WRC RESEARCH PROGRAMME ON ENDOCRINE DISRUPTING COMPOUNDS (EDCs)

VOLUME 2

Implementation of a Research Programme for Investigating Endocrine Disrupting Contaminants in South African Water Systems

Reports to the
Water Research Commission
consolidated by

AEC Burger

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This report emanates from nine projects and consultancies undertaken for the WRC *Programme on Endocrine Disrupting Contaminants* (WRC Project No. K5/1402 and K5/1402 II). You will find a summary report and 13 contributing reports. A full list of the research titles and authors is provided in the List of Appendices.

This is the second volume on the topic.

Volume one is titled: *"Strategic Research Plan for Endocrine Disrupters in South African Water Systems"* (WRC Report No. KV143/05).

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FOREWORD

EDC (Endocrine Disrupter Chemicals) research has become of great importance worldwide. It was imperative that South Africa become involved in this field. The EDC research programme was compiled in order to coordinate and extend research done by several groups of researchers in the country. This was the first effort to conduct an integrated research programme involving Universities, Technikons, the water treatment sector, government departments and parastatal research institutions. Internationally the program was assisted by the Global Water Research Coalition (GWRC) and its members as well as consultants from Japan and Norway.

It is intended to present the EDC research programme in 4 volumes. This report represents the work completed in Volume 2.

Volume 1:
The development of a strategic research plan to determine the occurrence of EDCs in South African water systems (report printed as WRC Report KV 143/1/05)

Volume 2:
Implementation of the research programme for investigating endocrine disrupting contaminants in South African water systems

Volume 3:
Extension of the strategic plan for future research actions on EDCs in South Africa, including research into other effects of EDCs and remedial actions to limit the risk to the human population and animals.

Volume 4:
Implementation of revised strategic plan on extended research of EDCs and recommendation on remedial actions.

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The author would like to express her appreciation to all researchers, as well as laboratory and department heads, for their time and valuable input. Special thanks to Prof CJ (Tiaan) De Jager for providing the background to this report. The team would also like to express our gratitude to the Global Water Research Coalition (GWRC) for contributing on the methodology of detecting Endocrine Disrupting Contaminant (EDC) activity by biological means.

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EXECUTIVE SUMMARY

Endocrine Disrupting Chemicals (EDCs) are defined as chemicals that interfere with the structure or function of hormone-receptor complexes. They can cause endocrine disruptive effects at exposure levels up to a million times lower than carcinogen exposure levels of concern. Internationally, the negative impact of EDCs on health is evident and no longer an issue of dispute. Before 1999 only limited research was done on possible contamination of EDCs in South Africa. A need has been identified for a coordinated research program involving all researchers and stakeholders such as government departments and water suppliers. EDC research requires a multidisciplinary approach involving research in disciplines such as zoology, physiology, toxicology and analytical chemistry to be able to assess the risk to the human and wildlife populations and the state of contamination of the water resources.

The main objective of the research programme is to develop the tools to be able to determine the risk to the population. In order to conduct risk assessments, reliable data is needed on occurrence, magnitude and frequency of pollution. Very little data existed in the country on pollution of EDCs. Most of the existing data was collected with toxicity in mind and could not be used because the detection limits in the studies were too high.

During a workshop held in Pretoria in 1999, a need was expressed by the stakeholders for reliable and relevant data on the levels of EDCs in South African water systems. The development of a strategy for EDC research was proposed in order to lay the foundation for a programme to develop the tools to investigate the fate and occurrence of Endocrine Disrupter Chemicals (EDCs) in South African water systems to be able to determine the risk to human and animal populations. The research programme should also include recommendations for remediation, removal, manage options and policy development.

In the strategic research report (Volume 1, WRC Report KV 143/05) a survey was conducted on the state of the science in South Africa and existing data was evaluated. A list of priority compounds to be investigated was compiled, analytical methods for determining EDC activity and levels of individual EDC components were evaluated and selected. A survey of laboratory capability and capacity was conducted. Recommendations on a protocol for a research programme were suggested.

The first phase of the implementation of the programme (Volume 2) consisted of capacity building in research facilities for activity testing as well as chemical analysis. A workshop was held to select appropriate activity tests to be used in the study, because no single test could be used to determine the EDC activity. Two workshops were presented where students received training in activity testing and chemical analysis of EDCs in aquatic systems.

A limited surveillance study was conducted at four selected sites during four sampling events. The samples (water and sediment) were sent to the different laboratories for activity testing and chemical analysis. These sites were selected at a workshop held in

Stellenbosch during May 2003. The sampling events were chosen to cover the different seasons and rainfall events during one year. Ten samples were taken at different sampling points at each site during a specific sampling event. For activity testing some of the samples were combined because of the limited analysis capacity of the laboratories conducting these analyses. The hormone-, mineral and pesticide analysis on the samples were done individually. Pesticide analysis on water was conducted by the ARC (PPRI), industrial chemicals and pesticides on sediment by the SABS and CSIR, mineral analysis by the ARC (IGWC) and hormone analysis by AMPATH. Activity tests were done at University of Pretoria, CSIR (Environmentek) and University of Stellenbosch.

