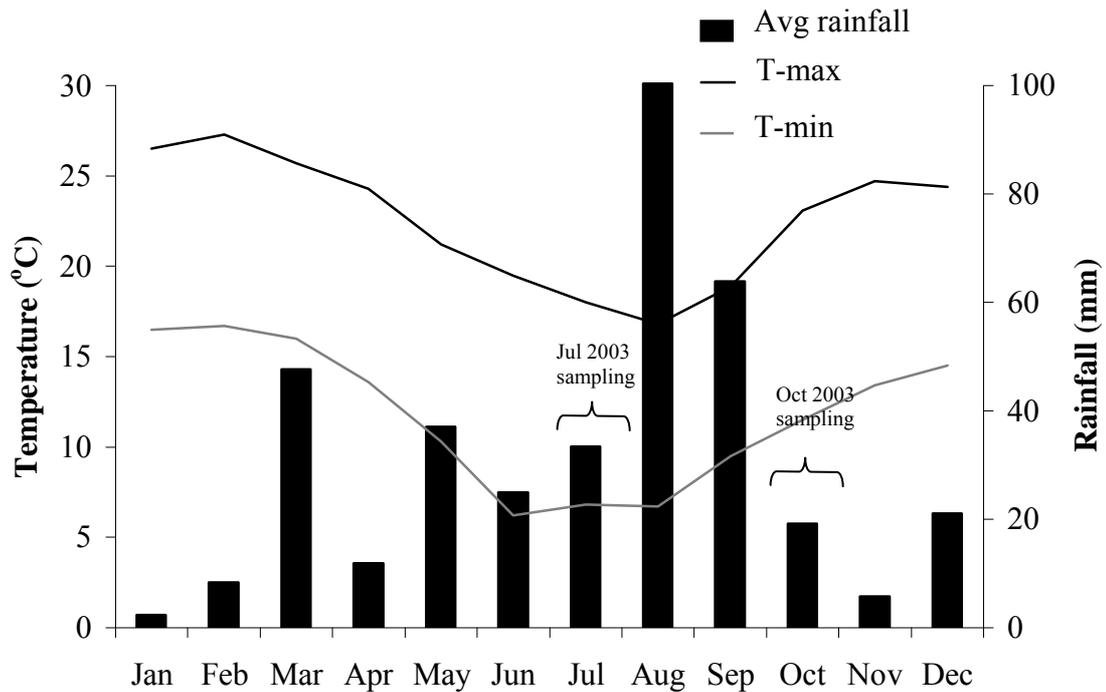


Figure 2.3: Total monthly rainfall (mm) and maximum (T-max)/minimum (T-min) temperatures (°C) recorded for the months of January 2003 to December 2003 within the Cape Metropolitan Area (CMA), Cape Town. Sampling periods for this study, as indicated, followed summer (July) and winter (October) rainfall periods.

Ten sites allocated along the reaches of the river, as indicated in Figure 2.2, were selected as sampling sites and correspond to the sites as selected by the Scientific Services Department of the City of Cape Town. Samples cover high, low and middle reaches of the Eerste/Kuils River catchment system. A description of each site is provided in Table 1.

Table 1: Description of samples points allocated along the Eerste/Kuils River catchment system. Provided by the City of Cape Town, Water Services Department.

Sample code:	Sample site:	Site name:	Sample point key:	Site description:
EK01	Site 1	Kuils River Industrial	next to R300 on Kuilsriver side	Wetland scenery/reeds Banks covered in grass Terraces at bridge
EK02	Site 2	Bottelary River	at Amandel Road	Surrounding urbanisation Branch of Kuils River - winery along upper reaches Stormwater discharge pipe
EK03	Site 3	Carinus Street	Stormwater discharge at Rietvlei Road Upstream of Bellville WWTWs	Cement channelling Housing - dogs/recreational use Stormwater discharge pipe
EK04	Site 4	Stellenbosch Arterial	at Stellenbosch Arterial Road Downstream of Bellville WWTWs	Nooiensfontein pump station - overflow into river Cement channelling High levels of pollution (plastic/food/clothes) Housing - shacks to brick houses Grazing of horses & cows along banks
EK05	Site 5	Faure	Kleinvlei Canal	Itemba lab facility Channeling & Urbanization
EK06	Site 6	Baden Powell Drive	at Baden Powell Drive	Wetland setting Surrounding farming community
EK07	Site 7	Zandvliet Treatment Works	below Zandvliet WWTW final effluent discharge	Farming setting - cows
EK08	Site 8	Macassar Treatment Works	in estuary d/s of Macassar WWTW	Estuary - outlet into ocean Situated close to beach Grazing cows directly in estuary
EK09	Site 9	Moddergatsp ruit	"spruit" at Macassar Road	Brick terraces High pollution (plastic)
EK10	Site 10	Eerste River	at N2 freeway	Bridged area Ravine habitat Surrounding pasture fields

2.2.2 Water collection and transportation

Glass containers

Hysil glass bottles (1L) were used to collect water samples. Each bottle was washed with a mixture of warm water and soap (Contrad concentrate, Merck (PTY) LTD, South Africa), using a bottlebrush. The bottles were then rinsed with clean hot water until no more soap could be detected in the bottle, followed by rinsing bottles 3 times with hot water and then 3 times with reverse osmosis water (ROH₂O). The final step saw bottles rinsed with 70% methanol, left to dry overnight (upside down) and immediately sealed with lids (washed accordingly) to prevent any contamination until the collection period. Caution was taken to prevent touching the inside of bottles or lids.

Sample Collection

Water samples were collected at 10 sites located on the Kuils River and Eerste River Catchment System (See Figure 2.2). Upon reaching the laboratory, duplicate aliquots of 1.5ml per site were prepared and stored at -80°C for future assays. The rest of the water sample was stored at 4°C in the dark. Samples stored at 4°C were used within 2 days after collection.

2.2.3 Water Analyses

2.2.3.1 Chemical analysis

Water samples were measured on site (YSI Portable system) for physical-chemical determinants (Temperature (°C), Dissolved Oxygen (mg/l), O₂ Saturation (%), pH, Conductivity (mS/m)). Chemical Oxygen Demand (mg/l) was measured using the Hach system and Total Nitrogen (mg/l N), Soluble Ammonia (mg/l N), Nitrate Nitrite (mg/l N), Phosphorus (mg/l P) and Orthophosphate (mg/l P) were analysed using a Lachat QuikChem 8000 Flow Injection Analyzer (FIA) which automatically conduct the chemical analyses system (Water Services Department, City of Cape Town). Samples are filtered through 0.45-micron filters with the exception of total phosphorus and total organic nitrogen samples, as they are pre-exposed to a specific digestion method before being subjected to the FIA system.

Coliform and Escherichia coli analysis

A complete description of the analytical methods, comprising the analyses of coliform counts is summarized by Clesceri *et al.* (1998) in the Standard Methods for the Examination of Water and Wastewater (Clesceri *et al.*, 1998).

2.2.3.2 Human Whole blood cultures: cytotoxicity and inflammatory activity in water

Water samples were tested for cytotoxic properties by incubation with whole blood cultures. Blood was collected from healthy volunteers, not on any medication for two weeks prior to donation. Blood was collected in 4.5ml Vacutainer (Preanalytical Systems, UK) and stored at ambient temperature. Blood was used within 48 hours for assays. Human blood was diluted in RPMI 1640 tissue culture medium, containing L-glutamine (BioWhittaker, USA) at a ratio 1:9.

Cytotoxicity: Lactate dehydrogenase activity (LDH)

Leaching of lactate dehydrogenase (LDH) from the erythrocytes was measured as an indication of cell wall damage. Assays were done in 96-well plates (Nunclon Surface, NalgeNunc, Denmark). Water samples were added at volumes of 20µl each to the wells and 200µl of the diluted blood was added to each water sample. A positive control sample containing 0.05% (m/v) Sodium dodecyl sulphate solution (Sigma, South Africa) in sterile pharmaceutical-grade water was included for each assay run. Negative control consisted of laboratory water (RO H₂O). The plate was incubated at 37°C for 18 hours. The tissue culture medium was then aspirated and immediately assayed for LDH using a commercial colorimetric assay kit (Sigma Diagnostics, INC, USA).

The LDH bioassay is based on the ability of LDH to catalyse the conversion of pyruvic acid to lactic acid. Pyruvic acid reacts with colour reagent to form a coloured hydrazone that has a peak absorbance at 400-550nm. Kit reagents consisted of working substrate (sodium pyruvate; 0.75 mmol/L in buffer, pH 7.5), colour reagent (2,4-Dinitrophenylhydrazine; 20mg % in 1N hydrochloric acid) and nicotinamide adenine dinucleotide (NADH). 10µl from each of the whole blood cultures were placed on a multi-well plate (Maxisorp; NalgeNunc, Denmark). A working substrate was prepared by adding 1ml pyruvate to 1mg of NADH, was added to each sample and incubated at 37°C for 30 minutes. Colour reagent (50µl) was added and the plate incubated for a further 20 minutes at room temperature. The reaction was stopped by

adding 50µl of NaOH (1M NaOH) per well. Absorbance was then measured with a Labsystems Multiscan MS spectrophotometric plate reader at 450nm. LDH concentrations were calculated from a standard curve, prepared using samples containing known concentrations of pyruvic acid. LDH activity is measured in (Berger-Broida) BB units/ml and can be converted to international units (IU) of LDH by multiplying with 0.48 (Positive control for lactate activity was established at 1474 BB units and a 10% variance based on the negative control level created a range between 500 – 700 BB units as negative impact zone).

Inflammatory activity: Interleukin-6 (IL-6) synthesis

Water samples were tested for their ability to elicit immunological (inflammatory) responses through incubation with whole blood cultures. The synthesis of interleukin-6 (IL-6) by the macrophages was measured as an indication of the presence of pathogens in the samples. Human blood (200 µl), diluted in RPMI 1640 tissue culture medium (BioWhittaker, USA), was loaded into each well of a 96-well plate. Water samples were added at volumes of 8µl each to the wells. Positive controls were prepared by adding 8µl of a 10ng/ml Control Standard Endotoxin (CSE) from *E. coli* (Charles River Laboratories, USA), while the negative controls consisted of sterile pharmaceutical-grade water. The plate was incubated at 37°C for 18 hours. The culture supernatant was then aspirated and immediately assayed for IL-6 content.

IL-6 was detected by a validated ELISA, described by Pool *et al.* (1999). All steps were performed at room temperature. Maxisorp micro plates were coated with 100µl rabbit anti-IL-6 primary antibody and placed on an orbital mixer to incubate overnight. After aspirating the coating antibody, non-specific binding sites on the plate were blocked for 20 minutes by the addition of 200µl of 0.1% (v/v) human serum albumin to each well. The plate was then washed with 0.9% (m/v) NaCl (saline) and 50µl of samples and standards were added along with 50µl of anti IL-6 biotinylated secondary antibody (Sigma, USA) and incubated for 2 hours. The plate was then washed with saline, followed by the addition of 100µl enzyme conjugate (Streptavidin-horse radish peroxidase; Boehringer-Mannheim, GmbH) and incubated for 20 minutes. The plate was washed again as before. Substrate (100µl) (BM Blue POD Substrate, soluble, Roche, South Africa) was added to the wells. The chromogenic reaction was stopped after 10 minutes through the addition of 50µl/well of 0.5 M H₂SO₄. The optical densities were read on a plate reader at 450nm. IL-6

standard were included on each ELISA plate. Samples were read off a standard curve constructed using Excel package.

2.2.3.3 *Salmonella* antigen ELISA

An in-house ELISA for *Salmonella* antigens was set up to screen the water samples. All samples were assayed in duplicate on a 96-well ELISA plate (Maxisorp. Nunc, Denmark). A positive control consisting of *Salmonella typhiriumium* (ATCC® 14028, Christophe Technologies, Hampshire, England) and a negative control consisting of ROH₂O was included on each plate. Samples (50µl) were transferred to wells of the ELISA plate. The plate was then incubated for 2 hours at room temperature. The plate was then washed with 0.9% m/v NaCl. The wells were then blocked with 1% albumin in 0.9% NaCl for 20 minutes. This was followed by incubating each well with 50µl of rabbit anti-*Salmonella* (Biogenesis, Poole, England) diluted 1/1000 in blocking solution for 1 hour. The plate was washed again followed by addition of 50µl per well of peroxidase linked sheep anti-rabbit IgG (Boehringer-Mannheim GmbH, Germany) and incubated for 45 minutes. The plate was washed again as before. Substrate (100µl) (BM Blue POD Substrate, soluble, Roche, South Africa) was added to the wells. The chromogenic reaction was stopped after 10 minutes through the addition of 50µl per well of 0.5 M H₂SO₄. The optical densities were read on a plate reader at 450nm.

2.3 Statistical analysis

Variation in data among sampling sites (spatial, representing different effluent and runoff mixtures) and months (temporal, with different preceding rainfall regimes) collected in, were assessed by using two-way analysis of variance (ANOVA) and a multiple-comparison test procedure (Holm-Sidak test). Data were tested for homogeneity of variation and normality prior to the ANOVA. In the case of non-parametric conditions, analysis of variance by ranks, Kruskal-Wallis test along with the Newman-Keuls multi-comparison test was used. A p-value < 0.05 was considered significant. Statistical calculations were performed using SigmaStat computer software (SPSS, Inc.).

2.4 Results

2.4.1 Water chemistry parameters

The physical-chemical and bio-organic measurements of the Eerste/Kuils River Catchment System for two different sampling seasons (July and October) are presented in Table 2. The mean values of three selected physical (temperature, total suspended solids (TSS), conductivity) is presented in Figure 2.4a and four chemical parameters (Nitrogen, Ammonia, Phosphorus, Nitrate Nitrite) indicating high concentration variation (Figure 2.4b). Illustrated for each parameter is the target water quality range (TWQR) recommended by DWAF (1996) as criteria to maintain good and ideal water quality. The analytical results in Figure 2.4a and 2.4b also depict seasonal variance for the selected parameters at the various sampling sites.

Table 2: Water quality measurements conducted by the Scientific Services Department of the City of Cape Town for seasons July and October 2003. Site 1 excluded from data analysis – restructuring of selected sample sites prior to sampling periods. Determands (discussed in text) are summarised according to site and physical-chemical and bio-organic measurements.

Sample Date: July 2003										
Determand	Unit	EK02	EK03	EK04	EK05	EK06	EK07	EK08	EK09	EK10
Physical-chemical measurements										
Temperature	°C	8.6	19.1	14.1	9.9	8.3	9.3	9.3	8.2	8.6
Total Suspended Solids	mg/l	7	4	27	6	3	6	9		6
Conductivity	mS/m	134	88.4	87	54.1	86.9	79.1	74.3	62.7	47.4
Bio-organic measurements										
Total Nitrogen	mg/l									
	N	22.02	6.484	15.85	1.695	5.104	5.369	6.183	1.064	6.03
Soluble Ammonia	mg/l									
	N	0.058	0.04	4.492	0.016	0.02	0.058	0.18	0.033	0.597
Soluble Nitrate Nitrite	mg/l									
	N	11.08	0.543	3.284	0.217	2.654	4.615	4.853	0.183	2.725
Soluble Phosphorus	mg/l									
	P	1.18	0.139	2.754	0.052	2.23	2.562	2.358	0.05	1.554
Sample Date: October 2003										
Determand	Unit	EK02	EK03	EK04	EK05	EK06	EK07	EK08	EK09	EK10
Physical-chemical measurements										
Temperature	°C	15.2	16.5	16.5	16.7	14.3	15.7	16.1	14.1	14.4
Total Suspended Solids	mg/l	114	222	43	66	4	13	26	10	602
Conductivity	mS/m	109.4	77.3	72.5	66.5	104.5	94.9	73.1	62.6	30.3
Bio-organic measurements										
Total Nitrogen	mg/l									
	N	9.46	5.94	5.519	1.996	2.613	3.944	5.054	1.251	2.156
Soluble Ammonia	mg/l									
	N	0.901	2.363	2.304	0.09	0.289	1.454	1.626	0.026	0.342
Soluble Nitrate Nitrite	mg/l									
	N	7.22	2.542	2.212	1.149	1.359	1.562	2.475	0.664	0.946
Soluble Phosphorus	mg/l									
	P	2.268	7.151	2.917	0.312	3.366	3.008	2.574	0.224	1.346

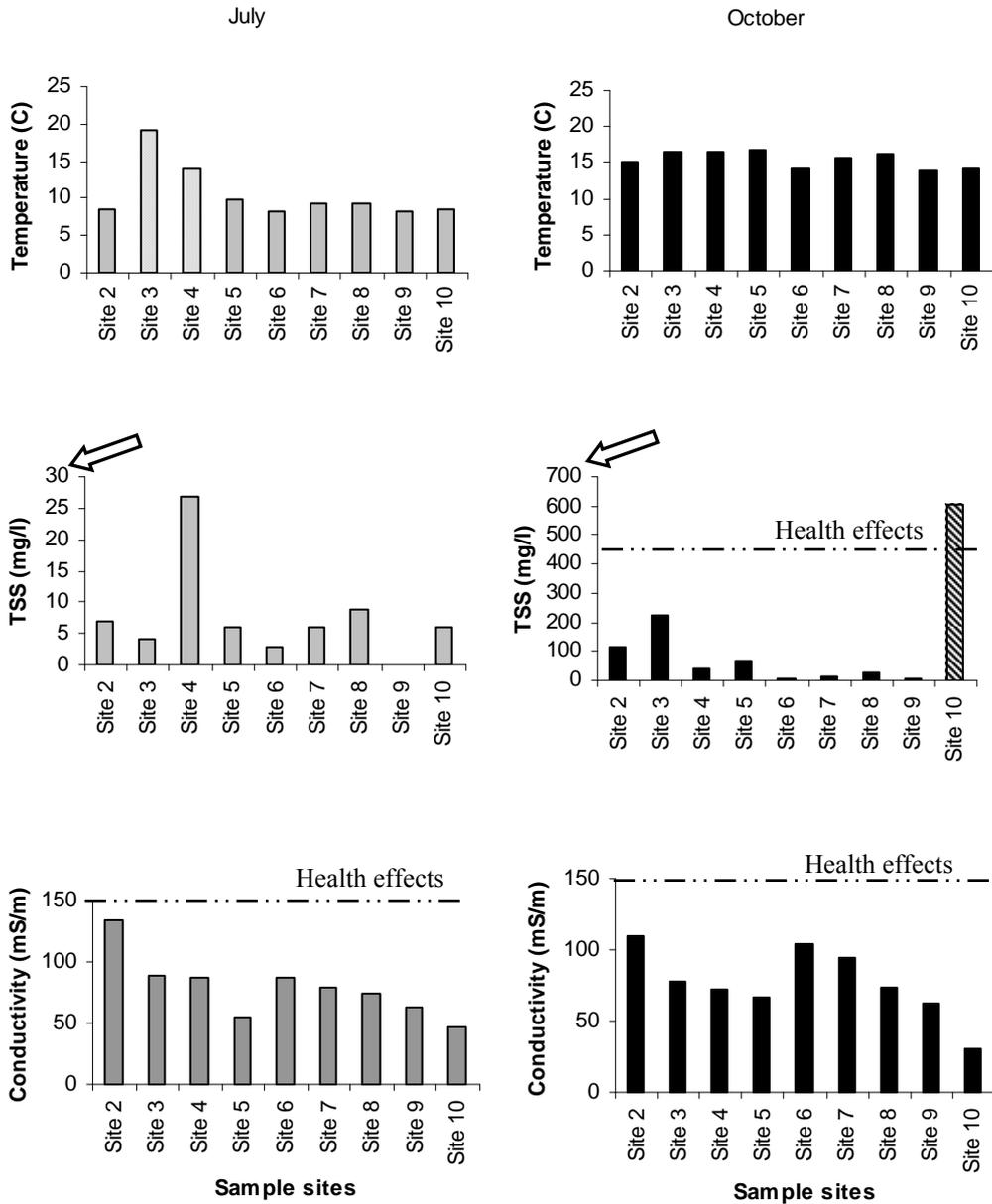


Figure 2.4a: Physical-chemical parameters as measured by the Water Services Department of the City of Cape Town for sites 2 to 10 allocated for the Eerste/Kuils River Catchment System (Site 1 excluded from City of Cape Town analysis data). Selected parameters of Temperature (°C), Total Suspended Solids (TSS) (mg/l) and Conductivity (mS/m) recorded at levels concerning high variation. Hatched bars indicate level at which human health is at risk (Conductivity, 150 mS/m and TSS, 450 mg/l) (DWAF, 1996; SABS, 1984). Arrows indicate scaling variance in the Total Suspended Solids between the months sampled. Scaling varies for each parameter.