The results of the different laboratories participating in the study are summarized and presented in this report. Water and sediment samples were analyzed for pesticides, minerals and industrial chemicals. Hormone analysis was only conducted on water samples. Analysis of minerals was done on both water and sediment samples. Although only few minerals have EDC properties, it is known that some of them have a synergistic effect with some pesticides.

EDC activity was detected at all the sites and the presence of EDCs was confirmed by chemical analysis. Hormones (Estrone, ethinylestradiol and estriol) were found in water. Pesticides (Endosulfan, terbutylazine, DDT and dieldrin) were found in low concentrations. Industrial compounds (p-Nonyl phenol, Phthalates and PCBs) were detected at some sites.

All the individual reports of the participants are on the CD attached to the report.

No human health risk assessment was undertaken because there is not yet a model available for risk assessment of EDC pollution. The available risk assessment models use the onset of cancer as an endpoint which could not be used for EDCs. Recommendations for future research needs and updating of the strategic research programme were also made.

Capacity building:
Sophisticated instrumentation is required, which also requires the knowledge and experience of highly qualified scientists. It was outside the scope of this project to embark on a training exercise. Researchers were, however, encouraged to train and utilise previously disadvantaged individuals (PDIs) in their individual projects. During the period 2002-2003, 11 PDIs were actively involved in the projects as scientists, analysts and assistants. It was decided to conduct two workshops for post-graduate students during 2002-2003 in order to promote interest in this field of science and an awareness of what research in this field entails. The students attending these workshops were encouraged to further their studies in this field. The overview of the two workshops is as follows:

- A short course on environmental analysis was presented at the University of Stellenbosch on 11 December 2002, just after the ANALYTIKA congress. Overseas experts on chemical analysis at trace levels (Prof. J. Roerade from

Sweden and Prof. P. Sandra from Germany), as well as local experts (Prof. E. Rohwer of the University of Pretoria, Mrs H. Meyer of the SABS and Mr R. Meinardt of the Agricultural Research Council), were invited to conduct the course. Unfortunately, Prof. Sandra could not attend for personal reasons. The topics addressed included:

- Sampling in the environment
- Novel analytical procedures
- Quality assurance in the laboratory
- Community involvement in research projects.

Eighteen students attended the course, nine of whom were from previously disadvantaged communities.

- A workshop on EDCs and toxicants was held in Stellenbosch from 5-7 May 2003. Fifty-five participants registered for the training courses, 20 of whom (37%) were PDIs. Three separate workshop sessions took place, namely:
  - WRC/EDC programme review (refinement and overview)
  - Bioassay workshop
  - Chemprop workshop.

  The current status of EDC research and development was presented by the following three overseas experts:
  - Prof. Lou Guillette, University of Florida, USA
  - Prof. Gerrit Schuurman, Department of Chemical Ecotoxicology, Germany
  - Prof. Taisen Iguchi, Okazaki National Research Institute, Japan.

  These experts presented a series of lectures to students and representatives of organisations that are currently involved in EDC analysis and research.

The focus of the bioassay workshop, presented by Dr Edmond Pool and Prof. Hannes van Wyk, was biochemical assays. Nineteen PDI students attended the workshop. The feedback was very positive, and a number of students indicated their intention to pursue postgraduate studies in this field.

Prof CJ de Jager attended the Global Water Research Coalition Workshop on Analytical Methods for EDC in Water Systems in Karlsruhe, Germany (31 March–2 April 2003). Dr IEJ Barnhoorn was trained at the Hatherley Laboratories in Exeter, UK in the development and validation of catfish VTG. She subsequently mentored a student (Ms S Mbazo) in repeating the procedure in a different fish species. Ms C van Zijl was trained in the YES assay and assisted with the new EPA cell lines. Ms L Rhavadalala assisted Prof. Fatoki of the University of Venda in the phthalate analyses.

During June 2003, Mrs M Roseman (Du Buisson and Partners Laboratories) attended a training session on the analysis of samples with LC-MS-MS at the University of Stellenbosch.

Publications:

ANECK-HAHN NH (2002) Screening for anti-oxidant pollutants and estrogenic activity in drinking water in poverty stricken areas of South Africa. DTech thesis, Technikon Pretoria, South Africa.

BARNHOORN IEJ, PIETERSE GM, BORNMAN MS, VAN VUREN JHJ and VAN DYK C (2003) Intersex in feral sharptooth catfish (*Clarias gariepinus*). Proceedings of the 38th South African Society of Aquatic Scientists (SASAqS) Annual Congress held in conjunction with the Zoological Society of South Africa (ZSSA). University of Cape Town, Cape Town, South Africa.

FATOKI OS and AWOFULA RO (2003) Methods for selective determination of persistent organochlorine pesticide in residues in water and sediments by capillary gas chromatography and electron-capture detection. *J. Chromatogr. A*. 983 225-236.