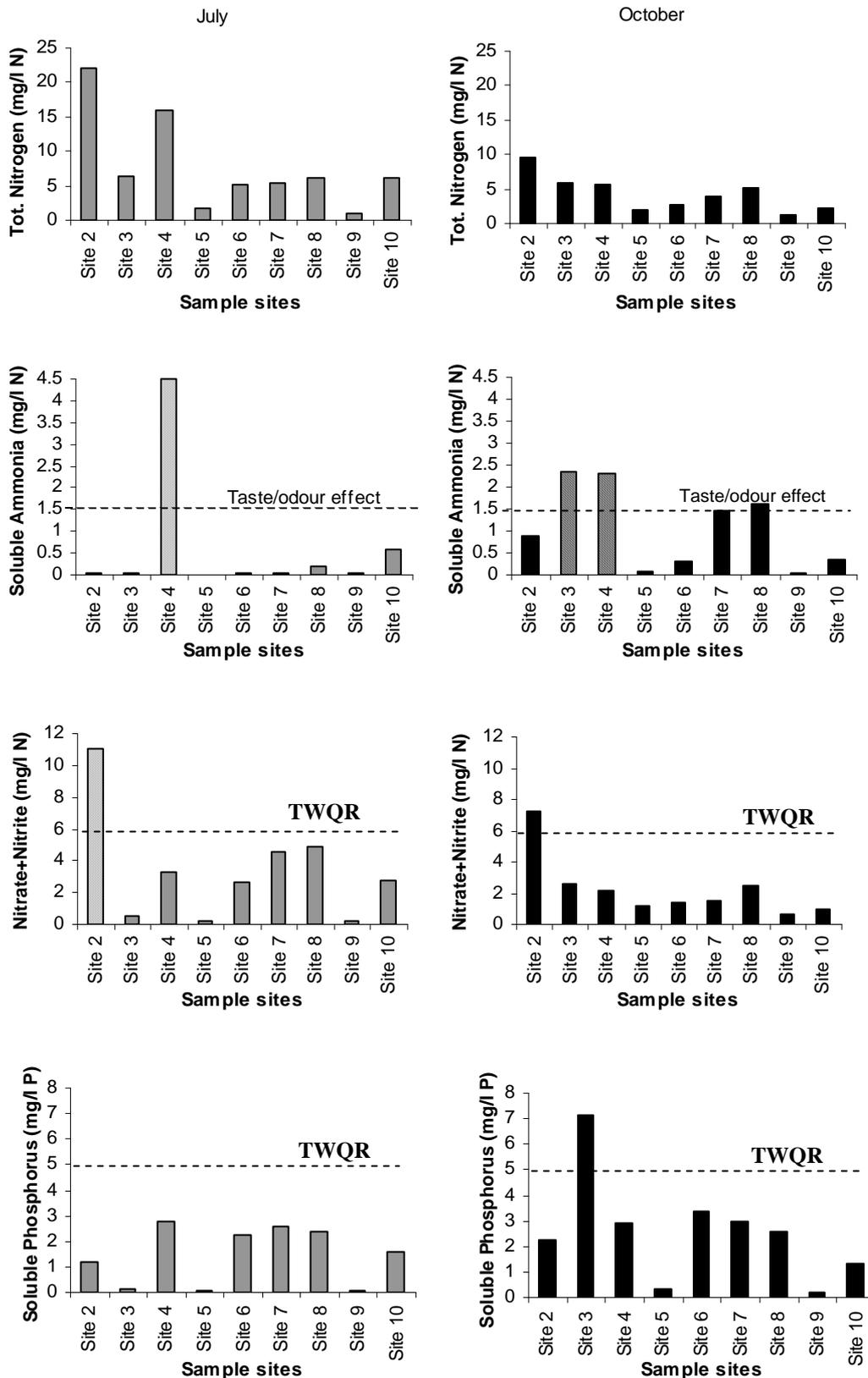


Figure 2.4b: Bio-organic measurements for Total Nitrogen (mg/l N) and Soluble Ammonia (mg/l N), Nitrate Nitrite (mg/l N) and Phosphorus (mg/l P) for sampling periods of July and October 2003. TWQR as set by DWAF (1996) for human health risk (hatched bars). Site 2 to site 10 as allocated on reaches of the Eerste/Kuils river catchment.

2.4.1.1 Physical-chemical measurements

Temperature: Temperature on average varied significantly between the two sampling periods, July (an average of 9°C) and October (an average of 15°C) (ANOVA; $p < 0.05$; $F_{1:17} = 22.19$), but did not vary significantly among sites along the catchment system (ANOVA; $p > 0.05$; $F_{8:17} = 1.98$). The highest water temperature was recorded at Site 3 (19.1°C) during July 2003 (Figure 2.4a). The overall average water temperature was recorded at 13.05°C (SD = 3.5).

Total Suspended Solids (TSS): Significant temporal variation (ANOVA: $F_{1:16} = 12.190$ $p < 0.05$) was recorded between the July and October sampling periods. TSS range for July varied between levels of 3-30 mg/l compared to October range values of 4-610 mg/l (Figure 2.4a). Although no significant difference was recorded for among site variations (ANOVA; $F_{8:16} = 1.230$; $p = 0.399$ ($p > 0.05$)), TSS at site 10 during October, exceeded the TWQR of 450 mg/l, recording a TSS level of 602 mg/l.

Conductivity (EC): Conductivity values for both sampling seasons (July and October) were recorded within acceptable health limits (Figure 2.4a). No significant temporal variation was recorded between July and October (ANOVA: $F_{1:17} = 0.245$ $P = 0.634$ ($p > 0.05$)). A significant difference was recorded within site variations (ANOVA: $F_{8:17} = 9.388$; $p < 0.05$). Multiple comparison procedures indicated significant variance within each sampling period for sites 2, 6 and 7. South African limit for conductivity in domestic water supply is set at 150.0 mS/m (Morrison *et al.*, 2001) with the TWQR set for a no effect range at < 45 mS/m. All sites exceeded the no effect range during both sampling periods.

2.4.1.2 Bio-organic measurements

Soluble Ammonia: No significant temporal variation or site variations were recorded for levels of soluble ammonia (Date – ANOVA: $F_{1:17} = 1.011$ $P = 0.344$ ($p > 0.05$)/Site – ANOVA: $F_{8:17} = 2.599$; $p > 0.05$). At concentration levels higher than 1.5 mg/l N for July (site 4: 4.492 mg/l N) and October (site 3: 2.363 mg/l N, site 4: 2.304 mg/l N, site 8: 1.626 mg/l N) ammonium exerts odour and taste impacts but presents no human health or aesthetic effects (Figure 2.4b).

Soluble Nitrate Nitrite: No significant temporal variance was recorded for levels of Nitrate Nitrite (ANOVA: $F_{8:17} = 2.998$ $p > 0.05$), but indicated significant variance between in site recordings (ANOVA: $F_{8:17} = 7.306$ $p < 0.05$). In both seasons site 2 stood out with the highest nitrate/nitrite values (Figure 2.4b). TWQR maximum limit is set at 6 mg/l N for human health consumption.

Total Nitrogen: No significant temporal variance was recorded for total nitrogen levels during sampling periods July and October 2003 (ANOVA: $F_{8:17} = 5.111$; $p > 0.05$). During July 2003, Total Nitrogen values varied significantly among sites (ANOVA; $F_{8:17} = 3.799$ $p < 0.05$). In particular, site 2 (22.02 mg/l N) and site 4 (15.85 mg/l N) were noted as high values (Figure 2.4b). This trend is also established for October although overall nitrogen levels is lower (1-10 mg/l N) than that of July (1-22 mg/l N).

Soluble Phosphorus: The levels of phosphate ranged from 0.05-2.754 mg/l P in July to 0.224-7.151 mg/l P during October. No significant temporal (ANOVA: $F_{8:17} = 3.972$; $p = 0.034$) or site variance (ANOVA: $F_{1:17} = 4.646$; $p = 0.063$) was recorded for soluble phosphorus during sampling. Site 3 (October 2003) recorded at an elevated level of 7.151 mg/l P in comparison with 0.139 mg/l P during July (Figure 2.4b). Sites 2, 4, 6-8 and 10 averaged around similar levels, respectively, for both sampling seasons with the exception of site 3 (October).

2.4.2 Microbial activity

Results are presented as the count of coliforms/100 ml of the water sample. Data were divided into faecal coliform and *E. coli* counts to establish possible origins of coliform contamination for each of the sampling seasons of July and October (Figure 2.5).

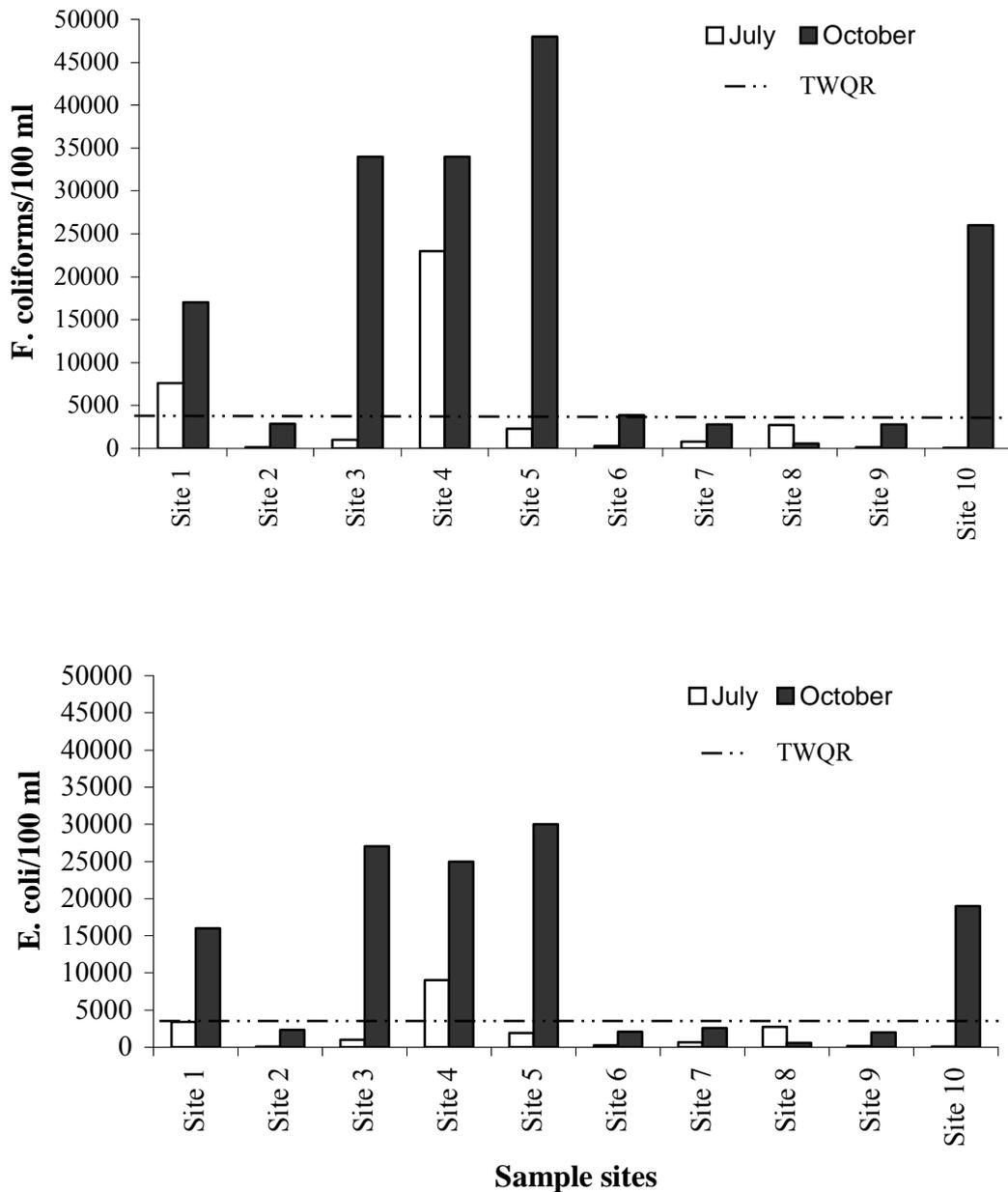


Figure 2.5: Faecal coliform and *E. coli* counts (per 100ml) recorded for July and October 2003, Eerste/Kuils River Catchment System, Western Cape. Target Water Quality Range (TWQR) is indicated for both parameters as suggested by DWAF (DWAF, 1996).

Faecal coliform counts exceeded recorded TWQR set for domestic use of water (100 counts/100 ml) for all sites during July and October. Recreational use limits (1000 counts/100 ml) were exceeded for sites 1 (7600/100 ml), site 4 (23000/100 ml), site 5 (2300/100 ml) and site 8 (2700/100 ml) during July and exceeded the 1000/100 ml count for October, except that of site 8 (600/100 ml).

TWQR for *E. coli* set at 130/100 ml for recreational use of water was exceeded during both sampling periods at all sites with the exception of site 6 (120/100 ml) and 10 (90/100 ml) during July. Human health hazard limits set at 400/100 ml for *E. coli* was exceeded at site 1 (3400/100 ml), site 3 (1000/100 ml), site 4 (9000/100 ml), site 5 (1900/100 ml), site 7 (640/100 ml) and site 8 (2700/100 ml) during October.

Sampling recordings indicated a seasonal trend for both faecal coliform and *E. coli* counts, as the sampling season of July recorded at approximately 80% overall less than during October. Similar patterns for both *E. coli* and faecal coliform was established for each sampling period with an approximately 20% variance between the two counts.

2.4.3 Bioassays

2.4.3.1 Lactate dehydrogenase (LDH)

During the *in vitro* exposure of white blood cells to water samples collected from the selected sites along the Eerste/Kuils River catchment, no significant leakage of LDH from cells were measured. No significant statistical variance was recorded between the two sampling seasons ($F_{1:47} = 0.364$; $p = 0.552$). Recorded samples during July for site 2 (453/ml), site 3 (380/ml), site 4 (399/ml), site 5 (376/ml), Site 6 (403/ml) and site 7 (418/ml) indicates lyses activity of cells (< 500 BB units/ml). LDH was also recorded at sites 1 (445/ml), site 2 (312/ml) and site 4 (451/ml) during the October season. No LDH activity was recorded for the sampling seasons as all levels reclined around negative values of 500 BB units/ml (Figure 2.6) and positive levels was recorded at 1500 BB units/ml ($F_{11:24} = 25.221$ $p < 0.001$) with no site recordings exceeding this level (not statistically significant).

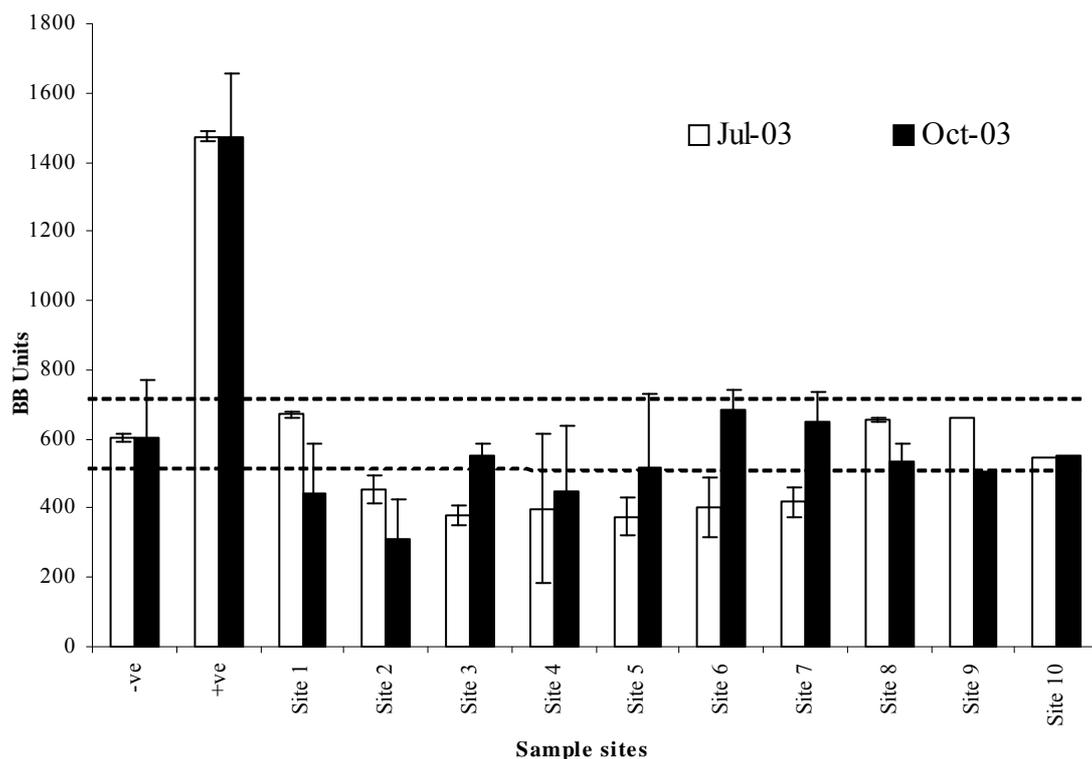


Figure 2.6: Catalytic action of LDH conversing pyricic acid to lactic acid in human blood cells and the leaching from the damaged cells. Range between 500 – 700 BB units indicates no cell wall damage to white blood cells occurs (area between horizontal dotted lines).

2.4.3.2 *Salmonella* biomarker

All sites recorded at less than 50% of the positive control during both sampling seasons. Significant variation was recorded for both spatial (ANOVA; $F_{9:19} = 5.646$; $P < 0.05$) and seasonal (ANOVA: $F_{2:19} = 79.179$; $P < 0.001$) variance data (Figure 2.7).