FATOKI OS and NOMA A (2002) Solid phase extraction method for selective determination of phthalate esters in the aquatic environment. *Water, Air, Soil, Poll.* 140 (1-4) 85-98.

HURTER E, POOL EJ and VAN WYK JH (2002) Validation of an ex vivo Xenopus liver slice bioassay for environmental estrogens and estrogen mimis. *Ecotoxicology and Environmental Safety* 53 178-187.

POOL EJ, JAGALS C, VAN WYK JH and JAGALS P (2003) The use of IL-6 induction as a human biomarker for inflammatory agents in water. *Water Science and Technology* 47 71-75.

SLABBERT JL and VENTER EA (2005) Recombinant yeast screen for the detection of estrogens and estrogen mimicking chemicals. CSIR Report No. ENV-P-I 2005-042. Pretoria: Division of Water Environment and Forestry Technology, CSIR.

Conference Presentation:

AEC Burger; APM Moolman. First Phase of an Endocrine Research Programme for South African Water Systems. Platform presentation, IWA World Water Congress, Beijing, China, 10-14 September 2006.

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Development of biomarkers in selected sentinel species as indicators of EDC exposure

Report 2A: University of Stellenbosch (K5/1471)
Water resource monitoring and method development and validation of biochemical methods to determine endocrine disrupting activity in water systems

Report 2B: University of Stellenbosch (K8/471)
Development of biomarkers for determining EDC contamination in surface water

Report 2C: University of Stellenbosch (K8/1471)
Literature review: Sex determination and gonad differentiation control pathways in fish as bio-indicators for endocrine disruption in aquatic systems

Report 3A: CSIR (Environmentek) (K8/478 & K5/1472)
Method development for biochemical procedures related to estrogen and androgen screening of water and sediment samples

Report 3B: CSIR (Environmentek) (K5/1555)
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The chemical, biological and immunological evaluation of selected estrogens found in sewage effluent in the GaRankuwa /CRC area

Report 5A: Agricultural Research Council (Plant Protection Research Institute) (K8/480)
Sampling and chemical analysis of water from the Josini dam for EDC pesticides

Report 5B: Agricultural Research Council (Plant Protection Research Institute) (K5/1470)
Sampling and analysis for selected EDCs in selected water environments in the Makhatini flats, Hartbeespoort dam, Vaal river barrage and Jukskei river (Rietvlei dam)

Report 6: AMPATH
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abs	Absorbance
AC	Activated charcoal
AEV	Acute effect value
AF	activation function
AhR	Aryl hydrocarbon receptor
ALP	Alkali-labile phosphate
ANOVA	Analysis of variance
ANSA	8-anilino-1-naphthalenesulphonic acid
AR	Androgen receptor
ARC	Agricultural Research Council
ARC-ISCW	Institute for Soil, Climate and Water of the Agricultural Research Council
ARC-PPRI	Plant Protection Research Institute of the Agricultural Research Council
As	Arsenic
ASE	Accelerated solvent extraction
ATCC	American Type Culture Collection
AUCC	Animal Use and Care Committee
BA	Bisphenol A
BB	Berger-Broida units
BBP	Butyl benzyl phthalate
BHC	Benzene hexachloride
Bo	Maximum binding
$CaCO_3$	Calcium carbonate
$CaSO_4.2H_20$	Gypsum
Cd	Cadmium
cDNA	Complementary DNA
CEV	Chronic effect value
cf-VTG	Catfish-vitellogenin
CNS	Central nervous system
CPRG	Chlorophenol red-ß-D-galactopyranoside
CRM	Certified reference materials
CSIR	Council for Scientific and Industrial Research
Cu	Copper
CUP1	Copper inducible promotor
CV	Coefficient of variation
CYP1A	Cytochrome P450
DBP	di-n-butyl phthalate/ Dibutyl phthalate
DCM	Dichloromethane
DDD	Dichloro-diphenyl-dichloroethane
DDE	Dichloro-diphenyl-dichloroethylene
DDT	Dichloro-diphenyl-trichloroethane
DEHP	di(2-ethyl hexyl) phthalate/ Diethyl hexyl phthalate
DEP	Diethyl phthalate
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DM	DAX/Mab3
DMP	Dimethyl phthalate
DMRT1	*Doublesex/mab-3* related transcription factor 1
DMSO	Dimethylsulphoxide

DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E_1	Estrone
E_2	17β-estradiol
E_3	Estriol
EC_{50}	Median effective concentration required to induce a 50% effect
ED	Endocrine disrupting
EDC	Endocrine disrupting chemical/ compound/ contaminant
EDTA	Ethylenediamine tetraacetic acid
EE	Ethinylestradiol
EEQ	Estradiol equivalent
EI	Electron ionisation
E-KRCA	Eerste-Kuils River water catchment area
ELISA	Enzyme linked immunosorbent assay
EPA	US Environmental Protection Agency
ER	Estrogen receptors
ER-CALUX	Estrogen receptor-mediated chemical activated luciferase gene expression
ERE	Eestrogen responsive element
EU	European Union
FAO	UN Food and Agriculture Organisation
FID	Flame ionisation detector
FSH	Follicle stimulating hormone
g	Gravitational
GC	Gas chromatography
GC-MS	Gas chromatograph mass spectrometry
GC-MSTOF	Time-of-flight mass spectrometer
GLP	Good laboratory practice
H_2SO_4	Sulphuric acid
HCB	Hexachlorobenzene
HD	Samples from Hartbeespoort dam
hER	Human estrogen receptor
hERα	Human androgen receptor
Hg	Mercury
HRPO	Horseradish peroxidise
ICP-AES	Inductively coupled plasma-atomic emission spectrometer
ICP-MS	Inductively coupled plasma mass spectroscopy
IgG	Immunoglobulin
IL	Interleukin
ITD	Ion trap detector
ITS	Ion trap sensor
JC	Samples from confluence of Jukskei and Crocodile Rivers
KCl	Potassium chloride
LC-MS-MS	Performance liquid chromatography, mass spectrometry, mass spectrometry
LDH	Lactate dehydrogenase
LH	Luteinising hormone
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LP	Liquid partitioning
LVI	Large volume injection

m/z	Ion mass
MAE	Microwave-assisted extraction
MCL	Maximum contaminant level
MDL	Method detection limit
Medunsa	Medical University of Southern Africa
MF	Samples from Makhatini flats
$MgSO_4$	Magnesium sulphate
mRNA	Messenger Ribonucleic acid
MS	Mass spectrometer
MT	Metallothioneins
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NIEHS	National Institute of Environmental Health Sciences
NP	p-nonylphenol
NPn2EO	p-nonylphenol ethoxylate
NSB	Non-specific
OC	Organochlorines
OD	Optical density
OE_2	17ß-estradiol
OECD	Organisation for Economic Cooperation and Development
OHC	Organohalogenated compounds
OP	p-octylphenol
OPnEO	p-octylphenol ethoxylate
OTT	Open tubular trapping
PAH	Polyaromatic hydrocarbons
Pb	Lead
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
p-NP	p-Nonylphenol
POP	Persistent organic pollutants
PTS	Persistent toxic substances
QC	Quality control
QPCR	Quantitative PCR
QSAR	Quantitative structure–activity relationship
RCBA	Recombinant yeast bioassay
RD	Samples from Rietvlei dam
RF	Response factor
RIE	Relative induction efficiency
RNA	Ribonucleic acid
RO	Reverse osmosis
RP	Relative potency
RPMI	Roswell Park Memorial Institute
RT-PCR	Real-time reverse transcription PCR
SABS	South African Bureau of Standards
SBSE	Stir bar sorbtive extraction
SD	Standard deviation
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Soxhlet extraction

SFE	Super critical fluid extraction
SIM	Single ion monitoring
siRNA	Short inhibitory RNA
SOP	Standard operating procedure
SPE	Solid phase extraction
SPME	Solid phase micro extraction
SRY	Sex-determining region Y
STW	Sewage treatment works
TCA	Trichloroacetic acid
TDS	Total dissolved solids
TEQ	Testosterone equivalents
TOF	Time of flight
TWQR	Target water quality range
TZW	Technologie Zentrum Wasser
UN	United Nations
UNEP	United Nations Environmental Programme
US	United States of America
VB	Samples from Vaal barrage
VOC	Volatile organic compounds
WHO	World Health Organisation
WRC	Water Research Commission
XFL	Xikundu fish ladder
YAS	Yeast androgen screen
YES	Yeast estrogen screen
Zn	Zinc
ZR	Zona pellucida
γ-BHC	Benzenehexachloride

UNITS OF MEASUREMENT

'	minute
µm	micrometre
°C	degree Celsius
µamp	microamp
µg	microgram
µℓ	microlitre
cm	centimetre
cm^3	cubic centrimetre
d	day
g	gram
ha	hectare
kD	kilodalton
kg	kilogram
ℓ	litre
M	molar
m	metre
m/v	mass:volume
mg	milligram
min	minute
mℓ	millilitre
mm	millimetre
mM	millimolar
mm^3	cubic millimetre
mN	milliNewtons
N	normality
ng	nanogram
nm	nanometre
Nmol	nanomole
pg	picogram
ppm	parts per million
rpm	Revolutions per minute
upm	Umdrehung pro minute (rotations per minute)
V	Volt / volume
v/m	volts per metre
v/v	volume:volume

1. BACKGROUND

Endocrine disrupting chemicals (EDCs) are defined as chemicals that interfere with the structure or function of hormone-receptor complexes. They can cause endocrine disruptive effects at exposure levels up to a million times *lower* than carcinogen exposure levels of concern. Internationally, the negative impact of EDCs on health is evident and is no longer disputed. (Compredo Credo, 2004) Examples include the increase in testicular and prostate cancers (Toppari et al., 1996; Bergstrom et al., 1996; Møller, 1998), the higher incidence of undescended testes and hypospadia (Møller and Weidner, 1999; Skakeback, 2001), the decline in male reproductive health and fertility, and a possible impact on the cognitive and immunological development of children (Tilson, 1998). Female reproductive health and fertility, particularly conditions such as endometriosis, breast adenomyosis and reproductive tract cancer, and possibly also polycystic ovarian syndrome, seem to be mediated by EDCs (Gerhard and Runnebaum, 1992). Fetal exposure to EDCs has been found to influence reproductive and general health. It would also appear that environmental EDCs contribute to the decline of some wildlife populations (Guillette et al., 1995; Johnstone et al., 1996; Vos et al., 2000).