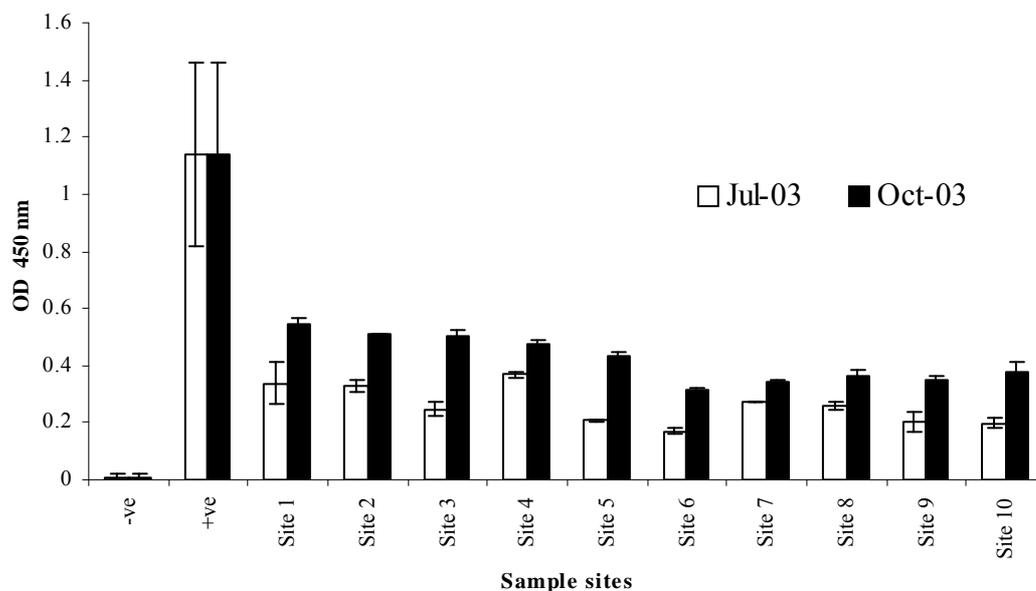


Figure 2.7: Levels of *Salmonella* detected by antibodies against O and H antigens of *Salmonella* through an ELISA assay. The positive control (+ve) consisted of *Salmonella thyphiriumium*. Negative control (-ve) laboratory water (ROH₂O).

All sites indicated a temporal variance with seasonal variance established for the upper reaches of the Kuils River. Site 1 (0.338), site 2 (0.327) and site 4 (0.368) recorded highest *Salmonella* levels (OD Units) during July and again for October sampling period (site 1: 0.542, site 2: 0.51, site 4: 0.475).

Final *Salmonella* data was subsequently expressed as a percentage of the positive control (Figure 2.8). Both sampling seasons July and October did not exceed the 50% level compared to the positive control.

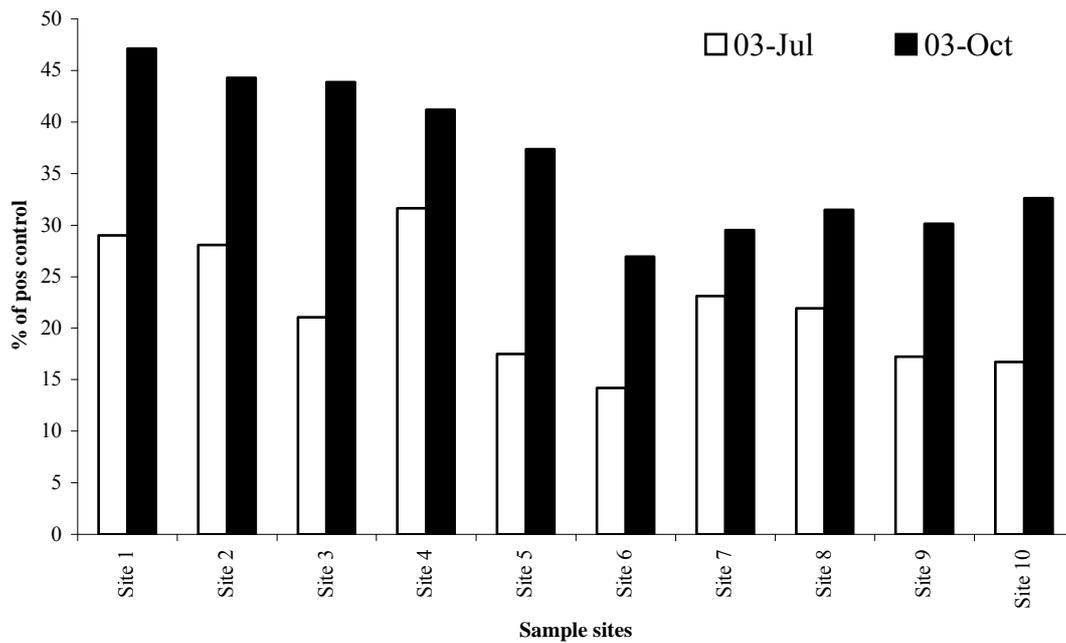


Figure 2.8: Levels of *Salmonella* expressed as a percentage (%) of the positive control (+ve) (100%). Values for both sampling seasons do not exceed the 50% level in comparison with +ve.

2.4.3.3 Inflammatory activity

Significant spatial variance was recorded for IL-6 (ANOVA: $F_{9,23} = 4.357$; $p = 0.011$). Site variance was linked to specifically sites 3 and 4 for both sampling periods (exceeding 200pg/ml). IL-6 activity levels recorded below safe limit (100 pg/ml), set for human contact, for site 9 during both sampling seasons at effective levels of 17 pg/ml (July) and 66 pg/ml (October).

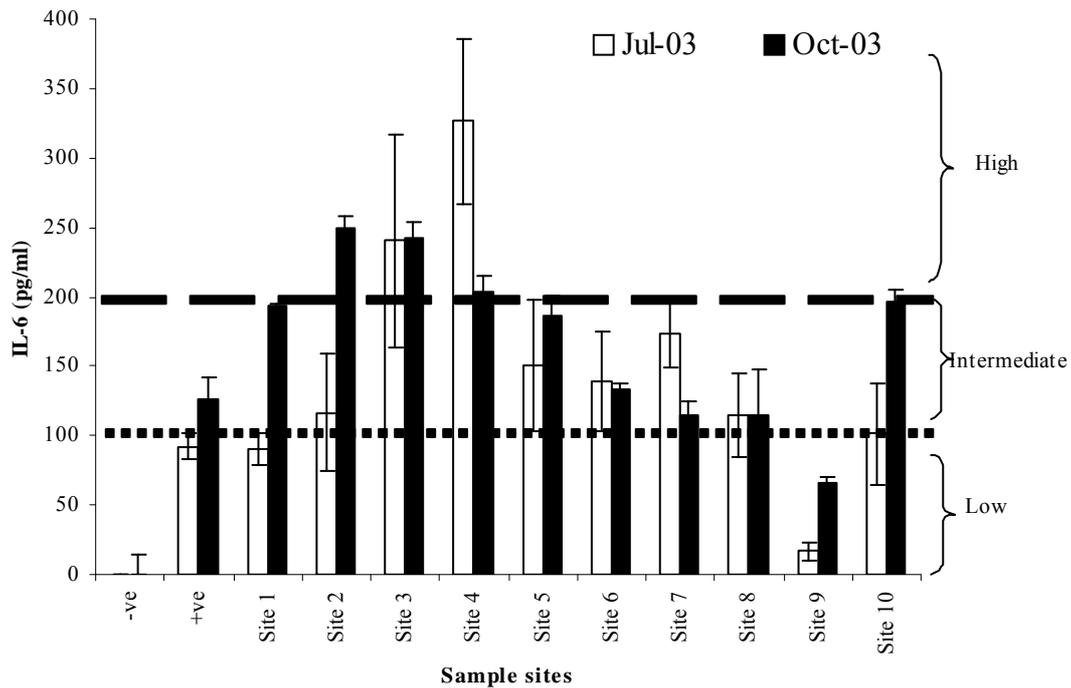


Figure 2.9: Inflammatory activity of water samples (1-10). The IL-6 induction by the positive control (+ve) was attained with 20 E.U. *E. coli* lipopolysaccharide. Negative control (-ve) laboratory water (ROH₂O). Low or safe concentration levels established at up to 100pg/ml (for human use), intermediate levels between 100pg/ml – 200pg/ml (human health risk association). Levels exceeding 200pg/ml (high) are considered dangerous to human health.

No significant seasonal variance was recorded for the sampling seasons of July and October (ANOVA: $F_{1:23} = 1.165$; $p = 0.304$ ($P > 0.05$)). A similar seasonal trend pattern could be established for the two seasons with site 2 (117 pg/ml), site 5 (151 pg/ml), site 6 (139 pg/ml), site 7 (174 pg/ml), site 8 (115 pg/ml) during July and sites 1 (193 pg/ml), site 5 (186 pg/ml) and site 6 (134 pg/ml) during October, fluctuating within intermediate active IL-6 levels. Both sites 3 and 4 exceed the maximum human health hazard (200pg/ml) for July (site 3: 241 pg/ml, site 4: 326 pg/ml) and October (site 3: 243 pg/ml, site 4: 204 pg/ml).

2.5 Discussion

Rivers are described as confined, uni-directional systems that accumulate materials (drains) brought in by wind, water and surrounding urban and rural communities (Dallas and Day, 2004). The draining effect is reflected directly in the river of a catchment and is capable of altering the entire length of the river through the build-up of pollutants in the water and sediment (Dallas and Day, 2004). As a limited resource and with the dependency of all South African communities, water quality regulations and National acts have become an essential tool in the decision-making and management consideration of rivers in a semi-arid South Africa. The National Water Act (Act 38 of 1998) incorporates receiving water quality objectives (RWQO) on a river-by-river and/or catchment-by-catchment basis, focussing on the preservation of water quality in natural rivers. Criteria and guidelines for water quality standards are well established internationally and in South Africa (SABS, 1984; DWAF, 1993; WHO, 1993; US EPA, 1996).

Water quality as management tool is based on the integration of various natural, physical and chemical variables. Climate, geomorphology, geology, soils and biotic composition differ from continent to region and influences water quality parameters (Dallas and Day, 2004). Within the South African context, Day and King (1995) linked regional differences in water quality to the combined effect of geological and climatic factors. Within the Eerste/Kuils River catchment a diverse community of urban and rural settlements, extending to the banks of the river, impacts on the system. Rural populations utilise the river largely as a recreational and drinking source, comprising of activities such as swimming, fishing and to a lesser extent domestic use purposes. Growing concern surrounding water extraction have been linked to the increasing potential for the outbreak of water-borne diseases due to human development and various point source contamination (WWTWs, effluent discharge, storm water runoff, agricultural runoff, stock pollution) (DWAF, 1996; Jonnalagadda and Mhere, 2001; Coetzee *et al.*, 2002; Levite and Sally, 2002; Kamara and Sally, 2003; Dallas and Day, 2004).

Analytical results for water quality analysis have been obtained through applied methodologies of known quality and accuracy and have been supplemented in the current study through the use of quantified bioassays. Water quality variables included temperature, total concentration of suspended solids (TSS), conductance, toxins and nutrient concentrations including soluble ammonia, nitrate and nitrite, total nitrogen and phosphorus and provides an overall view on the health of a river.

Temporal changes over a seasonal climax vary continuously for natural river systems and it is the temporal effects of pollutants in a river which is of concern. In the past the Kuils River was classified as a seasonal river with continuous flow during winter (July – August) and no or very little flow in summer months (October – March). Currently the river can be classified as perennial due to manmade changes (effluent discharge and canalisation) to the system (Cape Metropolitan Council, 2003). The Kuils River recorded varying temperatures over the longitudinal reaches of the river for each of the sites. July averaged around 13°C and October at 16°C. These changes can be linked to the summer flow periods of the Kuils River consisting almost entirely of treated sewage effluent from wastewater treatment plants located at Stellenbosch, Bellville and Zandvliet (Cape Metropolitan Council, 2003). Problematic is the fact that higher temperatures favour the growth of sewage fungus. Surrounding informal settlements utilise these waters for domestic and recreational activities and thus contribute to the spread of hazardous water borne diseases through the contagious effect of microbial contamination (Barnes *et al.*, 2003).

One of the more obvious characteristics of water quality to an observer lies within the visual clarity of the water, defined as the conductivity (electrical conductance) of the water or light penetration ability. Conductivity, along with Total Dissolved Solids (TDS), is a way to measure the presence of all “anions” and “cations” in your drinking water. Most of these compounds are harmless, but in very large quantities may cause potential issues. TSS recordings for the Kuils River indicated a clear seasonal trend coinciding with the onset of the summer period (dry season) as July averaged at 30 mg/l, increasing to 700 mg/l during October as rain storm events and thus water flow increased. Site 4 marked the highest level (27 mg/l) during July, due to organic and human pollutants originating from the surrounding rural community, with sites 2, 3, 5-8 and 10 averaging only between 3-9 mg/l, as pollutants became diluted and spread out along the reaches of the river. Conductivity and TSS influence mainly aquatic organisms (diversity and abundance) and fish populations (diseases, gill function, reproduction, habitat loss, food availability) (DWAF, 1996). With the exception of site 10 (655 mg/l) during October, no human health effects could be linked to TSS in the Kuils River (Kempster *et al.*, 1997). Maximum recommended concentration for TSS in drinking waters by SABS (1984) is 450 mg/l compared to the 500 mg/l by US EPA (1996) and the 1000 mg/l by WHO (1993).

Plant nutrients are required for the natural growth of riverine plants and include nutrients such as nitrogen, potassium, calcium, phosphorus and magnesium (Dallas and Day, 2004). Various factors such as climate and catchment characteristics contribute towards nutrients occurring within rivers. Water quality problems concerning nutrients derive mainly from eutrophication (nutrient enrichment) caused by human impacts on the system and can be of point-source (sewage treatment works, intensive animal enterprises, storm water runoff) or non point source in origin (agricultural runoff, urban runoff). According to Dallas and Day (2004), phosphorus (P) and nitrogen (N) as nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) is responsible for nutrient enrichment and stimulates excessive plant growth.

Nitrate-nitrite and ammonia levels, present as a nitrogen component (Rast and Thornton, 1996), exceeded threshold nutrient levels during both July and October. Although nitrate occurs naturally in water, it can also be introduced using high levels of fertilizer or industrial pollution. Too much nitrate turns to nitrite in the bloodstream as indicated for site 2 (July and October) and can cause methemoglobinemia (blue baby syndrome), a condition that hampers the body's ability to carry oxygen through the bloodstream (Faniran *et al.*, 2001). Nitrite is manufactured as a preservative and levels are particularly toxic causing death through anoxia (Dallas and Day, 2004). Ammonia toxicity affects the respiratory system of aquatic animals, causing cell damage, decreased metabolism and loss of equilibrium, coma and death in individual fish populations. An unstable compound, ammonia quickly turns into nitrate and then to nitrite, which can lead to methemoglobinemia. In the case of the current study (Site 2) high nitrate-nitrite levels of 11.08 mg/l N (July) and 7.22 mg/l N (October) can potentially be linked to the agricultural runoff from surrounding vineyards and grazing fields on the banks of the Bottelary River. Increased nitrate-nitrite concentration levels during July have also been linked to agricultural runoff (site 2 and 6), sewage effluent (site 7 – Zandvliet WWTW and site 8 – Macassar WWTW) and urban runoff (site 4 and 10) from neighbouring communities. TWQR for ammonia concentrations set for domestic use falls within 0-6 mg/l N (DWAF, 1996) with taste and odour problems arising as levels exceed 1.5 mg/l N. Ammonia levels for the Eerste/Kuils River maintained TWQR levels, impacting only on an aesthetic level at site 4 (July) and sites 3, 4, 7 and 8 (October). As temperature levels rise during October (summer) free ammonia levels increase with the associated increase in toxicity of NH_3 .

Phosphates normally do not pose a health threat in domestic water but will limit eutrophication and result in adverse ecological effects (WRC, 2000; Fatoki *et al.*, 2001). The South African standard for P in water resources that will reduce the growth of algal and other plants is set 5 µg/l (DWAF, 1996). Eutrophication supports a dense plant (aquatic) population, of which the decomposition kills animal life by depriving it of oxygen. It has also been reported that eutrophication-related problems increase in warmer water systems (0.34 mg/l to 0.70 mg/l P) (Fatoki *et al.*, 2001). The associated N concentration would be of the order of 0.34 mg/l N to 0.70 mg/l N (Rast and Thornton, 1996). Eutrophication in the Eerste/Kuils River system is high through-out the year (July, mean = 1.431 mg/l P; October, mean = 2.574 mg/l P), leading to the growth of blue-green algae, unsightly and malodorous scum and the associated release of toxic substances impairing the domestic and recreational use of the river (Fatoki *et al.*, 2001; Cape Metropolitan Council, 2003).

In summary, it seems that the chemical data for the Eerste/Kuils River catchment system is influenced by agricultural and storm water runoff, humans and anthropogenic non point-source activities causing serious problems for the aquatic system (Jonnalagadda and Mhere, 2001). Bio-organic parameters detected the occurrence of seasonal variance between the onset of winter and summer periods, including the presence of high nutrient levels during all seasons. Physical-chemical parameters sampled for the sites of the Eerste/Kuils River catchment system fell within the TWQR suggested by DWAF for recreational and domestic use (DWAF, 1996).

Along with the various indicator parameters used by the City of Cape Town, which include water quality parameters (pH, dissolved oxygen, total nitrogen, total phosphorus) and faecal coliforms counts, it has also become essential to classify waters through a more cost and time effective methodology. *In vitro* bioassays have proven to provide additional bio-activity data that can be used in addition to other monitoring programmes.

Microbial activity

Microbial quality of surface waters may be seriously affected by discharges from sewage works as well as runoff from informal settlements. Indicator organisms and faecal coliforms are used as bacterial indicators of faecal pollution (DWAF, 1996). General hygienic quality of the water was established through the calculation of density indicators (total coliform, faecal coliform and *E. coli*) (Clesceri *et al.*,

1998). Hence testing for pathogens within the Eerste/Kuils River system was based on the measuring of faecal coliform (as total coliform counts) and *E. coli* counts. Faecal coliform bacteria data for the reaches of the Kuils River followed a seasonal pattern with high overall counts per millilitre, particularly along the upper reaches of the river. High levels of coliforms were mainly found during the summer period (October) (Site 3, 4, 5 and 10). Based on the South African guideline value of 10 counts/ 100ml coliforms (DWAF, 1996), water from the river is unsuitable for domestic use purposes during summer seasons. Recreational guidelines of 0 to 130 counts/100ml set for full-contact recreation is also exceeded for October (Sites 3, 4, 5 and 10). This level of coliform concentrations poses a risk of contracting gastrointestinal illness as a result of contact with untreated water (Fatoki *et al.*, 2001). The faecal coliform level in the Eerste/Kuils River during October (Sites 1, 3, 4, 5 and 10) are higher than the South African guideline of 200 counts/100ml (DWAF, 1996) for water used for livestock watering and could pose a health risk to animals (Fatoki *et al.*, 2001). This is not surprising, as sources of faecal contamination for the river have been linked to storm water discharges and animal excretions. In particular, the Rietvlei storm water discharge point (site 3) and the Nooiensfontein pump station (site 4) have been identified as problematic areas as rural encroachment, grazing stock and commercial pollution produces high pollution quantities (Cape Metropolitan Council, 2003). Essential is testing the presence of *E. coli*, similar to that of the faecal coliform patterns for both July and October. *E. coli* counts for the Kuils River is thus representative of approximately 85% of the total coliform count. A worrying trend as 80-90% of faecal coliform counts consists of *E. coli*, particularly since *E. coli* is indicative of faecal contamination of waters by animals and humans.

Lactatedehydrogenase (LDH)

During the *in vitro* exposure of white blood cells to water samples collected from the selected sites along the Eerste/Kuils River catchment, no significant leakage of LDH from cells were measured. An LDH recording is based on the breakdown of cell walls and release of LDH from eukaryotes into the surrounding medium.

Salmonella biomarker

Salmonella as bacterium is associated with faecal wastes, causing gastroidal infections and disease in mammals on ingesting contaminated waters. *Salmonella* content for water within the Eerste/Kuils River system recorded < 50% of our positive

control for the whole study period with an increase in *Salmonella* content for samples during the summer sampling period (October). Seasonal variance exists and future studies on the particular cause and effect of this scenario will have to be established. Of interest is particular trend recorded for the two sampling seasons as counts of the bacterium decreased down stream towards site 6. Various factors contribute to this trend as site descriptions change from the upper to lower reaches e.g. site 1 is located within an industrial area, site 2 consists of the confluence of the Bottelary River (agricultural surroundings) and site 3 to 6 are highly populated, polluted and over grazed. Of concern is the fact that the downstream sites (site 7 to 10) increase in count instead of decrease as would be expected from the dilution effect on the river moving towards the ocean. Further investigation and regular monitoring programmes will contribute towards the pinpointing of the particular source of contamination of *Salmonella* linked to the WWTWs, storm water discharges or pollution.

Inflammatory activity

Inflammatory activity (IL-6 bioassay) has also been used to assess water quality linked to the impact of human activity on the quality of water in the Eerste/Kuils River catchment system. Microbes and their breakdown products cause severe reactions in humans such as fever, diarrhoea and anaphylactic shock in severe cases (Pool *et al.*, 2000). All samples collected from the Eerste/Kuils River catchment indicated inflammatory activity. Of the 10 selected samples, sites for July (sites 2, 5, 6, 7 and 8) and October (sites 5, 6, 7, 8 and 10) fell within intermediate levels (< 200 pg/ml) of inflammatory activity, posing a health risk to users of the water. Although unsatisfactory at intermediate level, this level of contamination is manageable and functional as a low health risk. The high levels of inflammatory activity in the river can be linked to the specific site location. For example, site 2 collects storm water runoff from the surrounding Kuils River urban area, site 3 is situated within the Rietvlei storm water discharge point and site 4 directly next to the Nooiensfontein pump station surrounded by rural encroachment and grazing stock on the banks of the river. It is of no consequence that through organic and inorganic pollution, microbes find then within exceptional breeding conditions and due to the natural watercourse, gets deposited downstream at lower intermediate concentrations. With the exception of site 9 (Moddergatspruit) acting as a natural filter through its man-made terraces and local community involvement in cleaning up the area, the Eerste/Kuils River catchment system is a source of high inflammatory activity.

Sources, not only linked to polluted sites (sites 2, 3, 4), but to the treatment works of Zandvliet and Macassar, attributes to unsafe inflammatory levels (100 pg/ml) in the river. It is strongly suggested that further studies be conducted on a seasonal (monthly) base for the Eerste/Kuils River, not only to identify sources of contamination, but to implement measures of prevention (removal of stock, storm water discharging control) and community involvement as management strategies. Both IL-6 and *Salmonella* antigen assays have proved to be effective test assays in the monitoring and assessment of water quality in local rivers. The *Salmonella* and IL-6 bioassays provides a clear indication of existing situations along the reaches of rivers as the *Salmonella* ELISA can detect dead organisms capable of inducing inflammatory reaction (Pool *et al.*, 2003b). These assays will aid in the assessment of water quality for factors that can potentially be a risk to human health.

2.6 Conclusion

As stated by the City of Cape Town, there exist a number of environmental challenges that face the Eerste/Kuils River catchment area, including that of organic pollution (bacterial contamination), recreational risk, habitat destruction, water treatment (WWTWs), rural encroachment and increasing catchment urbanisation. The main problem is associated with the increasing population growth along the reaches of the river and also the discharging of effluent into the surrounding estuarine and local aquatic environment. It is essential to address these problems to avoid further environmental degradation and associated human health risks. Substantial change through human influence has caused severe impact on the Kuils River as urban and storm water runoff continuously debilitates riverine and aquatic ecosystems (Dallas and Day, 2004). Continuous effluent discharge from storm water runoff and treatment plants have caused changes in the water regime and effectively caused severe flooding of the banks (Zhegar, 2001). Nutrient rich sewage effluent pollutes the river increasing microbial contamination. Urban runoff have been identified as containing twice the biological oxygen demand, fifteen times the P and N concentrations, and more suspended solids than well treated sewage effluent (Dallas and Day, 2004). Stated by Walsh (2000), a catchment affected by >25% of anthropogenic and human activities can only be appropriately monitored by an impact assessment focussing on pollution loading. Along with the various indicator parameters used by the City of Cape Town which include water quality parameters (pH, dissolved oxygen, total nitrogen, total phosphorus) and faecal coliforms counts,

it has also become essential to classify waters with a more cost and time effective methodology.

The demand for clean water has grown significantly and not only due to population growth, but increasing demands for water through domestic, agricultural and industrial use (Brown, 2002; Perret, 2002; Pollard, 2002; Deksissa *et al.*, 2003; Kamara and Sally, 2003; Dallas and Day, 2004). In the end the only possible solution to the problem lies within the prevention of further degradation of water sources, the cleaning of polluted waters and the involvement of management policies and strategies collaborating with local communities and national government ensuring the success and implementation of monitoring and assessment programmes.

CHAPTER 3

An assessment of estrogenic activity in the Eerste/Kuils River Catchment System, Western Cape, using an *in vitro* frog (*Xenopus laevis*) liver assay and an *in vivo* Zebrafish (*Danio rerio*) vitellogenin assay.*

3.1 Introduction

The Endocrine Disruptor Hypothesis suggests that a variety of chemicals (natural or synthetic) released into the environment modulate or disrupt the endocrine system in humans and wildlife (Guillette and Gunderson, 2001). This hypothesis originates from reports that certain natural and synthetic environmental chemicals are associated with adverse reproductive and developmental effects in wildlife and humans. Initial evidence suggested that environmental chemicals may mimic the action of female sex hormone, estradiol (estrogenic) or prevent this hormone from controlling its target organs (anti-estrogenic) (Rodgers-Gray *et al.*, 2000; Guillette and Gunderson, 2001; Takatsuki and Yamaguchi, 2001; Kirk *et al.*, 2002; Mosconi *et al.*, 2002). However, reports soon showed that other endocrine systems, including the androgenic control system in male reproductions as well as the thyroid system may also be targets of environmental chemicals (Hayes, 1997a; Gray, 1998; Kloas, 2002; Kloas *et al.*, 2002). Although the link to human health is still controversial, the health threat posed to wildlife populations has been shown in several case studies as well as laboratory exposures of selected bio-indicator species. It is therefore generally accepted that EDCs in natural water resources and drinking water potentially pose a health threat to both humans and wildlife populations (Damstra *et al.*, 2002).

The origins of endocrine active substances in water resources, including drinking water can be categorized into three broad environmental sources, namely 1) synthetic man-made pharmaceuticals, for example, contraceptives (17 α -ethinylestradiol), 2) natural compounds with hormone modulatory effects (female steroids, such as, 17 β -estradiol and phytoestrogens found in plants) and 3) chemical compounds originating from man-made industrial chemicals (alkylphenol polyethoxylates (APEs), phthalates, bisphenol A, dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs)) (Harries *et al.*, 1999; Baker, 2001). Chemicals with endocrine modulatory activity potentially could enter the aquatic

* Submitted to Water SA, 2004

system via wastewater treatment plants, industrial effluents, agricultural run-off and spills (Jobling *et al.*, 1998; Guillette, 2000; Baker, 2001). Rivers and streams globally have become catchments of large amounts of chemicals (natural and man-made EDCs) originating from industrial, agricultural and domestic sewage waste sources (Jobling *et al.*, 1998).

Concern surrounding the occurrence of EDCs started (1970's) with interest in the presence of hormones in human and animal excrements (Brauner and Tappeser, 1995), for example, fish (as biomarkers) (Sumpter and Jobling, 1995; Jobling *et al.*, 1998; Rodgers-Gray *et al.*, 2000; Takatsuki and Yamguchi, 2001), reptiles and birds (Guillette, 2000) and epidemiological studies of humans (Brauner and Tappeser, 1995; Damstra *et al.*, 2002). The US Environmental Protection Agency (US EPA) was mandated by the US government to develop screening and testing programmes for EDCs and their possible effects in humans (US EPA, 1996).

The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) were formed in 1996 presenting recommendations to the US EPA on the development of screening and testing programs. Outlined in 1998, these recommendations were incorporated in the Endocrine Disruptor Screening Programme (EDSP) created to screen potential environmental contaminants for endocrine modulating activities (agonistic and antagonistic) on estrogen, androgen and thyroid hormone systems (US EPA, 1996).

Although the EDSP is mainly aimed at identifying and categorizing chemicals as EDCs, the analytical detection of such chemicals at low concentrations in the environment remains problematic. Compounds may also bioaccumulate within sediments, organs or tissues or interact with other chemicals resulting in synergetic effects. These difficulties lead to (resulted in) the development of highly sensitive and specific bioassays based on biomarkers associated with specific hormone actions. Although bioassays initially identifies hormone modulatory activity and may not identify specific chemical compounds, expensive analytical methodology could be used only when hormone modulatory activity has been shown.

EDSTAC recommended a tiered screening and testing approach to assessment screenings towards early applicable and effective priority systems: Tier 1 screening focuses on assessment and priority setting of chemicals for which there is insufficient scientific data. The bioassays recommended include three *in vitro* assays and five *in vivo* assays. The *in vitro* assays included estrogen and androgen receptor binding (transcriptional activation assays) and a steroidogenesis assay using minced testes. The

five *in vivo* assays include a rodent 3-day uterotrophic assay; a rodent 20-day pubertal female assay for effects on thyroidal function, a male rodent 5-7 day Hershberger assay, a frog metamorphosis assay for thyroid effects and a fish gonadal recrudescence or vitellogenin production assay (US EPA, 1996; Sherry *et al.*, 1999; Smeets *et al.*, 1999). Tier 2 testing is designed to characterize more defined responses and therefore includes endpoints that will give decisive evidence whether or not tested chemicals may be an endocrine disruptor. Tier 2 tests include several wildlife species and are longer-term tests. The battery of tests suggested, include a two-generation mammalian reproductive toxicity assay, an avian reproductive toxicity assay, a fish life cycle toxicity assay, an invertebrate life cycle toxicity assay and an amphibian development and reproductive assay. It has been suggested that the weight of evidence approach will be used to evaluate the results obtained from these tests (US EPA, 1996). Following Tier 2 testing, positive chemicals and water samples could be tested for hazard assessment and eventually risk to human and wildlife health can be determined.

Endocrine disrupting effects manifests as antagonistic/agonistic activity in androgenic and estrogenic reproductive systems. These include evidence of masculinization (Vos *et al.*, 2000; Baker, 2001; Larsson *et al.*, 2002), anti-androgenic effects in secondary sexual traits (Foran *et al.*, 2000; Uglem *et al.*, 2002), abnormal phallic development and steroid hormones detected in alligators of Lake Apopka (Guillette *et al.*, 2002) and estrogenic activity in male fish exposed to sewage effluents resulting in decreased fish populations (Harries *et al.*, 1997; Harries *et al.*, 1999; Haung and Sedlak, 2001; Kirk *et al.*, 2002), etc. Although the wildlife to human link remains speculative, suggestions are that reproductive pathologies like altered sex ratios of offspring, developmental abnormalities in reproductive organs, temporal reduction in sperm counts and quality, and effects on neurological and intellectual function in young children could be EDC related (Guillette *et al.*, 1998; Guillette, 2000; Baker, 2001).

The detection of biological responses to exogenous environmental estrogenic chemicals was employed through the development of biomarkers in relevant indicator species (Schmieder *et al.*, 2000; Hurter *et al.*, 2002), for example, frogs (*Xenopus laevis*) and fish (trout, medaka, zebrafish). Vitellogenin (Vtg) as bioindicator of exogenous estrogens in contaminated water formed the basis of bioassays. Oviparous bio-indicator species, fish and amphibians, using Vtg as biomarker for estrogenic activity have been widely employed in screening and testing programmes. Vtg

production is a natural process, under multi-hormonal control, dominated by the stimulatory effect of 17β -estradiol (E_2) and is active during seasonal reproduction cycles of vertebrates (Sumpter and Jobling, 1995). During periods of increased E_2 production, Vtg (as a yolk precursor) is synthesised by the liver and released into the bloodstream. As the egg yolk precursor (Vtg) circulating in the blood, Vtg is taken up by the ovaries and transformed to egg-yolk in the developing oocytes (Allen *et al.*, 1999). This natural process is normally absent in males because of low circulating E_2 . However, the genes for expressing Vtg synthesis is present in the liver of males and when exposed to estrogen or estrogen mimics these are stimulated to produce Vtg (into plasma) (Sumpter and Jobling, 1995). Vtg stimulation, therefore, acts as an efficient biomarker to show the presence of estrogenic pollution in the environment. The question of whether it is harmful to male individuals however still remains unanswered (Kime, 1998).

UK scientists conducted a caged fish exposure study to detect estrogenic activity in wastewater treatment work (WWTW) effluent (Harries *et al.*, 1997; Harries *et al.*, 1999; Kirk *et al.*, 2002). Exposure studies utilising lower vertebrate species followed and paved the way for the collection of experimental organisms directly from the environment. This included organisms such as fish (Beyer *et al.*, 1996; Beullens *et al.*, 1997; Chen *et al.*, 2001; De la Tore *et al.*, 2002), frogs (Kloas, 2002; Van Wyk *et al.*, 2003) and alligators (Crain and Guillette, 1998; Guillette *et al.*, 2002; Katsu *et al.*, 2004). The Zebrafish (*Danio rerio*) and the Japanese medaka (*Oryzias latipes*) have proven to be effective models due to the fact that they are small fish species with short generation times (Tong *et al.*, 2004).

Zebrafish assays are currently being validated under Tier 2 of the US EPA, as suggested by the Organization for Economic Cooperation and Development (OECD) and EDSTAC, as robust and sensitive methods for Vtg quantification (Fenske *et al.*, 2001). Although fish screens or *in vivo* tests prove sensitive and reliable, they are labour intensive and time-consuming (Kime, 1998; Hurter *et al.*, 2002). The correlation of *in vivo* and *in vitro* assays (Jones *et al.*, 2000; Mosconi *et al.*, 2002) would therefore be more efficient in establishing a complete image of the environmental impacts. This has led to the development of *in vitro* assays (screens) (Krner *et al.*, 1999; Sherry *et al.*, 1999; Smeets *et al.*, 1999; Baker, 2001; Folmar *et al.*, 2002; Van den Belt *et al.*, 2004) including transgenic yeast and animal cell screens (Li *et al.*, 2004; Pawloski *et al.*, 2004; Schultis and Metzger, 2004). Recently the developments of a *Xenopus laevis* liver slice bioassay along with a specific

ELISA detection system for *Xenopus* Vtg allows for exposure of tissue slices rather than using single cell culture systems (Hurter *et al.*, 2002).

United Kingdom (UK) estrogen research in rivers and streams (Rodgers-Gray *et al.*, 2000) receives wide-spread attention, specifically where wastewater effluents and treated sewage effluent are concerned. Case studies include fish species such as roach (Jobling *et al.*, 1998), gudgeon (Rodgers-Gray *et al.*, 2000) and flounder (Allen *et al.*, 1999) screened for Vtg. Further studies tested estrogen activity on male fertility (Purdom *et al.*, 1994), the influence of pesticides on tilapia (*Oreochromis mossambicus*) (Panday and Shukla, 1980) and Medaka (Gray and Metcalf, 1997) and various other effects such as testicular and ovarian morphology, steroid genesis, sperm viability, ovulation, eggs, embryos, larvae and juvenile fish (Kime, 1998).

In South Africa, environmental regulation is done through several national monitoring programmes, including microbial, chemical, eutrophication and river health programmes (DWAF, 1994; DWAF, 2002a, b). Recently, with the establishment of a revised National Water Act (DWAF, 1998), the development of a National Toxicants Monitoring Programme (NTMP) was initiated for implementation in 2007 (Murray *et al.*, 2003). Considering the list of potential EDCs released into the environment through various sources (industrial, agricultural, sewage effluents) it is of no consequence that EDC activity may exist in the South African aquatic environment (Hayes, 2002). It is therefore important to include bioassays to assess EDC activity in South African waters. However, most bioassays available still needs validation and assessment of use under local environmental conditions.

The City of Cape Town, an authoritative municipal body for the urban areas of Cape Town (Western Cape), has the task of monitoring and controlling various catchments systems under their control. Currently the City of Cape Town, through their Scientific Services Department, directorate Water and Waste conduct water quality studies within their major and minor catchments. The specific state of the rivers in the City of Cape Town varies greatly between catchments, depending largely on the degree of urbanization (e.g. sewage and industrial effluents, storm water runoff). City of Cape Town catchments include the Salt, Diep, Lourens, Houtbay, Zeekoei and Noordhoek catchment areas, of which the Eerste/Kuils catchment stimulated interest due to three sewage treatment works located along the banks of the river. Various factors such as physical pollution, canalisation due to urbanization, loss of habitat, rural encroachment and hydrological physical alterations to the river have increased dramatically over the past 5 years (Cape Metropolitan Council, 2003).

The aim of the present study was to assess the practical aspects of using an *in vitro* *Xenopus laevis* Vtg liver slice bioassay and *in vivo* Zebrafish exposures as screening tools to assess estrogenic activity in the Eerste/Kuils River catchment system and determine the potential estrogenic activity for point's sources along the reaches of the river.

3.2 Material and methods

3.2.1 Study area, sampling sites and sampling handling

Water samples to assess estrogenic activity in the Eerste/Kuils River Catchment system was collected during July 2003 and October 2003. Study description, site selection, sample collecting and handling have been detailed in Chapter 2. Water samples were selected from 5 specific sites (sites 2, 4, 7, 8, 10) (Figure 3.1) allocated along the Kuils River.