Endocrine disrupting (ED) effects are not restricted to a small group of therapeutic agents such as oral contraceptives or hormone replacements. On the contrary, many compounds that are in daily use in industry, agriculture and households have ED effects. These include the alkylphenols, polychlorinated biphenyls, dioxins and furans, and organochlorine compounds, which are used in various forms as plasticisers, lubricants, packaging material, pesticides and insecticides. Dichloro-diphenyl trichloroethane (DDT) and other organochlorine pesticides are well known for their ED effects. South Africa's scarce water resources, the limited health budget, the likelihood of significant pollution by industry, the lack of proper waste control and the need to use DDT for malaria vector control emphasise the need for the authorities to adopt timely measures.

Persistent organic pollutants (POPs), a subgroup of EDCs, are hazardous chemical substances that do not break down naturally, or do so extremely slowly. They accumulate in fatty tissue, becoming more concentrated higher up in the food chain over time and thereby putting the environment and human health at risk. Of all the pollutants released into the environment every year by human activity, POPs are among the most harmful. They are highly toxic, causing an array of adverse effects, notably death, disease and birth defects, among both humans and animals. Specific effects include cancer, allergies and hypersensitivity, damage to the central and peripheral nervous systems, reproductive disorders and disruption of the immune system. POPs present a special risk to children because they are conveyed through the placenta and in breast milk, and can have a critical effect on the fetus and infant at key stages in its development (WHO, 2002).

The long-lasting POPs travel in multiple cycles of evaporation and condensation and are transported by air to remote areas far from the source of their release, necessitating international regulation. The international POPs convention, signed in May 2001 in Stockholm, defined measures to reduce global concentration levels of the 12 identified POPs, which are grouped into the following three categories:

- Pesticides: aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex and toxaphene
- Industrial chemicals: hexachlorobenzene (HCB) and polychlorinated biphenyls (PCBs)
- Unintended by-products: dioxins and furans.

The EDCs are an even larger group of chemicals. Apart from the POPs, the other chemicals included in this group are:

- Alkyl phenols and their ethoxylate: p-nonylphenol (NP), p-octylphenol (OP), p-nonylphenol ethoxylate (NPn2EO) and p-octylphenol ethoxylate (OPnEO).
- Phthalate: butyl benzyl phthalate (BBP), di-n-butyl phthalate (DBP) and di(2-ethyl hexyl) phthalate (DEHP)
- Plasticisers: bisphenol A (BA)
- Herbicides: atrazine, simazine, 2,4-D
- Fungicides: vinclozolin
- Organophosphate pesticides: azinphos-methyl, parathion
- Pharmaceuticals: diethylstilbestrol (DES), tamoxifen and raloxifen
- Certain heavy metals: cadmium (Cd), arsenic (As), lead (Pb) and mercury (Hg)
- Flame retardants: polybromobiphenyl ethers
- Natural and synthetic hormones: 17β-estradiol, ethinyl-estradiol, estrone, estriol
- Other industrial chemicals: benzene, styrene.

These EDCs do not all remain sufficiently stable to travel across international borders and to be classified as POPs. Clearly, the less stable ones are harmful in the particular area of their release, where exposure levels are high. These therefore require specific local monitoring and regulation, as they are not covered by the international convention.

Public fear of EDCs can be likened to public fear of radioactivity and disease-causing bacteria and viruses, since EDCs are not readily detectable and introduce a risk of unknown magnitude against which governments have to protect their populations. However, industry is involved in producing and releasing EDCs, and proper controls will undoubtedly come at a cost. EDCs are thus a politically sensitive issue. Even *perceived* threats to human health and to the environment can threaten the survival of the South African economy. South Africa could easily fall victim to international activism (either engineered or spontaneous) to ban export products from South Africa as a result of the incidence of high levels of one or other of the identified pollutants. The only defence against such threats is an improved knowledge of the state of the South African environment, so that the information can be used to promote clean industrial production and to ensure that present and future legislation is strictly enforced as far as banning – or controlled use – of certain chemicals is concerned. For this to happen, facilities are needed to monitor both the target chemicals and the endocrine disrupting effects.

2. AIM OF PROJECT

The aims of this programme were driven primarily by the need identified by stakeholders for reliable and relevant data, as well as the data needed for risk assessment models, namely:

- To determine the occurrence of EDCs in South African water systems

- To attempt to establish the sources of the contamination
- To determine whether there is evidence of EDC effects on humans and wildlife
- To recommend possible actions to minimise the effect on humans and wildlife.