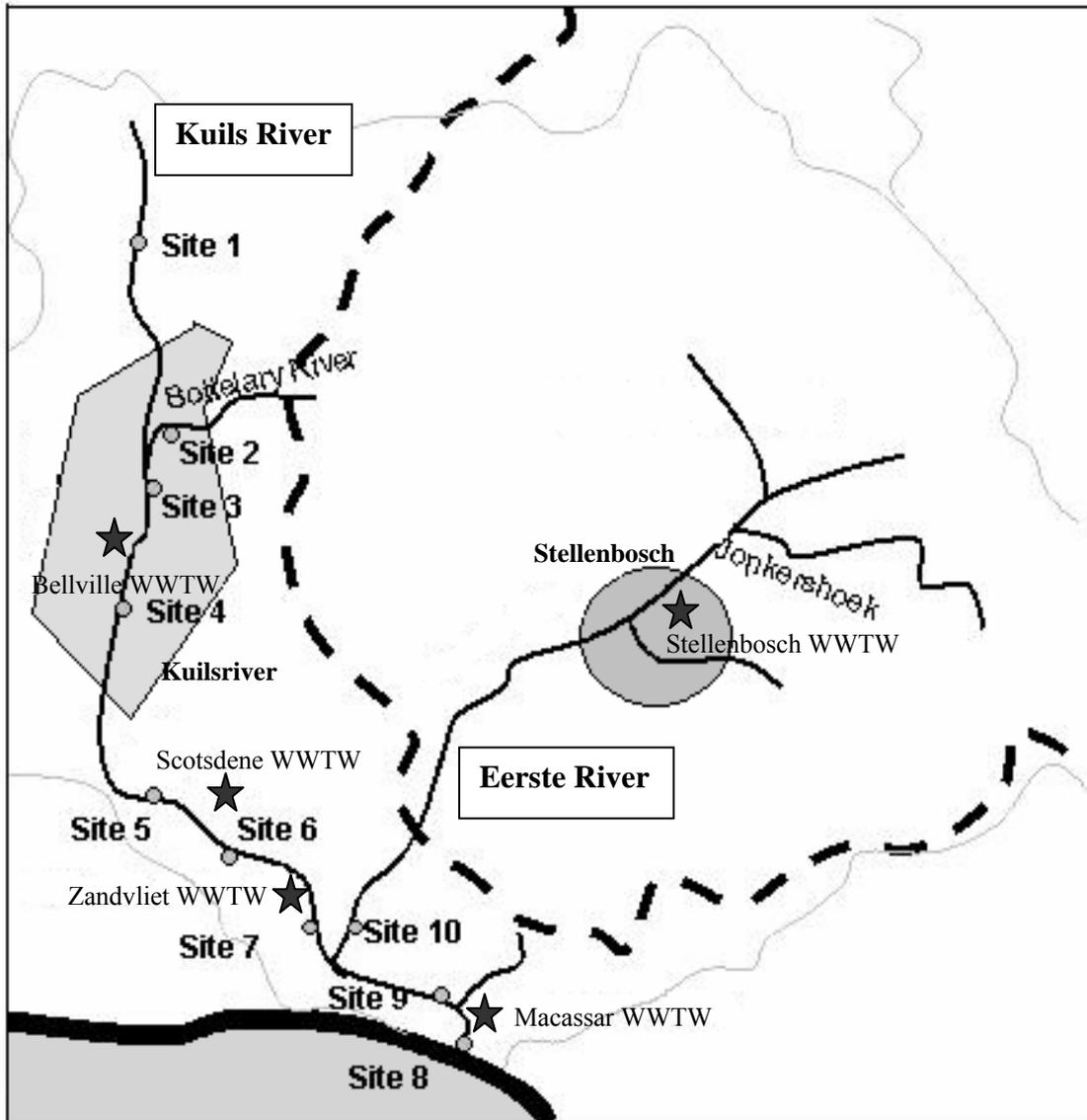


Figure 3.1: Sampling sites 1-10 located along the reaches of the Eerste/Kuils River Catchment System (Table 1), Western Cape, for sampling periods July and October 2003. Location allocated according to the impacts along the reaches, including the Stellenbosch, Bellville, Zandvliet, Scotsdene and Macassar sewage treatment works (STWs) (effluent discharge points).

3.2.2 Liver slice bioassay

Animals

The *in vitro Xenopus laevis* liver slice bioassay for Vtg production was performed according to the protocols described in Hurter *et al.* (2002). Adult *Xenopus laevis* frogs were purchased from a commercial supplier (African Xenopus, Knysna), collecting *Xenopus* from water bodies in pristine areas in the George-Knysna area (34°01'S; 22°23'E).

In the laboratory frogs were maintained in a climate-controlled room set at 25°C (±2°C) temperature and fitted with a 14:10 LD cycle. Frogs were fed every week *ad libitum* with commercial fish pellets (AquaNutro (Pty) Ltd, South Africa).

Preparation of C18-extracted water samples

Sub-samples (200ml) were filtered through 185mm diameter circle filter paper (Whatman). Hydrophobic (lacking affinity for water) molecules in the water were extracted using Isolute SPE 6ml C18 solid-phase columns (Anachem, South Africa). Solvent mixture (40% hexane, 45% methanol, 15% 2-propanol) was utilised to elute the bound hydrophobic substances. The eluate was collected in sterile 2ml glass tubes, dried under air and finally re-dissolved in ethanol to 1/100 of the original volume.

Tissue Culture Medium Preparation

RPMI 1640 containing L-glutamine (BioWhittaker, Cambrex Bio Science, USA) was used as tissue medium. A mixture of antibiotics consisting of streptomycin, fungizone and penicillin (Highveld Biologicals) was added to the medium, in accordance to the manufacturer's instructions, and stored at +4°C for future use. For *Xenopus laevis* liver culture this medium was diluted 7:3 with sterile pharmaceutical grade water. Subsequently the diluted RPMI medium will be referred to only as "liver culture medium".

Tissue Culture Preparations

A male *Xenopus laevis* frog was selected as a donor for liver tissue and placed in 70% ethanol for decontamination of the skin. Subsequent procedures were carried out in laminar flow cabinet using aseptic techniques. Liver tissue was removed and placed in a culture dish (Nunclon Surface, Denmark) containing "liver culture medium" to prevent dehydration of liver tissue. Cubes of approximately 1mm³ were cut from

liver. Cubes (1 per well) were transferred to 96-well culture plates (NalgeNunc, Denmark). Wells containing a single cube of liver tissue was then filled with 200µl “liver culture medium” and 50µl prepared water sample media. Four replicates of each sample were prepared. After 72 hours of incubation at 27°C, wells were refilled, following removal of medium, with fresh medium containing reconstituted water samples. The plates were then incubated for a further 72 hours. Medium was finally collected on day 6 and stored at -80°C.

Xenopus Vtg ELISA

Vitellogenin production was detected by a validated ELISA, as described by Hurter *et al.* (2002). In summary, samples were assayed in duplicate on a 96-well ELISA plate (Maxisorp, Nunc, Denmark). A positive (+ve) and negative (-ve) control was included on each plate. The +ve control was a sample with a known Vtg concentration. The -ve control consisted of a sample containing no Vtg. Wells were coated with 50µl of sample diluted 1/50 in phosphate buffered saline (PBS) and incubated for 2 hours. The coat was then aspirated and wells were blocked with 200µl of 5% milk powder in PBS for 30 minutes. The plates were then washed with 0.9% v/m NaCl and 50µl mouse anti-*Xenopus* Vtg diluted 1/1000 with PBS added to each well and incubated for 2 hours at room temperature. The plate was washed followed by the addition of 50µl/well of peroxidase linked goat anti-mouse immunoglobulin conjugate complex (Amersham, South Africa) and incubated for 1 hour. The plate was washed again as before. 100µl of substrate (POD Substrate, Roche, Germany) was added to each well. The reaction was stopped after 20 minutes through the addition of 50µl/well of 0.5 M H₂SO₄. The optical densities were read on a plate reader at 450nm.

3.2.3 *In vivo* Zebrafish (*Danio rerio*) exposure

Zebrafish (adult males) was obtained from a commercial supplier (Bromley’s Fish and Pet Centres) and placed in laboratory water (reverse osmosis water containing 3.75 g/l NaCl and 1.2 g/l NaHCO₃). Fishes were kept in aerated and filtered water in a glass-holding tank (50L). The fish were left for 2 days to acclimatise to laboratory conditions of temperatures between 24-26°C prior to experiment. The zebrafish (20 fish per water sample) were then transferred to 1.5L glass bottles, filled with environmental waters selected according to their possible estrogenic activity based on physical-chemical measurements (Temp, DO, Total Suspended Solids, pH). Sites

were selected to cover and incorporate all possible effects on the river, for example, wastewater treatment works (WWTWs). A negative control containing buffered laboratory water only and a positive control containing water spiked with 100µg/l 17β-estradiol was included with each run. After 7 days of exposure, blood was collected from the tail vein (tailfin removed) into an anti-coagulate buffer solution (0,01 % Phenylmethylsulfonyl fluoride (PMSF) in saline containing 5 IU/ml heparin). The blood samples were then centrifuged (8000 rpm) for 5 minutes and then transferred to a 96-well ELISA plate (Maxisorp, Nunc, Denmark) at 50µl/well.

3.2.4 Zebrafish Vtg ELISA

Blood samples were diluted to give a protein concentration of 100µg/ml. Maxisorp micro plates (Nunc, Denmark) were coated with 100µl of the diluted blood samples from exposed fishes. The plate was placed on an orbital mixer to incubate overnight. After aspirating the coating antibody, non-specific binding sites on the plate were blocked for 20 minutes by the addition of 200µl of 1% (v/v) albumin in 0.9g/100 ml NaCl to each well. The plate was then washed with 0.9% (m/v) NaCl (saline). This was followed by the addition of 50µl of mouse monoclonal antibody against *Xenopus* Vtg dilute 1/1000 in 0.1% albumin in saline and incubated for 2 hours. The plate was then washed with saline, followed by the addition of 100µl enzyme conjugate (sheep anti-mouse IgG horseradish peroxidase conjugate) and incubated for 20 minutes. The plate was washed again as before. Substrate (100µl) (BM Blue POD Substrate, soluble, Roche, South Africa) was added to the wells. The chromogenic reaction was stopped after 10 minutes through the addition of 50µl/well of 0.5 M H₂SO₄. The optical densities were read on a plate reader (Labsystems Multiscan MS) at 450nm.

Following the Zebrafish Vtg ELISA, possible females present in groups were removed from the final results through histological investigation. Zebrafish samples, which were Bouin-fixed (Bankroft and Stevens, 1977), were dehydrated and embedded in Paraplast-plus (56°C melting point). Microtome cut sections of 5-10 µm was stained with haematoxylin and eosin (H&E staining method, Bankroft and Stevens, 1977) for histological discrimination between male and female zebrafish based on gonad inspection. Visual discrimination of gonads was based on histology slides as presented in Figure 3.2.

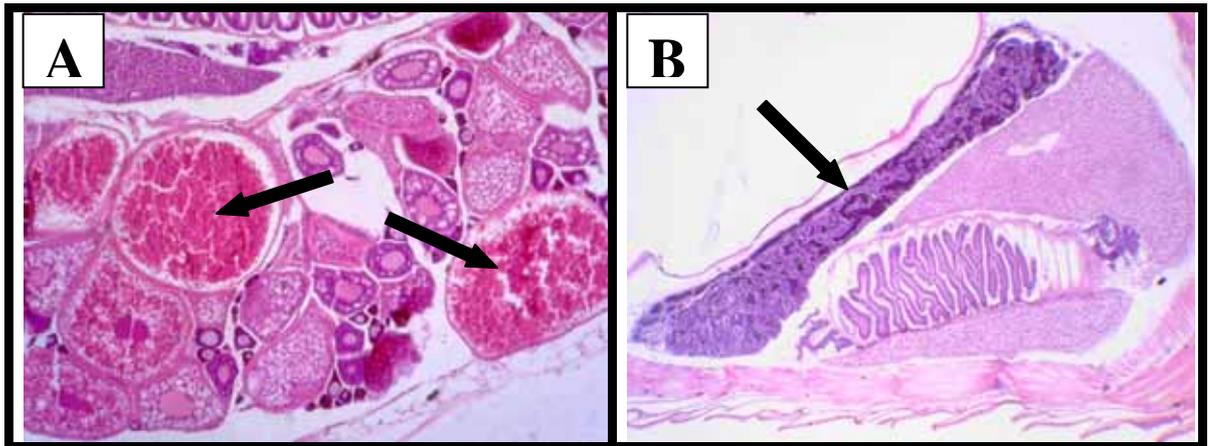


Figure 3.2: Photomicrographs of longitudinal histology sections (10um) of Zebrafish (*Danio rerio*) to determine the sex of individuals. A) Female specimen showing large vitellogenic ovarian follicles, B) Male specimen showing testicular tissue.

3.3 Statistical analysis

Statistically significant differences were confirmed by using one-way analysis of variance (ANOVA). Following an ANOVA, a multiple comparison test (Holm-Sidak) was used to indicate between sample differences. Data were tested for homogeneity of variation and normality prior to ANOVA. In the case of non-parametric conditions, analysis of variance by ranks, Kruskal-Wallis test along with the Newman-Keuls multi-comparison test was used. Two-way analysis of variation was conducted to see weather season had and effect on site variation. A p-value < 0.05 was considered significant. Statistical calculations were performed using SigmaStat computer software (SPSS, Inc.).

3.4 Results

3.4.1 Liver slice bioassay

Results recorded for estrogenic activity in water from the Eerste/Kuils River (July 2003 and October 2003), using a *Xenopus laevis* liver slice Vtg assay, indicated low vitellogenin (Vtg) (average = 3.8 µg/ml) (Figure 3.3, sites 1 to 10). Internal controls showed that the assays negative control liver slices (exposed to laboratory RO water) resulted in relatively low Vtg production, whereas the positive control (laboratory water spiked with estrogen) resulted in high Vtg production responses.

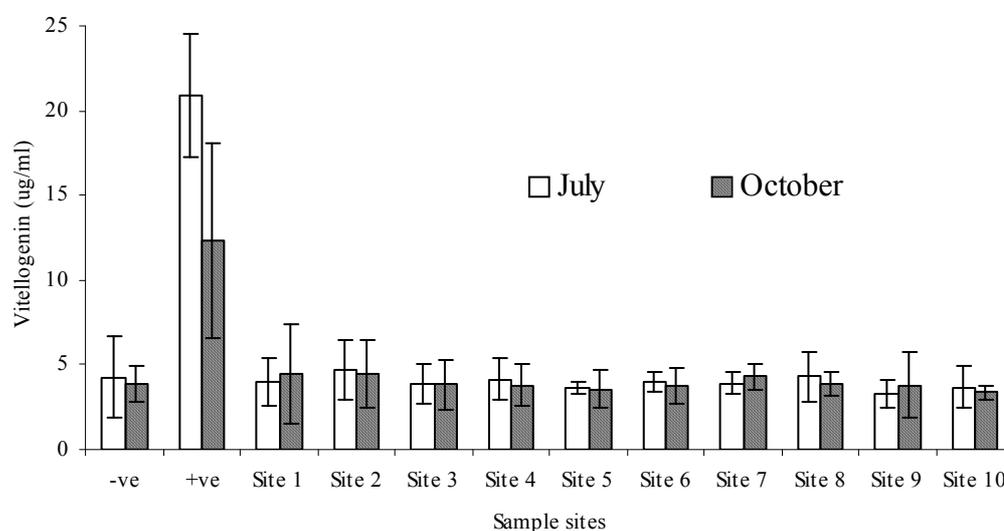


Figure 3.3: *In vitro* mean vitellogenin (Vtg) concentration induction by water samples extracts using *X. laevis* liver slices (N = 4 replicates per sample site). Y Bars indicated standard error of deviation for each site. Negative control (-ve) consisted of buffered laboratory H₂O. Estrogen spiked (1000 EU/ml) (European Units) laboratory water served as a positive control (+ve) for this assay. Site 1 excluded from City of Cape Town data, subjected to bioassay analysis of water from the Eerste/Kuils River Catchment System.

No significant spatial variation in Vtg response following exposure of liver slices to water from the different sampling sites during July 2003 (ANOVA: $F_{10:43} = 1.223$; $P = 0.313$). Recorded Vtg levels for July, exceeded -ve assay level ($4.25\mu\text{g/ml}$) at site 2 ($4.65\mu\text{g/ml}$) and site 7 ($4.31\mu\text{g/ml}$), but these proved not to differ significantly ($P > 0.05$) from the negative control or any of the samples collected during October (ANOVA: $F_{10:43} = 0.547$; $P = 0.844$). Although signs of increased Vtg production were noted at site 1 ($4.49\mu\text{g/ml}$), site 2 ($4.45\mu\text{g/ml}$) and site 7 ($4.29\mu\text{g/ml}$), none of these proved too significantly ($P > 0.05$) different from the Vtg production measured in the negative control or any other sites (Figure 3.3).

From Figure 3.3 it is clear that between months (seasonal) variation in Vtg production was small with no significant variation in Vtg production recorded between the July and October exposures (ANOVA: $F_{1:95} = 1.171$; $p = 0.283$).

3.4.2 Zebrafish exposure (*in vivo*)

Male zebrafish ($N = 20$ per sample) was exposed to five water samples (a sub set of the complete sample set) collected during the October 2003 sample period (Figure 3.4, Sites 2,4,7,8 and 10). No statistically significant difference was recorded (ANOVA: $F_{4:90} = 20.021$; $P = 0.098$) between the five water sample sites. The zebrafish Vtg assay proved to be responsive showing significant differences between the Vtg response when comparing the negative control (laboratory RO water) and a positive control (estrogen spiked laboratory RO water) (Figure 3.4).

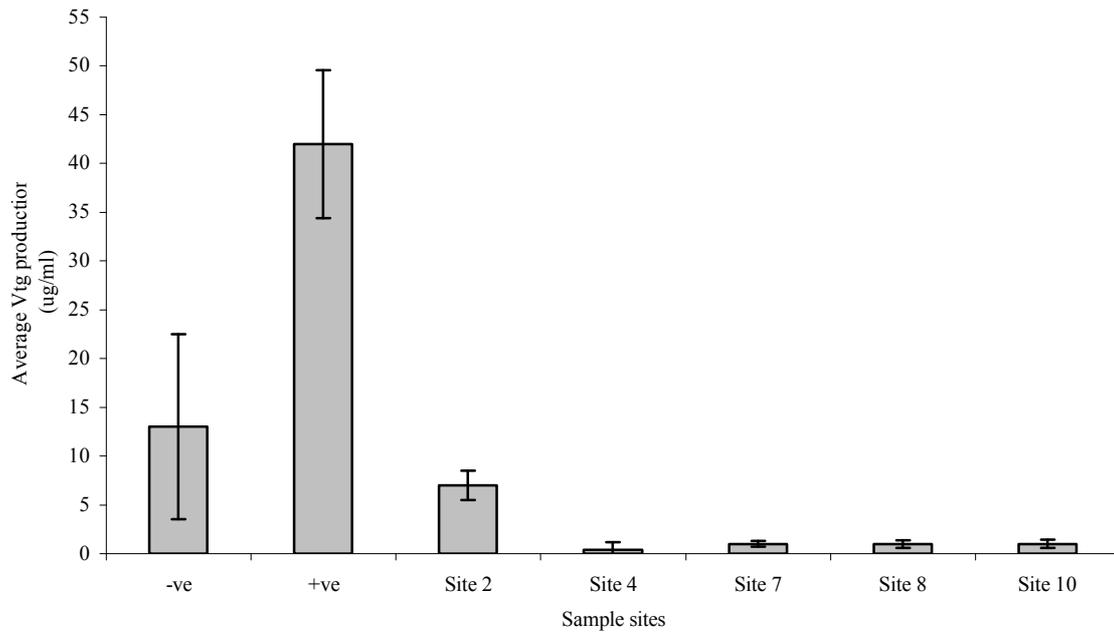


Figure 3.4: The *in vivo* induction of vitellogenin (Vtg) synthesis (mean concentration) using Zebrafish (N = 20 per sample site) exposed (8 days) to environmental water samples collected at five selected sites in the Eerste/Kuils River Catchment System. Y Bars indicate standard error of deviation for each site. Negative control (-ve) consisted of buffered laboratory water. Positive control (+ve) 50 μ g/l (17 β -estradiol). Site 2 varies significantly from sites 4, 7, 8 and 12, but not significantly from the negative control.

Initial results, however, showed increased Vtg levels in the negative control fish, but following histological examination all the high outliers proved to be female fish. In spite of the removal of these samples from the experimental group, plasma Vtg levels in the remaining male fish were still relatively high (Figure 3.4; average at 13 μ g/ml). Following the removal of female fish (histological investigation) the remaining male fish showed significant increased plasma Vtg levels in water collected from site 2 (7 μ g/ml) (ANOVA: $F_{6,134} = 149.24$; $P < 0.001$, Figure 3.4).

3.5 Discussion

Endocrine disrupting compounds (EDCs) are chemicals that have been suggested to interfere with, or adversely affect the reproductive system of fish and other wildlife populations (Johnson *et al.*, 2001). In the present study two bioassays, *Xenopus laevis* *in vitro* liver assay and an *in vivo* Zebrafish (*Danio rerio*) exposure were used to assess the possibility of estrogenic activity at selected points in the Eerste/Kuils River catchment system. Using the vitellogenin (Vtg) response of the liver as biomarker, results from both these assays showed that little estrogenicity (a positive response only at one site), occurred in the Eerste/Kuils River catchment system during the two sample periods, July and October 2003.

The US EPA, through the EDSTAC report on the development of an EDC screening programme, suggested a tiered approach to screen for EDC activity of environmental chemical (Gelbke *et al.*, 2004). Assessing potential estrogenic activity at Tier 1 level may be done by using *in vitro* bioassay utilizing Vtg, a yolk precursor lipoprotein complex production in the liver as biomarker for estrogen stimulation (Sumpter and Jobling, 1995; Harries *et al.*, 1997; Jones *et al.*, 2000). The recent development of a *Xenopus laevis* Vtg ELISA detection assay (Hurter *et al.*, 2002) allows for the use of the local *Xenopus laevis* in both *in vivo* and *in vitro* bioassays. For this study the liver slice *in vitro* culture system was selected and proved to respond to estrogenic activity in water samples (spiked positive control vs. negative control). The study corroborates the findings by Hurter *et al* (2002) that the assay has a detection range between 62.5 and 1000 ng/ml with sensitivity for detecting natural estrogens (as low as 1 ppb), as well as man-made estrogen mimics present in the environment. Although the *X. laevis in vitro* liver slice assay proved effective in its detection of estrogenic activity in spiked water samples, no or very low estrogen activity was detected in the water collected from the study sites. These results seem surprising considering the effluent discharge received by this catchment (including three sewage treatment plants). Similar results were reported by Hayes (2002) regarding three other Western Cape rivers. However, when collecting fish from these catchments, Hayes (2002) found a significant number of male fish showing the presence of Vtg in their plasma. They concluded that low level pollutants may bioaccumulate over a period of the life time of the fish and that point assessments may lead to false negatives because of low concentrations or high dilution rates in the catchments.

Xenopus laevis liver assay results were confirmed by the zebrafish Vtg exposure assay. For the Zebrafish assay water collected at selected points in the catchment were used undiluted. Following an *in vivo* exposure of seven to eight days, Vtg production levels of exposed male zebrafish were measured using a fish Vtg ELISA. The zebrafish Vtg exposure bioassay has been included in the battery of EDC screens recommended by the OECD (Gelbke *et al.*, 2004). In support of the validation studies, the Vtg response in the male zebrafish exposed to estrogen spiked water suggested that this *in vivo* assay is sensitive to low estrogen (<1ppb) environmental concentrations. Moreover, the fact that male fish exposed to laboratory RO water, stored in a plastic container, showed some Vtg response suggest that the zebrafish assay is indeed sensitive to low levels of environmental estrogens. The zebrafish assay showed a positive Vtg response when exposed to water collected from site 2 (Bottelary river - urbanisation, storm water discharge point, vineyards) in the catchment. Although this sampling site was upstream of the main industrial and sewage discharge points, this particular incidence may be linked to the surrounding agricultural runoff associated with the vineyards along the banks of the Bottelary River. Several reports suggest that herbicides and insecticides can act as weak estrogens (Andersen *et al.*, 2002; Brown and Fairchild, 2003; Hamers *et al.*, 2003).

As a biomarker, Vtg has been repeatedly used in the identification of estrogenic contamination in aquatic environment (Sumpter and Jobling, 1995). Various international studies (Sumpter and Jobling, 1995; Harries *et al.*, 1997; Allen *et al.*, 1999; Harries *et al.*, 1999; Haung and Sedlak, 2001; Kirk *et al.*, 2002; Pawlowski *et al.*, 2004), focussed on the assessment of estrogenic activity present in particularly wastewater and sewage effluent. As estrogens induce Vtg in fish species, zebrafish has been identified as a practical indicator species in screening for estrogenic activity (Örn *et al.*, 2003). One of the problems when using adult zebrafish in exposure studies is the accurate determination of sex. In the current study, histological inspection of individual specimens was needed to sex individuals and females were removed from the data-set before proceeding with the data analyses. Brion *et al* (2004), however, demonstrated that juvenile zebrafish are sensitive to low E₂ (17 β -estradiol) concentrations and that adequate amounts of Vtg could be produced to facilitate Vtg measurement by using existing ELISA techniques. Future exposure studies would benefit from using juvenile zebrafish in exposure studies since low plasma estrogen levels are associated with immature zebrafish and the problems regarding sex determination solved (Andersen *et al.*, 2003; Brion *et al.*, 2004).

Studies will have to be conducted to conclude that estrogenic activity in the Eerste/Kuils River is significantly low in spite of all the contributions or inflows. In particular, seasonal variance may be influential on other surrounding factors that may indirectly influence the stimulation or inhibitory effect of estrogen and/or estrogen mimics. Although effluents were not screened directly, discharging effluent has however been excluded as having adverse impacts linked with the estrogenic activity currently present in the Eerste/Kuils River System as sites, linked to the discharge points of effluents, indicated no significant levels of Vtg.

Future follow-up studies regarding the Eerste/Kuils River catchment need to include a more comprehensive seasonal monitoring programme, as well as exposing zebrafish and liver slices, to extracts from sediments (Kleinkauf *et al.*, 1998; Bolz *et al.*, 2001; López de Alda and Barceló, 2001; Bowman *et al.*, 2002). Moreover, plasma Vtg determination and histological studies on the reproductive organs need to be added to the list of potentially affected endpoints. Although effluents were not screened directly, discharging effluents need specific assessment.

3.6 Conclusion

The present study was conducted using the validated protocol for both the *X. laevis* liver slice and Zebrafish (*Danio rerio*) Vtg assay. Both assays worked well and were easy to implement. The liver assay was used on two occasions (July 2003 and October 2003) and the zebrafish Vtg assay on a single occasion (October 2003) to screen water resources for estrogenic activity. Data suggested that apart from a single site (site 2) no or very low levels of estrogenic stimulations were evident in the Eerste/Kuils River catchment system. Future studies should include the exposure of juvenile zebrafish to overcome the problem of sexing adult fish prior to exposures. It is important that local endemic fish species are collected in parallel with the screening activities. Moreover, the analyses should include exposure to soils at the sampling site. Finally, the study showed that the capacity to use *in vitro* and *in vivo* environmental assessments in terms of EDCs exists and should be utilized on a regulatory basis.

CHAPTER 4

Assessing endocrine disrupting activity related to thyroid function, at selected sites, in the Eerste/Kuils River Catchment System, in the Western Cape, South Africa.*

4.1 Introduction

The endocrine system in humans and wildlife regulates functions such as reproduction, sexual development, growth and homeostasis (Kime, 1998) and is therefore recognized as the main chemical messenger system in living organisms. Environmental compounds are suspected to cause chemical-induced alterations in the endocrine system of wildlife and humans and these compounds has been referred to as endocrine disrupting compounds (EDCs) (Kloas, 2002). Over the past decade, environmental chemicals suspected of being endocrine disruptors have received widespread attention (Jiménez, 1997; Lutz and Kloas, 1999; Kloas *et al.*, 1999; Sultan *et al.*, 2001; Kloas, 2002; Petrelli and Mantovani, 2002; Kuruto-Niwa *et al.*, 2004; Dalvie *et al.*, 2004).

Endocrine disrupting compounds (EDCs) include natural animal and plant hormones (e.g. 17β -estradiol and phytoestrogens, contraceptives, 17α -ethinylestradiol) as well as a range of synthetic man-made chemicals including industrial chemicals and general household products (e.g. endosulfan, dieldrin, atrazine, aldicarb, vinclozolin, DDT, metabolite DDE, bisphenol-A, phthalates, polychlorinated biphenyls and alkylphenols) (Preziosi, 1998; Harries *et al.*, 1999; Baker, 2001). These compounds are released into the aquatic environment through agricultural runoff, storm water runoff, sewage, industrial and household effluents and organic wastes. Compounds may act as hormone receptor agonists or antagonists or any other possible aspect in endocrine pathways, altering hormone production, pituitary or hypothalamic release of stimulatory or inhibiting hormones, hormonal inactivation pathways (biodegradation) as well as affecting secondary response or target systems (Asshuth, 1996; Crommentruijn *et al.*, 2000; Craven, 2000; Guillette and Gunderson, 2001; Babut *et al.*, 2003). Most of the initial research focus was targeted at the antagonistic/agonistic activity in androgenic and estrogenic reproductive systems (Gray, 1998; Hunt *et al.*, 2003). However, effects on the thyroid

* Submitted to Water SA, 2004

and immune systems have increasingly received attention (Davis, 1998; Kloas, 2002; Branchi *et al.*, 2003; Mendes, 2002; Colborn, 2004).

Natural hormones are known to function at low concentrations, and similarly, man-made EDCs show endocrine modulatory activity at low environmental concentrations (higher in sediments than water) (López and Barcelo, 2001; Legler *et al.*, 2002; Martin-Diaz *et al.*, 2002; Duft *et al.*, 2003). Moreover, interaction with other environmental chemicals as part of complex mixtures may lead to synergistic effects resulting in disruption of endocrine systems (Gray, 1998). Chemical detection of known EDCs or their metabolites at low concentrations, could prove to be difficult and close to the detection limits of analytical equipment. Therefore, analytical methods have been supplemented with the development of specific bioassays employing sensitive and very specific endocrine related biomarkers. For example bioassays developed for the screening of natural estrogens as well as various estrogen mimics interacting with the estrogen receptor include receptor binding assays, reporter gene assays and *in vitro* cell/tissue culture techniques to assess the equivalent estrogenic activity of compounds or water samples (Takatsuki and Yamaguchi, 2001). Most developed countries have initiated the development and standardizing of high through-put screens to identify potential EDCs. These programmes utilize screening batteries to include *in vitro* and *in vivo* testing as well as a range of different wildlife bio-indicator species (e.g. the report of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) of the US EPA, 1998) (Kavlock, 1999).

The Endocrine Disruptor Screening Programme (EDSP) established by the US EPA include a two tiered screening and testing approach to gain specific information on EDC interaction on endocrine systems (Kavlock, 1999). Tier 1 screening focuses on assessment and priority setting of chemicals for which there is insufficient scientific data. The bioassays recommended, include three *in vitro* assays and five *in vivo* assays. The *in vitro* assays are estrogen and androgen receptor binding (transcriptional activation assays) and a steroidogenesis assay using minced testes. The five *in vivo* assays include a rodent 3-day uterotrophic assay; a rodent 20-day pubertal female assay for effects on thyroidal function, a male rodent 5-7 day Hershberger Assay, a frog metamorphosis assay for thyroid effects and a fish gonadal recrudescence or vitellogenin production assay. Tier 2 testing is designed to characterize more defined responses and includes endpoints that will give decisive evidence whether or not a tested chemical may be an endocrine disruptor. The battery of tests suggested, include a two-generation mammalian reproductive toxicity assay,

an avian reproductive toxicity assay, a fish life cycle toxicity assay, an invertebrate life cycle toxicity assay and an amphibian development and reproductive assay. Following Tier 2 testing, positive chemicals and water samples will be tested for hazard assessment and risk to human and wildlife health.

Through the coordination of the Organisation for Economic Cooperation and Development (OECD), US EPA, the Japanese Environmental Agency and The European Union (EU) countries, standardized protocols and coordinate validation rounds in the form of inter laboratory testing (European Commission, 1996).

Initially the EDSTAC report recommended the use of an amphibian (African clawed frog, *Xenopus laevis*) *in vivo* metamorphosis bioassay to assess the effects of potential EDCs on the thyroid system (Fort *et al.*, 2000). Amphibian metamorphosis is a period of substantial morphological change and known to be under control of thyroid hormones (Shi, 2000). This phenomenon is characterized by three primary morphological changes, 1) resorption or regression of tissues that have primary functions in larval life, 2) the remodelling of larval organ systems to their adult form and 3) development of tissues in the adult that are not required by the larvae (Touart, 2002). Anuran metamorphosis is divided into three distinct periods: premetamorphosis, prometamorphosis and metamorphic climax (Kloas, 2002). Premetamorphosis refers to a period of embryonic and early larvae development that progress in the absence of thyroid hormone. One of the main developments that take place during this time is the development of the hind limb bud. The differentiation of the toes and elongation of the hind limbs occurs during prometamorphosis, characterized by the rising concentrations of endogenous thyroid hormone. Metamorphic climax is associated with a surge in thyroid hormones that triggers the final processes associated with metamorphosis, for example, forelimb development and the resorption of the tail (Touart, 2002; Shi, 2000).

Following the initial recommendation of Fort *et al* (2000) to use the rate of tail resorption during metamorphic climax as endpoint to study the effects on the thyroid system, Kloas (2002) and Touart (2002) proposed a revised XEMA (*Xenopus* Metamorphosis Assay) that include limb development endpoints and differential developmental stage assessments, rather than tail resorption during metamorphic climax (Kloas, 2002; Touart, 2002). Christian and Trenton (2003) reviewed evidence of thyroid effects by potential xenobiotics, most of which would have to be assessed by standardized protocols such as XEMA. Considering, the list of potential EDCs and their associated influence on the thyroid system (Brucker-Davies, 1998), it is clear

that the potential for similar disrupting effects associated with water resources in South Africa needs investigating and should not be ignored any longer. The African clawed frog, *Xenopus laevis*, is endemic to Southern Africa and occurs widespread in most natural and man-made water bodies in South Africa (King *et al.*, 1994).

South Africa implemented a revised National Water Act (Act 36) in 1998 to improve the regulation of water quality and quantity to local communities and implement better management and decision policies in terms of natural resource management (catchments, wetlands, rivers and streams) (DWAF, 1998). Although several national monitoring programmes have been established (e.g. microbial, chemical, eutrophication and river health programmes) (DWAF, 1996), it was only recently that the development of a National Toxicants Monitoring Programme (NTMP) was initiated with the vision to implement it in 2007 (Murray *et al.*, 2003). Preliminary recommendation for EDC related bioassays to be included in this programme does not include the XEMA protocol or any other thyroid related bioassays (Van Wyk, *pers comm.*; Murray *et al.*, 2003).

In the Western Cape province of South Africa, local authorities and municipalities implement and manage various monitoring programmes related to water quality (Cape Metropolitan Council, 2003). The specific state of the rivers in the City of Cape Town varies greatly between catchments, depending largely on the agricultural activities, industrial development and the degree of urbanization within the catchment, resulting in varied degrees of poor water quality as a result of sewage and industrial effluents, as well as storm water and agricultural runoff (Cape Metropolitan Council, 2003). Catchments include the Salt, Diep, Lourens, Houtbay, Zeekoei and Noordhoek catchment areas, of which the Eerste/Kuils catchment has stimulated interest due to three sewage treatment works located along the banks of the river. The quality of the water in the Eerste/Kuils River System has given cause to concern (Chapter 2) as various factors such as physical pollution, canalization due to urbanization, loss of habitat and hydrological physical alterations to the river have increased dramatically over the past 5 years (Cape Metropolitan Council, 2003).

The aim of this study was to firstly, set-up and run a XEMA using control chemicals, T4 (stimulatory), PTU (inhibitory) and buffered laboratory water and secondly, to use the XEMA protocol (Kloas, 2002) to screen environmental water collected along the Eerste/Kuils River Catchment system for potential EDC effects on the thyroid function.

4.2 Material and Methods

4.2.1 Study area and water sampling

Water samples to assess thyroid activity of in the Eerste/Kuils River Catchment system was collected during July 2003 and October 2003. Study area description, site selected sites, sample collecting and handling has been detailed in Chapter 2. Water samples were selected from 5 specific sites (sites 2, 4, 7, 8, 10) (Figure 4.1) allocated along the Kuils River and transported to the laboratory in cleaned (methanol) 25L steel drums. Water was the redistributed into smaller quantities as needed for further experiments e.g. toxicity exposure of tadpoles. The 5 sites were selected based on previous estrogen related studies (Chapter 3) potentially incorporating activity in the catchment system surrounding possible thyroid influence. Three sites (sites 4, 7 and 8) of the five sites are situated below wastewater treatment works (WWTWs) located at Bellville, Zandvliet and Macassar (Figure 4.1).

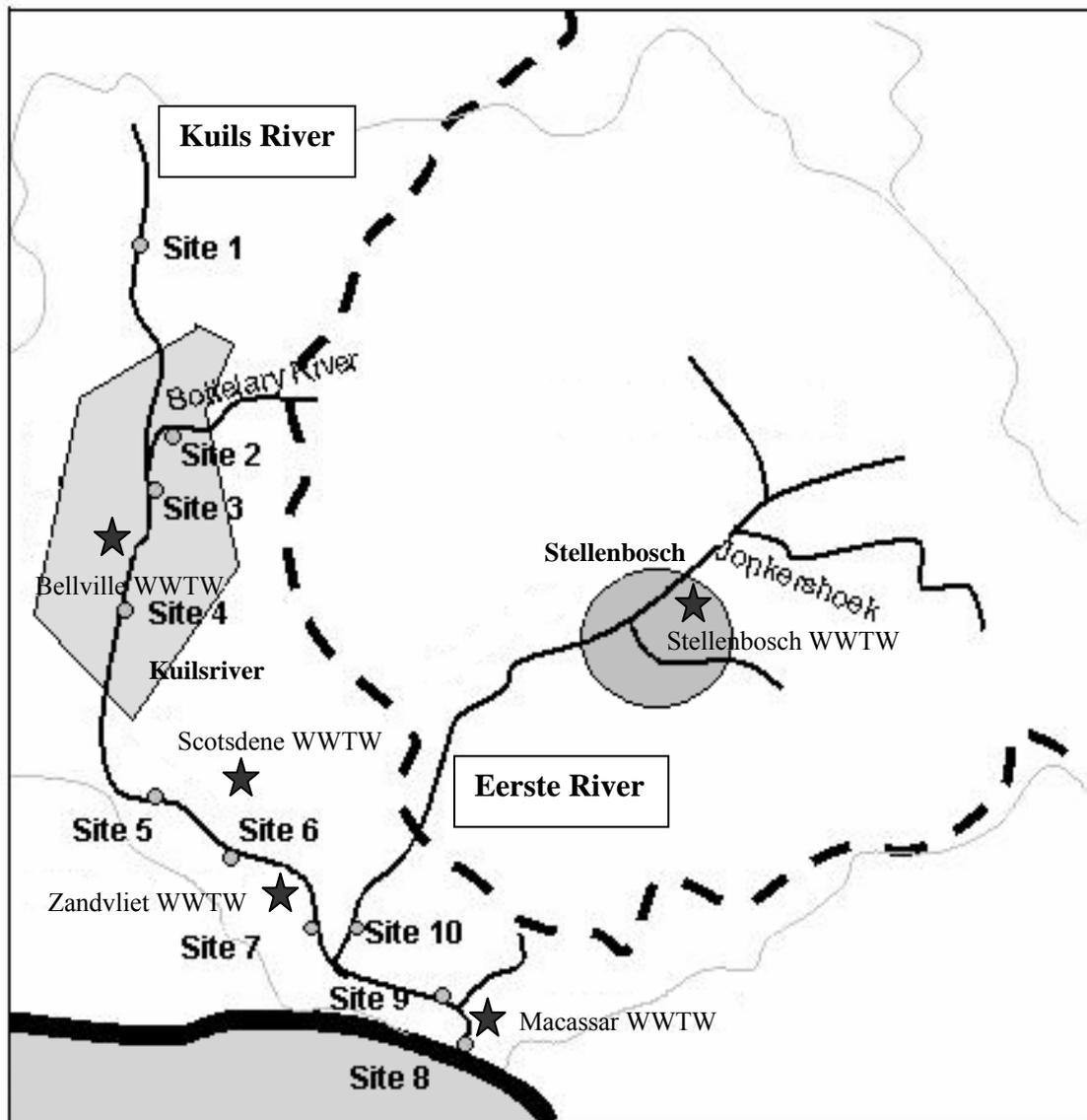


Figure 4.1: Locations of the five selected sampling sites along the reaches of the Eerste/Kuils River Catchment System. Sites were pre-selected based on potential EDC activity (e.g. effluents, pollution and storm water runoff) on the river.