Certain actions were needed to meet these aims, namely:

- Building human resource capacity, as well as capacity and capability in research institutions in areas where South African laboratories are lacking
- Creating awareness and interest among postgraduate students to qualify themselves in this field of science
- Conducting a small pilot study to ensure that the logistics were in place to embark on the main survey
- Conducting a limited surveillance study to determine whether EDCs are present in South African water systems.

3. METHODOLOGY

3.1 Selection of EDCs

The process of selecting EDC chemicals was described in volume 1 of this report (WRC Report No. KV 143/05). A list of selected chemicals is given in Table 1.

Table 1: Suggested list of priority EDC compounds

Compound	Chemical class	Potency relative to 17β-estradiol
17β-estradiol Estriol Estrone 17α-ethinylestradiol	Hormone	1 0.08-0.8 0.09-1 0.9-1.2
p-nonylphenol Nonylphenol ethoxylates p-Octylphenol Octylphenol ethoxylates	Alkylphenols	$7x10^{-3}$-$1x10^{-5}$ $1x10^{-5}$ $1.5x10^{-3}$-$1x10^{-4}$
PCBs	Polychlorinated biphenyls	$1x10^{-2}$-$1x10^{-4}$
DDT, DDE, DDD Dieldrin, aldrin, endrin α-endosulfan, β-endosulfan, Endosulfan-sulfate Heptachlor, heptachlor epoxide Lindane (γ-BHC)	Organochlorine pesticides	$1x10^{-7}$
Chlorpirifos Azinfos-methyl Parathion	Organophosphate pesticides	
Deltamethrin	Pyrethroid pesticide	
Atrazine Simazine Terbutylazine 2,4-D, 2,4,5-T Metoxychlor	Herbicides	 $1x10^{-4}$
DEHP DBP Bisphenol A	Plasticiser Raw material for resins	$1x10^{-5}$ $1x10^{-5}$

Compound	Chemical class	Potency relative to 17β-estradiol
Dioxins, dibenzofurans	Dioxins/furans	
Tributyltin, cyhexitin	Organo-tin compounds	
Lead, cadmium, mercury, arsenic	Toxic heavy metals	
Vinclozolin	Bactericide	

3.1.1 Elements of concern

Zinc, calcium, selenium, chromium, fluoride, bromide, boron, copper, iodine, molybdenum, strontium and vanadium are elements of concern. An inductively coupled plasma-atomic emission spectrometer (ICP-AES) scan may be used to determine whether they are present, and they may then be individually quantified where necessary.

3.2 Selection of analytical methods

EDC activity is generally determined by biochemical (*in vitro*) methods; EDC effect is determined by biological (*in vivo*) means; and the occurrence of individual chemicals is determined by chemical analysis. The selection of the appropriate and relevant method is of crucial importance when conducting research on EDCs. The process for selecting appropriate methods is described in volume 1 of this report (WRC Report No. KV143/05).

3.3 Capacity building in research facilities

The study of EDCs has two aspects:

- Determination of the EDC activity in water systems
- Determination of the chemicals responsible for the activity.

During the survey on laboratory capability and capacity, it became clear that capacity needs to be built in certain areas. (It is necessary to build both human resource capacity and research capability.) The methodology for determining EDC activity is still in the process of development worldwide. There is, as yet, no single test for all the aspects of endocrine disruption (estrogenicity, anti-androgenicity, thyroid function and neurological effect). It is therefore still essential to use a battery of tests to determine EDC activity in aquatic systems. The search for biomarkers also needs attention, because utilising biomarkers may prove to be the most efficient and cost-effective means of determining EDC activity in the environment. Much work has been done abroad on this aspect, but most of the species used are not indigenous to South Africa and are not suited to local conditions.

During the operation of the project, it also became apparent that capacity and capability need to be built in organisations that conduct chemical analysis. New methods are required to respond to the need to determine some of the EDC compounds at ultra-low levels. These methods are dependent on sophisticated instrumentation operated by highly skilled analysts.

3.3.1 Capacity building to determine EDC activity

The chemical analysis of all EDCs in aquatic systems would be very costly, since more than 4000 chemicals have EDC properties. It is therefore sensible to conduct chemical analyses only of samples that are EDC active. EDC activity is usually determined by biochemical means, making use of certain cell lines or using biomarkers such as fish and tadpoles. It would be costly and time-consuming to develop such tests in South Africa. It was therefore decided to verify and validate methods developed abroad and to modify those methods to suit South African conditions rather than developing new methods from scratch. Figure 1 illustrates, in schematic format, a plan for building the capacity and capability in research facilities to determine EDC activity.