4.2.2 Animals

Breeding pairs of the South African Clawed frog (*X. laevis*) were obtained from a local commercial supplier (African Xenopus, Knysna). General culture and care of adult *X. laevis* was conducted according to the standard operation procedures suggested for XEMA (Touart, 2002).

Breeding of tadpoles

Adult *X. laevis* males and females were injected with 100IU and 150IU hCG (Pregnyl) (Chronic Gonadotropin from Human Pregnancy Urine) respectively, four days before the required spawning (14 days prior to test initiation). Sexes were housed separately in 50L tanks filled with aged tap water. Three days later males received a second 100IU Pregnyl injection, while females received 200IU. Breeding pairs were housed individually in a mesh cage (to protect the eggs from being eaten by the adults) inside 10L tank in buffered laboratory water (Stock solution: 25g NaCl and 8g Na HCO₃/2L water) of 200ml/10L at 22°C. The breeding tanks were covered with secure lids, kept under low light conditions at 22°C with minimum disturbance. Spawning took place overnight and adult frogs were removed from the breeding tank the next day.

Eggs and tadpoles

Larvae hatched until day 3 of post fertilization (PF) and developed to stage 48 Nieuwkoop and Faber (NF) (Nieuwkoop and Faber, 1994) within 12-14 days post fertilization. During these pre-experimental stages, free swimming developing tadpoles were kept in holding tanks (30L glass tanks) containing buffered laboratory water, fitted with an activated charcoal filter. Tadpoles were fed from day 5 (PF), depending on amount of tadpoles in the holding tanks. Once dispensed, 200mg sera micron (sera GmbH, D-5218 Heinsberg) (100mg morning and evening) was provided to each tank of 30 tadpoles. Tadpoles were maintained for 7 days to reach NF developmental stages 48-50. As tadpoles developed to NF stages 46-48, selection according to the criteria set by the XEMA protocol (Touart, 2002) occurred and tadpoles were transferred to exposure tanks. Criteria for experimental animals included whole body length (17-24mm) and development stages 48-50 (20 tadpoles per tank).

4.2.3 XEMA Validation study

All XEMA protocol (Touart, 2002) was followed with the exception of water substitution, as water was replaced every 4th day (Monday and Friday) instead of every 2nd day as per XEMA. Controls consisted of a thyroid positive inhibitory control of PTU (6-n-propyl-2-thiouracil) at 75 mg/l concentration, a thyroid stimulator control thyroid hormone thyroxine (T₄) at 1 µg/l and a group of tadpoles exposed to buffered laboratory water. Tadpoles (Stage 51, Nieuwkoop and Faber, 1994) (n=15) were exposed for 21 days, following euthanasia with 10% benzocaine solution prepared at 1ml/L water (Stock solution: 100g/1L ethanol), fixed and preserved in 10% buffered formalin. Using Nieuwkoop and Faber (1994) as reference, all tadpoles were staged.

4.2.4 Preliminary study vs. exposure

In order to assess the toxicity of the undiluted water samples collected from the Kuils River, a 96hr survival test based on the protocol of the FETAX short-term (96hr) test protocol adapted from Saka (2004) was preformed. In summary, *X. laevis* embryos varying between NF stages 8 to 11 (midblastula to early gastrula) were exposed for 96hrs to the selected water samples and survival was checked every 12hrs. Approximately 30 embryos were placed into glass bottles containing 50ml water sample collected from the 10 selected sites along the Kuils River (Figure 2.2, Chapter 2). The tadpoles were maintained at 23°C and photo period regulated as a 12:12 day: night cycle. Surviving tadpoles were sacrificed in 10% benzocaine solution, subsequently measured to the nearest 1mm and fixed in 10% buffered formalin and kept for future reference.

4.2.5 XEMA screening of Eerste/Kuils River samples

Following the validation study, a new batch of tadpoles were bred and raised to NF stage 51. Tadpoles were randomly separated into exposure tanks (10L) and exposed to undiluted environmental water samples. Twelve, 10L tanks (6 duplicate tanks) (five river samples and one containing buffered laboratory water as control) were filled with 15 tadpoles selected from the holding tanks and placed in a light-cycle (12:12 hours) and temperature controlled room ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$). Fresh water samples were collected weekly from the five selected sites. Upon reaching the laboratory, two water samples were taken and split into two continuously aerated glass tanks (10L) housing 15 tadpoles each per sample. Tanks were cleaned and water exchanged every 7 days (day 7 and day 14). Following the 21 day exposure period, tadpoles were euthanized with a 1% benzocaine solution, fixed and preserved in 10% buffered formalin. Using Nieuwkoop and Faber (1994) as reference, all tadpoles were staged and full body length measurements recorded (head to tail) to the nearest mm.

4.3 Statistical analysis

Following the staging of the tadpoles, the occurrence of stages was expressed as a proportion (%) of the total exposure sample. Proportional data were transformed (arcsine of the square root) and analysed using analysis of variation (ANOVA) followed by multiple comparison testing (Holm-Sidak). Data were tested for homogeneity of variation and normality prior to ANOVA. A p-value < 0.05 was considered significant. Statistical calculations were performed using SigmaStat computer software (SPSS, Inc.).

4.4 Results

4.4.1 XEMA validation study

The validation of the test protocol was based on the results recorded by the three control groups: T₄, PTU and buffered laboratory water (Control) (Figure 4.3). A statistical significant difference (ANOVA: $F_{2:35} = 41.654$; $P < 0.001$) was recorded for the three control groups indicating the validity of protocol applied and followed. Multiple comparison testing (site versus the control) (river water) recorded the clear variance of T₄ ($t = 5.101$) as stimulatory and PTU ($t = 2.592$) as inhibitory on the metamorphosing tadpoles. Test assay is thus accepted as validated and within protocol procedures.

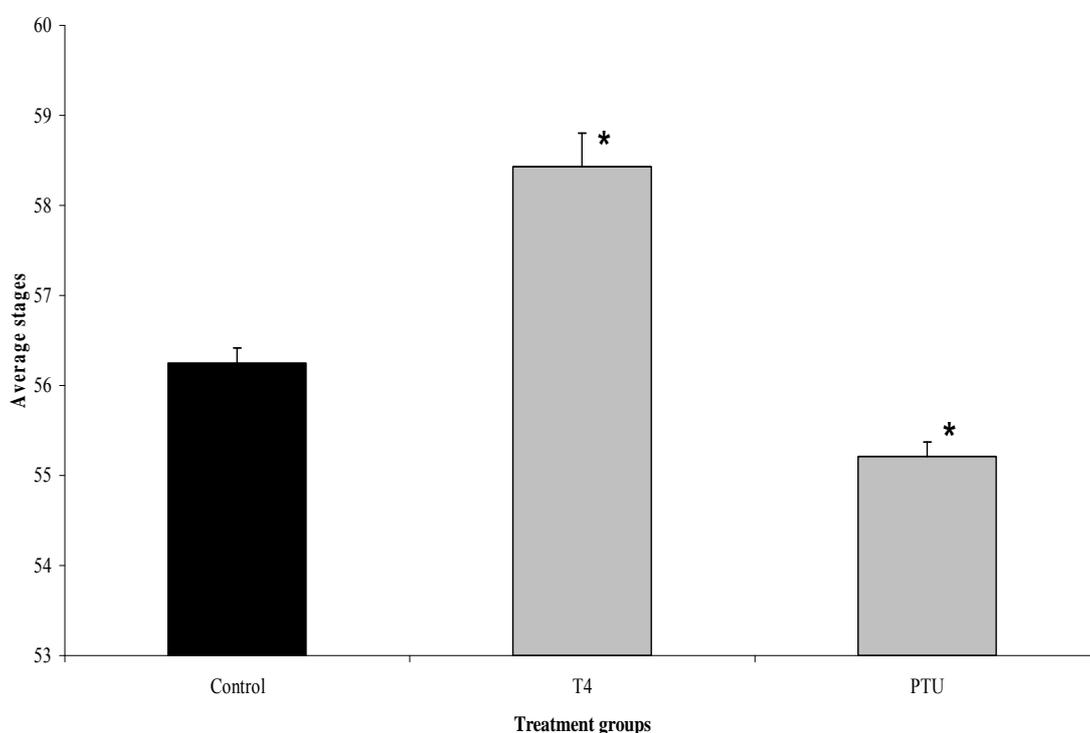


Figure 4.2: Validation results for study treatment groups. T₄ (thyroxine) acts as stimulator and PTU (6-*n*-propyl-2-thiouracil) as inhibitor of metamorphic development of tadpoles during a 21-day exposure regime. The control consisted of only buffered laboratory water (RO H₂O). (*) Indicate significant different ($p < 0.05$) from the control group.

4.4.2 Preliminary study vs. exposure

The initial short-term (96hr) test data (Figure 4.2) recorded no mortalities, but a induced growth effect at sites 1 (115%), site 2 (103%) and site 6 (121%) was recorded in terms of total body length percentage of negative (-ve) control. Site 8 indicated no stimulation or inhibitory effects on tadpole development toxicity (100%), as head-tail length data equalled that of the -ve control (100%). Inhibitory growth effects was found at sites 3 (95%), site 4 (92%), site 5 (91%), site 7 (86%) and site 9 (85%) of which site 9 indicated the most growth inhibition effect. A statistically significant variance was recorded amongst the treatment groups (ANOVA: $F_{9:116} = 6.885$; $P < 0.001$). Multiple comparison testing (sites versus the control) identified site 1 ($t = 2.684$), site 5 ($t = 3.948$), site 6 ($t = 3.154$), site 7 ($t = 3.092$) and site 9 ($t = 3.264$) as treatment groups significantly affected. Following this experiment Sites 2,4,7,8 and 10 were selected for the full XEMA exposure assay as determined by water quality parameters (Chapter 2).

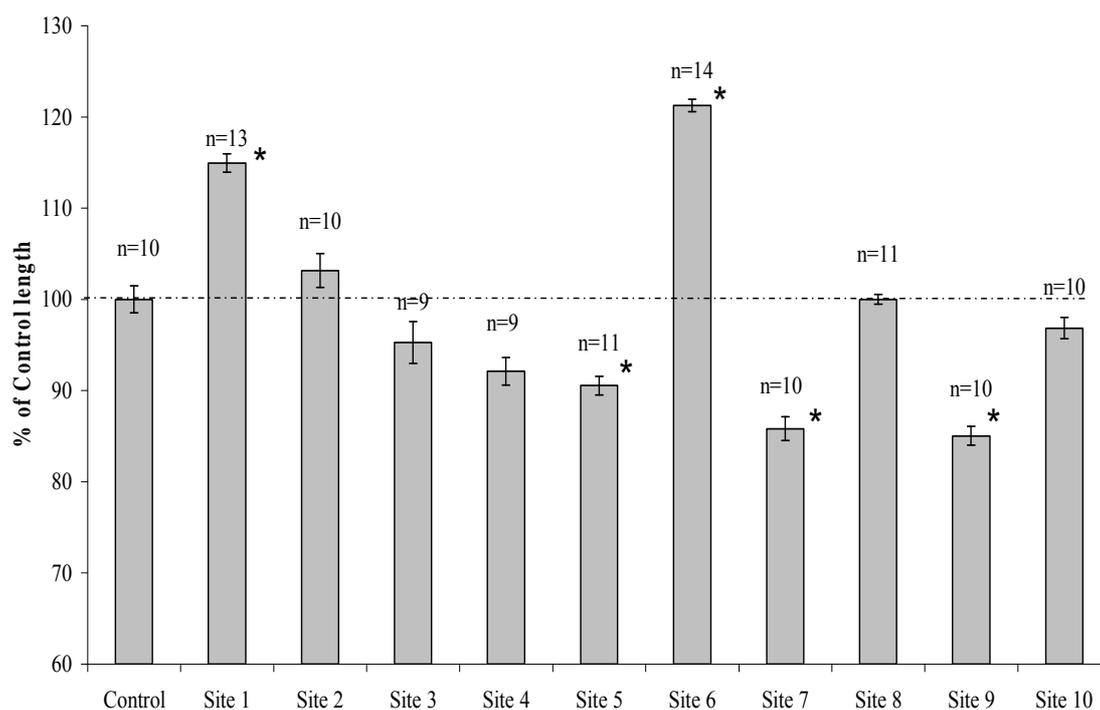


Figure 4.3 Total length of tadpole (Stage 57) expressed as percentage (%) of the control group (100%) following an initial short-term (96hr) exposure test. No mortalities were recorded. Significant variance was recorded for site 1 ($t = 2.684$), site 5 ($t = 3.948$), site 6 ($t = 3.154$), site 7 ($t = 3.092$) and site 9 ($t = 3.264$).

4.4.3 Eerste/Kuils River Catchment water

Data was analysed following the exposure period of 21 days of tadpoles to the water samples. Significant statistical variance for sites was recorded between the selected sites (ANOVA: $F_{5;9} = 1.846$; $P = 0.258$ ($P > 0.05$)). Multiple comparison testing show sites vary statistically (ANOVA: $F_{5;11} = 5.123$; $P = 0.036$ ($P < 0.05$)) from a comparative control, identifying sites 2 ($t = 4.232$) and 4 ($t = 4.323$) with significant variance. Average development stages reached for site 2 (Stage 59), site 4 (Stage 59), site 7 (Stage 57), site 8 (Stage 57) and site 10 (Stage 57), exceeded the average staging recorded for the control group (Stage 54) (Figure 4.5).

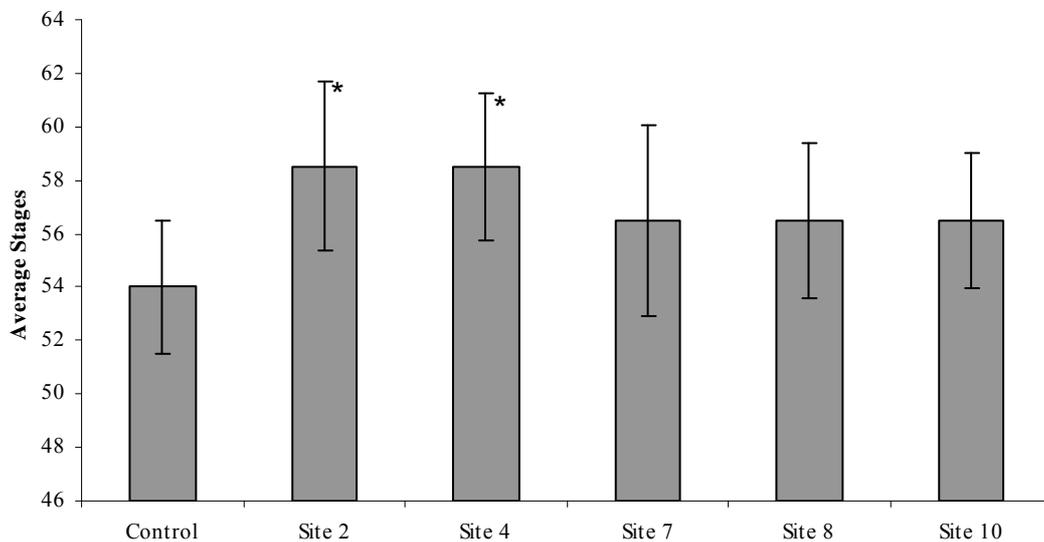


Figure 4.4: Average staging in exposure groups for *X. laevis* tadpole development during 21-day exposure regime for Eerste/Kuils River water samples (as per site indicated on graph). Buffered laboratory water acted a control group. Recorded average staging along the selected catchment sites indicated a stimulatory influence on tadpole metamorphic development with significant growth (*, $p < 0.05$) in water collected from Site 2 and Site 4.

Individual exposure groups per site are presented in Figure 4.5, indicating a trend for developmental influence on tadpole metamorphosis towards stimulated development per individual tadpole. Normality distribution (Kormogorov-Smirnov test) data for sites vary significantly. Site 2 (K-S Dist = 0.323) and Site 4 (K-S Dist = 0.423) metamorphic development ranges between NF stages 55-61 (above control average stage 54) with site 2 staging as high as NF stages 64 (10%) and 66 (10%) (Figure 4.5). Site 7 (K-S Dist = 0.330) provides a broader staging range (stages 49-59), with metamorphic development reaching stage 63 (5%). Site 8 (K-S Dist = 0.320) tadpole development ranges between stages 52-57 (stage 55 at 30%) with metamorphic development reaching stages 63 (5%) and 65 (5%) (Figure 4.5). Site 10 (K-S Dist = 0.287) ranges over a wider staging distribution (stages 52-65) with maximum tadpole development in stage 58 (20%). Overall metamorphic development distribution range expands above average control stage 54 (Figure 4.5), indicating stimulated developmental growth.

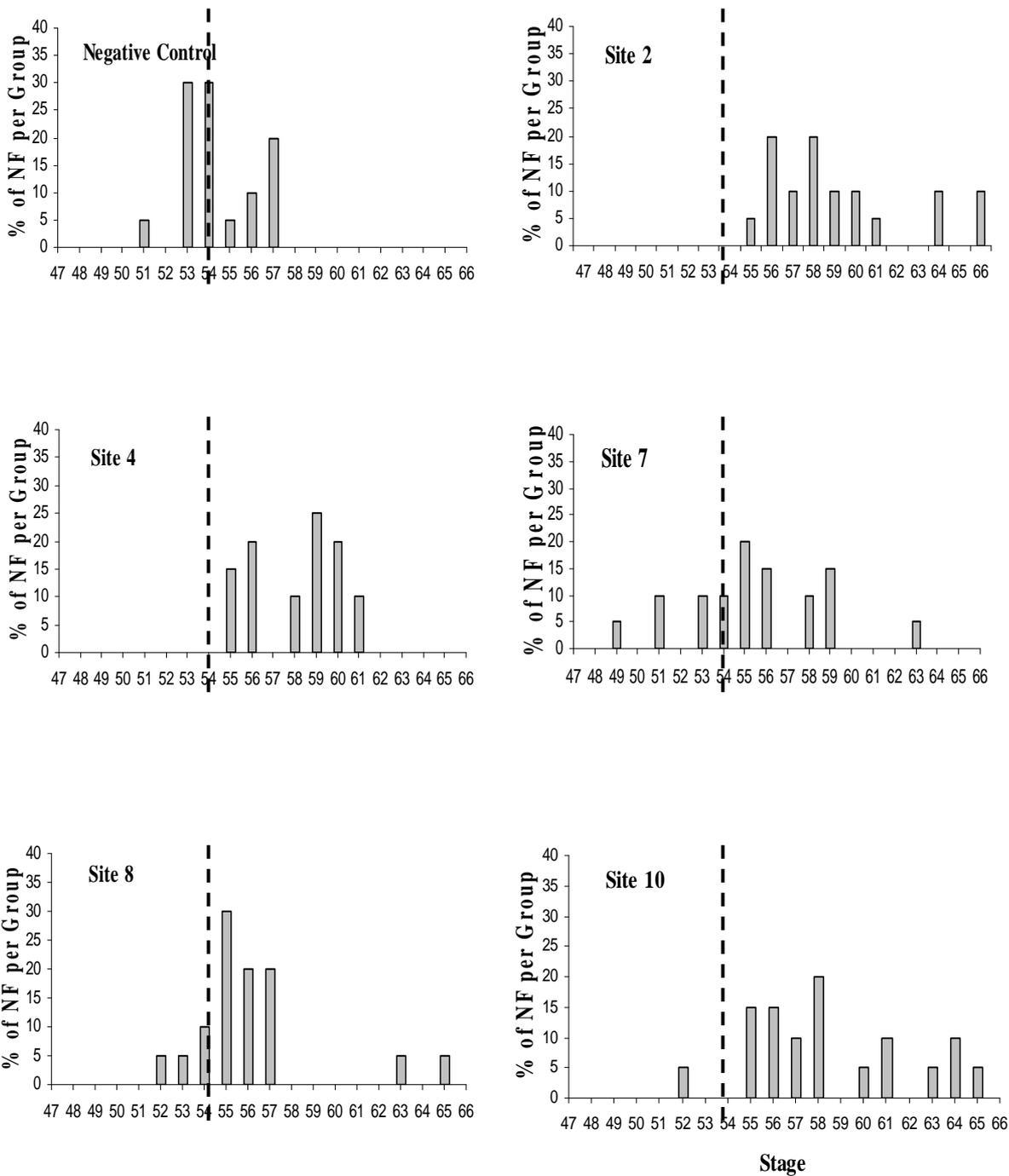


Figure 4.5: Total percentage (%) of tadpoles per NF stage following a 21-day exposure regime for selected sites along the Eerste/Kuils River. Metamorphic development as for the control group averages at stage 54 (vertical line). The distribution trend, sites averaging above stage 54 (control), provides an indication of thyroid stimulating activity present in the water samples.

4.5 Discussion

The results of the present study show *Xenopus laevis* as a model to assess endocrine modulatory activity in polluted water. *Xenopus laevis* as an amphibian model in endocrine disruptor assessment is a well-developed model system both in *in vitro* and *in vivo* exposures (Hurter *et al.*, 2002; Bogi *et al.*, 2003). The use of tadpoles and the progression of metamorphosis following exposure to potential endocrine disruptors during the sensitive stages of thyroid differentiation have been shown to have significant effects on the development of tadpoles (Kloas, 2002; Touart, 2002; Bogi *et al.*, 2003).

This study showed that during the initial short-term 96hr exposure, although no mortality occurred, growth of tadpoles was differentially affected. Tadpoles exposed to water from three of the sites along the river catchment (Sites 1, 2 and 6, Figure 4.3) stimulated growth relative to the tadpoles exposed to control water samples. Whether the observed differential growth at this early stage of development was associated with effects on the thyroid system is unknown. Samples 1 and 2 both originate from the upper catchment of the Kuils River, although within the urban area, suggesting some eutrophic activity in this area. Site 6 on the other hand is down stream of a large urban area as well as effluent points of sewage treatment works. Growth inhibition was noted in sites 5, 7 and 9. These were all down stream of the large urban as well as informal settlement areas. Water quality data (Chapter 2) indicated higher nutrient and organic levels at sites situated within urban and rural settlements than sites located upstream to these developments. Microbial and inflammatory data (Chapter 2) for both sample sites 3 and site 4 indicated high level of human impact on the Eerste/Kuils River system surrounding the urban and rural settlements on the bank of the river.

The results of the controlled XEMA bioassay using a 21 day exposure protocol showed that PTU (inhibitory control) and T₄ (stimulatory control) exposures significantly affected the development of the *Xenopus laevis* tadpoles when compared with the negative control exposures. The results were similar to the initial results reported by Kloas (2002) using the same chemicals and concentration ranges. The OECD's Amphibian Specialist Group recommended the use of a flow-through system, although a semi-static renewal exposure, similar to the protocol used in this experiment is acceptable (Kloas, 2002). For the present study wild caught adult frogs

were used to breed tadpoles for exposure, whereas, most international studies utilizes tadpoles bred from laboratory (captive) *Xenopus laevis* populations. The results obtained in the present study suggested that the responsiveness of tadpoles bred from wild caught adults was generally the same as reported in international studies. However, this is an aspect that needs further investigation in order to standardize exposure condition during screening tests.

Influences of various environmental waters on the developmental stages of the *Xenopus* tadpoles were recorded for both sites 2 and 4 (Figure 4.5), located in the upper reaches of the Kuils River, indicating agonistic effects on thyroid activity. This could be linked to the influx of agricultural runoff (pesticides, insecticides, herbicides) from the Bottelary River, specifically surrounding site 2 and the possible high organic and anthropogenic pollution present at site 4 (rural encroachment of banks). Previous studies by Hayes (1997b, 2002) indicated that thyroid hormones influence tail resorption and total body length during the metamorphic climax (Hayes, 1997b). The fact that the rate of development increased during exposure to water collected at these two sites suggest that some agent or mixture of agents affected the functionality of the thyroid either by premature thyroid activity or by increasing the rate of metamorphosis by increased interaction with the thyroid hormone receptors in the corresponding target tissues. Although mean developmental stage give an overall picture of the effect of exposure on the metamorphic development, individual developmental stage allow for the assessment of individual variation in exposure groups. Using this perspective, it was clear that tadpoles generally developed faster in water collected at most of the sites when compared with the negative control water (pristine mountain water). In several of the exposures did tadpoles develop to NF stages 60 -66 (Metamorphosis climax). It is well-known that several environmental factors (temperature, light, diet, iodine concentration in water, water volume and crowding) may influence the rate of thyroid hormone (TH) induced metamorphosis in amphibians (Shi, 2000). However, apart from iodine content of the water most of these influences were controlled for during the standardized XEMA protocol.

Brucker-Davis (1998) reviewed the effects of synthetic chemicals in the environment on thyroid function and confirmed the hypothesis that thyroid disruption in wildlife occur. Hyperthyroidism has been suggested as an outcome of exposure to environmental pollutants (Brucker-Davis, 1998; Jahnke *et al.*, 2004). In mammals and humans hyperthyroidism has been shown to be associated with disruption of the

reproductive system, for example, altered LH levels, early onset of menses and puberty (Jahnke *et al.*, 2004). In rats chemical induced hyperthyroidism lead to thyroid cancer, but in human's non-cancerous goiter were found (Jahnke *et al.*, 2004). At this point in time it is acknowledged that in spite of high homology between *Xenopus laevis* and mammalian thyroid hormones, receptors as well as co-activators and co-repressors (Dodd and Dodd, 1976; Lim *et al.*, 2002), altered thyroid function in wildlife species may not be predicative of human effects (Jahnke *et al.*, 2004). Future studies using molecular tools to assess relative expression of thyroid hormone receptors following exposure to water from these areas will throw more light on the possible modes of action involved and therefore the possible origin of stimulatory effects observed during the present exposures. .

Although several chemical compounds (EDCs) known to interact and affect the thyroid system (Brucker-Davis, 1998) have been studied in international laboratories, and of these some are being used in South Africa, no local study has explored the implementation of the XEMA protocol to screen for thyroid activity in our water resources. Using *Xenopus* metamorphosis as a simple and highly reproducible screen for biological active thyroid hormone agonists and antagonists has several advantages. For example, *X. laevis* thyroid hormones are identical in structure to mammalian thyroid hormones (Lim *et al.*, 2002), the initiation and completion of metamorphosis is dependent on thyroid hormones, target cell concentrations are regulated by the same deiodinase enzymes, and tadpoles readily take up thyroid hormones from the water and respond in a dose-response manner (Lim *et al.*, 2002). Thus, it is generally agreed that the use of *Xenopus* metamorphosis is a valid bioassay to assess thyroid activity in water resources.

4.6 Conclusion

There are compounds in the waters sampled from the Eerste/Kuils River System (Figure 4.5) that affect the metamorphic rate (i.e. thyroid activity) of exposed organisms. The effects of these chemicals on endemic aquatic species are not as of yet known and will need future research. Similarly in terms of human health, indications are that thyroid modulatory effects may result and in light of Colburn's (2004) warning that disruption of the thyroid hormone negatively influences fetal brain development, the precautionary principle will have to be applied regarding human consumption and recreational use. Seasonal variance, sex ratio determination, gonadal morphology, thyroid histology and the influence of an efficient flow through regime are the main factors in need of more research. XEMA have included these factors and can thus be successfully applied as a research tool for future interest and developments. It is the hope of the author that the current study has contributed to an expanding field of study towards the investigation of EDC effects on the environment and the state of ecotoxicology affecting aquatic organisms and human health.

CHAPTER 5

Summary and conclusion

South Africa has only recently, with the implementation of the National Water Act (No 36 of 1998), concentrated on the development of a National Toxicant Monitoring Programme. The South African Water Research Commission (WRC) plays a leading role in the funding of research projects related to potential EDC pollution and includes tests for estrogenicity (bioassays) of water resources located within the agricultural region in the Western Cape (Van Wyk *et al.*, 2002), validation of assays including androgenic, thyroid and immune system disruption, and the chemical analyses and implementation of first tier screening for estrogenicity (Van Wyk, 2002). Currently, South African focus on EDCs has shifted from the development and validation of bioassays to the successful implementation and practical application of screening test protocols and understanding the linkages between the cause and effect within and across the hydrological cycle. This has been incorporated in the WRC's four crosscutting domains, comprising mechanisms to address key strategic issues within each domain (McKay *et al.*, 2004). The domains cut across key strategic area's (KSAs) incorporating the ongoing programmes and projects of each KSA within the domains portfolio and aims to understand the ecological system, biodiversity in the system, prediction of society's impacts, endpoints of impacts, forward and backward linkages between ecological and governance/social systems and the design of a practical system for governance reflecting and responding to ecological change (McKay *et al.*, 2004).

Water quality management has become one of the most strategic management tools concerning the assessment of the local environment. Termed by Dallas and Day (2004) as the value or usefulness of water, water quality management forms the backbone of local government in managing water resources (rivers, streams, wetlands, catchments). Attention is given to the effluent discharge of WWTWs and storm water effluent discharging into rivers and streams. Concern surrounds the associated human health risk and spread of water-borne diseases through faecal contamination as SA consists of a high majority of rural settlements directly utilising water (domestic and recreational use) from rivers and streams surrounding their settlements (Cape Metropolitan Council, 2003). The issue surrounding WWTWs and associated EDC releases, is the tendency of contaminants to accumulate within estuaries and land fill

sites causing adverse effects such as “feminisation”, abnormal reproduction development, altered sex ratio’s and possibly cancer (breast, prostate) and lowered sperm counts in humans (European Commission, 1996; EHP, 1996; Baker, 2001; Juberg, 2000; Brown and Fairchild, 2003; Lemaire *et al.*, 2004).

In a South African context, the only studies exhibited in light of estrogenic contamination and its source of origin includes that of Schulz *et al.* (2001a, b, c) which focuses on pesticides as contaminants of surface waters, Meintjies *et al.* (2000) which assesses estrogen concentration in the environment and Hayes’ (2002) assessment of river health (Diep-, Palmiet-, Houtbay- and Lourens Rivers). The problem of estrogen contamination for SA lies within the complexity, expense and interpretation of the data (Dallas and Day, 2004) recorded in the environment. In essence, the solution establishes itself in a well-integrated and all-inclusive national ecotoxicological monitoring programme.

This study aims to draw a correlation between the water quality and the biological content or effects of EDC activity, inducing estrogenic and thyroidogenic, for a South African River, more specifically the Eerste/Kuils River in the Western Cape. The strategic assessment for this thesis was based on the potential link that exists between water quality standards and levels of estrogen activity present in the Eerste/Kuils River. In its entirety the process consisted of an overall assessment of the Eerste/Kuils River Catchment System located in Cape Town, Western Cape Province and was divided into (1) the assessment of existing water quality standards through *in vitro* assays, (2) determining estrogenic activity utilising *in vivo* assays and (3) the detection of thyroid influences on the endocrine system through an adapted *Xenopus* Metamorphosis Assay (XEMA).

The Eerste/Kuils River Catchment Systems falls under the administrative services of the City of Cape Town for the Western Cape, South Africa. The City of Cape Towns authority lies in the effective provision of sustainable water and sanitation services to local communities (Cape Metropolitan Council, 2003) and relies on the analysis of water quality measurements according to various parameters (pH, dissolved oxygen, nitrogen, phosphorus and chlorophyll-a levels) and collection of data as background information to continuously re-assess water quality standards.

Seasonal variability plays a vital role in the selection of sampling periods, as the Cape Metropolitan Area (CMA) receives most of its rain during the winter months

(June –August) (Cape Metropolitan Council, 2003). Incorporated in the study are the seasonal rainfall variance on the river and its possible influence on the related parameters. Although temporal changes over a seasonal climate vary continuously for natural river systems, it is the temporal effects of pollutants on a river which is of great concern. The various parameters analysed for the Eerste/Kuils River highlighted the human influence (pollution, sewage effluent) on the river. As surrounding communities of rural settlements continuously use the river as a convenient dump, and sewage works are not regularly upgraded, untreated storm water freely discharges into the river and continuously contaminates the river (domestic hazard). This aspect expands towards the recreational impacts of the Eerste/Kuils River as faecal contamination (*E. coli* and faecal counts) rises dramatically during summer seasons posing further recreational threats to humans.

Additional water quality parameters such as cytotoxic and inflammatory activity, faecal antigens, estrogen mimics and thyroid modulators were monitored in the study. The data obtained from cytotox (LDH) and faecal antigens (*Salmonella*) identified low to no impact levels. Inflammatory activity was recorded at levels above 200 pg/ml, which is above safe levels for human use (domestic or recreational use). This can be linked to the loss of natural river habitat due to canalisation (Cape Metropolitan Council, 2003) as 10% of the upper reaches of the Kuils River have been canalised for urban encroachment. The river acts as a drain for all pollutants and microbes (faecal contamination) as runoff and storm water drain from the surrounding areas. In the absence of riparian habitats acting as natural filters, removal or reduction of potential dangerous microbes becomes impossible. As 70% of the river runs through rural to low income communities it is not surprising that these communities are associated with a poor sanitary infrastructure and have very little consideration for the environment. Children of the surrounding suburbs utilise the same water source for recreational activities such as swimming and fishing. The river is also constantly utilised as watering holes for grazing animals. This immediately raises the stakes on public health and the control thereof and attention to such a health risk, including the absence of decent sanitary services to rural communities and the uneducated domestic and recreational use of these contaminated waters, will have to be addressed immediately. It is clear from current data that water quality attributes succeed in establishing efficient analysis of the Eerste/Kuils River catchment system. It is recommended that local authorities of catchment managements integrate reliable and

robust bioassays as essential tools for bioassessment or monitoring strategies of river health.

Although cytotoxic activity (LDH) for the system proved negative, the estrogenic milieu for the Kuils River influences the final assessment on the system. *In vitro* liver slice bioassay and *in vivo* exposure of Zebrafish was utilised to determine the estrogenic activity (vitellogenin levels) for the river and no activity was found. An interesting phenomenon could however be observed for both the exposure of Zebrafish and the liver slice assay for site 2 water samples. The Bottelary River (site 2) recorded the highest levels of vitellogenin (Vtg) production in both assays and this has been linked to possible estrogenic input from the surrounding agricultural development and vineyards of the river. The data supports the results obtained from *Salmonella* and LDH assays, as the WWTWs (Zandvliet, Scotsdene & Macassar) contributes minimally to the regime of the river and is therefore successful in its treatment process.

Thyroid impacts in the Eerste/Kuils River catchment system, as based on a recently validated *Xenopus* Metamorphosis Assay (XEMA), focussed on estrogen endpoints in the metamorphic amphibian *Xenopus laevis*. The present study implemented the exposure of tadpoles to environmental waters of the Kuils Rivers to determine their response. The exposure of these tadpoles not only proved successful as an analytical tool, but also indicated the presence of thyroid mimicking hormones in the river (stimulated development stages and increased growth rates). The extent of present thyroid elements in the water and the origin of the point sources will have to be established in future studies. The XEMA screen provides baseline information on the presence of thyroïdal activity in the Eerste/Kuils River water, but other environmental inputs and its associated impact will also have to be considered in extended studies in this field.

To summarise, this study suggests that the ecological status of the Eerste/Kuils River catchment system varies between moderate to high-modified environmental degradation levels. Most of the effects are caused by human interference and influence (urbanization) directly on the system. This is seen in the increase of domestic, recreational, industrial and partial agricultural (Bottelary river) activities surrounding the summer periods, directly and indirectly influencing bacterial growth in the river. The restoration of the river and the length of time necessary for the

restoration are still unknown as specific data on point source control is still too limited with a need for detailed environmental monitoring studies for future assessments.

Recommendations for the Eerste/Kuils River catchment system will include aspects concerning urban encroachment and pollution control management:

- ◆ Rural settlements serviced with poor sanitary services cause major impacts on rivers and wetlands, which accentuate the need of decent service provision addressed independently within a management strategy.
- ◆ Rural communities view local rivers and streams as rubbish dumps with the misconception of utilising the same polluted water downstream of the dumping zone for domestic and recreational function. It is essential to educate people and communities on the basics of “environmental hygiene”, spread of water-borne diseases and involve them in a sense of community pride for their environment.
- ◆ Canalisation of rivers is one of the main problems towards river degradation. People view cement-channelled rivers as modern drainpipes and to combat this it is imperative to change the social mindset.
- ◆ Prevention of the continuous loss of natural habitat.
- ◆ Cohesive urban planning should include the correct design of sewage networks and their associated capacity of service, which is essential due to the rate of expansion of urban areas within the City of Cape Town.
- ◆ Continuous upgrading of existing WWTWs to prevent spills and leakages into the surrounding environment.
- ◆ Urban areas are currently being constructed as impervious areas. Developing these areas to be more “penetrable” in its draining of surface runoff can essentially reduce the costs associated with treating polluted water as the water is naturally filtered.

Water quality attributes can be seen as an effective management tool in strategies surrounding catchment management, monitoring and assessment. However, as stated by Dallas and Day (2004), urbanization (rural settlements) and storm water runoff has the most severe impact on aquatic ecosystems and river health, and as urban developments continuously expand, catchments become runoff channels, accumulating pollutants in receiving rivers and streams. It would therefore prove a

more effective monitoring tool to analyse the amount of pollutants contaminating the system as additive scope to assessing catchment areas. In addition, estrogen activity or EDCs have developed as an international problem and have for the past two decades been assessed in as many aspects of wildlife and humans as possible. Unfortunately these assessments exclude developing countries, as studies have been limited and the potential risk of EDCs inadequately addressed. For example, in a growing field of study, toxicant assessment (including estrogen activities) integrates as an essential part of catchment management, in both wildlife and human cause-and-effect, and will therefore assist with both international and local management strategies.

If we claim to be a conservation sensitive nation, be it globally or nationally, knowledge of how we impact our surrounding environment, to what extent and how we will have to go about in remedying the problem becomes an essential assessment tool. As humans we interact with the environment on a daily basis and are exposed to a broad and unknown sector of man-made and naturally occurring chemicals hidden in the air we breathe, the water we drink and the food we eat. Taking care of the environment will mean taking care of ourselves.

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