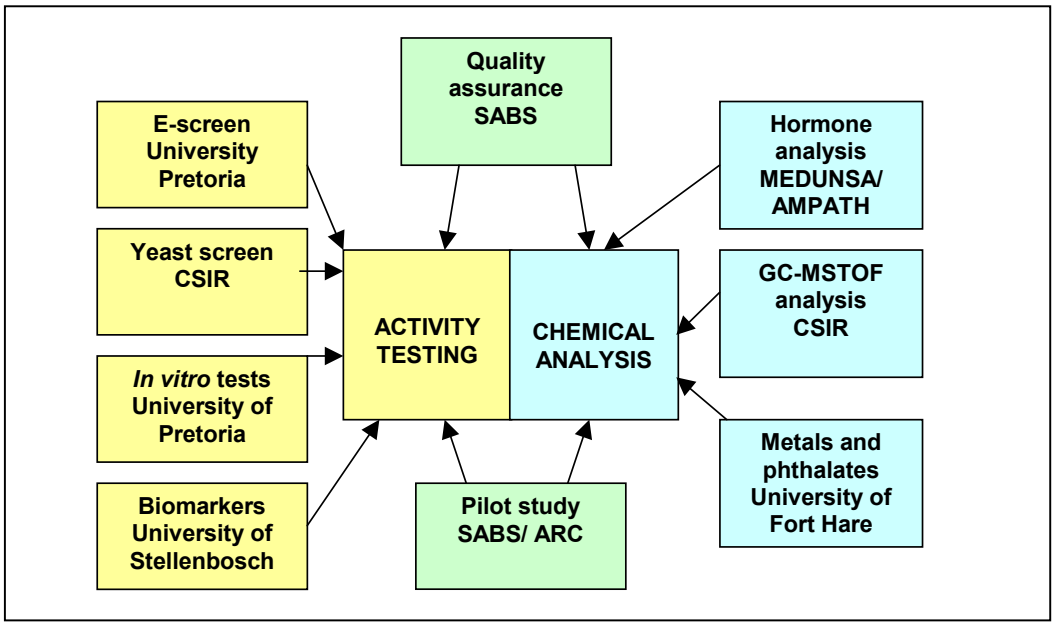


Figure 1: Plan for building capacity and capability in research facilities to determine EDC activity

Three laboratories were involved in developing (validating and verifying) tests to establish EDC activity in water and sediment.

3.3.1.1 Department of Urology, University of Pretoria

3.3.1.1.1 *In vitro* tests

The E-screen (MCF-7 cells) was successfully used to measure estrogenic activity. Technique development was needed to eliminate external estrogenic contamination from laboratory water used in the assay. However, a constant source of estrogen-free water for control purposes remains a problem.

The recombinant yeast bioassay (RCBA) was successfully used to measure estrogenic activity in water samples. Technique development was required to eliminate external

estrogenic contamination from laboratory water used in the assay. The validation of this test was also successfully carried out.

The MVLN reporter gene cell line was obtained as an alternative test to the estrogen receptor-binding (cell free) assay and the estrogen receptor transcriptional activation assay. This method still needs to be validated.

The uterotrophic assay was successfully used to determine estrogenic and anti-estrogenic activity. (Refer to Annexure A: Report 1A for analytical details.)

A methodology using catfish-vitellogenin (cf-VTG) coupled with an ELISA detection technique was developed and used successfully. (Refer to Annexure A: Report 1B for details.)

Although much research still needs to be done on bioassays, the developed methods should be sufficient to embark on a survey to determine EDC activity (estrogenic and anti-androgenic effect) in water and sediment samples in South Africa.

The problem of external estrogenic contamination of the available laboratory water negatively affected the schedule for the full development and implementation of the above-mentioned assays. Methodological problems have prevented the use or validation of the method for the extraction of sediment samples. The SABS consented to do the extraction of sediment for the quality control pilot study. The yeast screen and MVLN assays were used to analyse the samples.

3.3.1.1.2 Toxicity testing

Implementation of Organisation for Economic Cooperation and Development (OECD) protocols for one- or multi-generational studies and chronic low dose exposure in rodent models are in place.

The results of the ring test to determine recoveries in the extraction procedure and the results of the small pilot study are presented later in this report.

3.3.1.2 Department of Zoology, University of Stellenbosch

3.3.1.2.1 Monoclonal antibody development for VTG ELISAs for several fish species

Oviparous animals produce estrogen-induced yolk precursors (including vitellogenin) in the liver. These proteins are transported through the blood to oocytes to provide nutrients in support of early embryogenesis. Both male and female fish, including juveniles, have hepatic estrogen receptors, but normally only the livers of female fish will be exposed to estrogens. The production of vitellogenin by males and juveniles can therefore be utilised as a bioindicator for exposure to environmental estrogens. The yolk precursor protein, vitellogenin (VTG) is widely used as a biomarker in screening for endocrine disrupting compounds, specifically those with estrogenic effects. Throughout the world, several fish

species have been used for model systems, and VTG concentration is mostly measured in the plasma or whole body homogenates of exposed fish. Several small fish, including zebrafish (*Danio rerio*), have been recognised as good candidates for using VTG as biomarker for estrogenicity. This small oviparous cyprinid has a relatively short life cycle (four months), is a good experimental model and is easy to culture and breed. Both short-term and long-term multiple generation tests could be performed. Spawning may occur frequently, and as many as 400 eggs per female may be spawned at a time, with high fertilisation rates. Several zebrafish endpoints have been proposed for use in testing protocols in ecotoxicology. One of these, vitellogenin, is recognised as an important endpoint in detecting estrogenic activity. Another estrogen-inducible protein that has been recognised is the zona pellucida (ZR) protein, also produced in the liver and transported via the blood to the oocyte, to be incorporated in the zona radiata layer surrounding the oocyte. Although anti-zebrafish VTG antibodies and ELISA systems are available commercially (from BioSense, Norway) the costs of importing these make the routine use of these bioassays expensive.

In the light of the advantages of using zebrafish in screening studies to assess estrogenic activity in environmental samples, the objective of this project was (1) to develop monoclonal anti-VTG antibodies for zebrafish and screen other species for cross-reactivity and (2) to set-up and validate a VTG ELISA for zebrafish estrogen-inducible proteins. (Refer to Annexure A: Report 2A for details.)

Fourteen hybrids screened highly positive (OD>1.5 after 5 minutes) for estrogen-specific protein obtained from zebrafish. Three of these colonies also detected estrogen-specific protein from carp (ZFA1, ZFB1 and ZFB2). One of these monoclonal antibodies (ZFB1) also detected estrogen-specific protein from the Mozambique tilapia. Large volumes of monoclonal antibody were prepared from ZFA1, ZFB1 and ZFB2, and these antibody preparations were used for developing a standard ELISA protocol for screening zebrafish plasma for estrogen-induced protein synthesis. Although tested, no colony detected estrogen-specific proteins, including VTG, in catfish. However, universal VTG antibody (UniVTG) (developed inhouse), which is used in an ELISA, was used to measure plasma VTG levels in catfish. (Refer to Annexure A: Report 2B for details.)

3.3.1.2.2 Set up and validation of cytochrome P450 (CYP1A) enzymatic bioassays to evaluate pesticide (aryl hydrocarbons) contamination in fish

The induction of cytochrome P450 (CYP1A) in fish is increasingly being employed as a biomarker of exposure to environmental contaminants, including polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, petroleum products and halogenated dibenzo-p-dioxins and dibenzofurans. CYP1A is one of a superfamily of enzymes that catalyses mono-oxygenation reactions involving the conversion of endogenous and foreign compounds (mostly lipophilic substrates) to more water-soluble (hydrophilic) compounds, which may be conjugated and later excreted through the bile, urine or gills. The response to exposure to this group of environmental contaminants is therefore characterised by the induction of the CYP1A gene. This induction may be used as an early-warning signal of organic xenobiotic contaminants in the aquatic environment. The close link between the CYP1A enzymes and the synthesis and metabolism of steroid hormones suggests that if these enzymes preferentially deactivate pollutants, the natural synthesis and metabolism of

endogenous hormones will be compromised, resulting in endocrine disruption. Increased CYP1A expression may be a qualitative way of pointing to potential endocrine disruption activity that warrants further investigation. The objective of this project was to import the CYP1A antibodies and set up and validate an ELISA using tilapia fish. (Refer to Annexure A: Report 2B for details.)

This study successfully developed a competition ELISA for CYP1A. The ELISA is very sensitive, and the log-linear detection range is between 10 and 400 ng/mℓ CYP1A.

The antibodies were imported (from BioSense, Norway), but a custom peptide had to be synthesised in Belgium (Perbio). The ELISA was set up, and the exposure using tilapia to validate the ELISA was implemented successfully. (Refer to Annexure A: Report 2B for details.)

3.3.1.2.3 Set-up and validation of metallothionein ELISA for evaluating heavy metal contamination in fish

Metallothioneins (MT) are proteins that are induced as a result of metals such as copper and zinc and serve a homeostatic function under natural conditions. Excess of these metals, or the presence of other heavy metals, including cadmium and mercury, will induce increased production of metallothioneins. Induction of hepatic metallothioneins may therefore provide an indicator of potential endocrine disruption in exposed populations.

Because of the cost of the fish MT antibodies and preliminary data, which indicated that certain MT antibodies do not show cross reactivity with certain local fish species, it was decided to obtain anti-MT antibodies from a laboratory in Taiwan for the purpose of setting up the ELISA and checking for cross-reactivity with selected fish species used in exotoxicological studies (for example, zebrafish). All the components were obtained for setting up the ELISA. The ELISA was set up and validated with a standard curve. The initial exposure studies revealed that the tilapia MT ELISA could be used with zebrafish. Because very little published information is available on MT stimulation in tilapia fish, some range-finding studies for exposure had to be conducted initially. A second exposure was carried out using cadmium chloride. For this experiment, it was decided to use juvenile tilapia fish. The MT ELISA that was developed has a detection range of between 0.015 and 4 µg/mℓ. The normal value of MT in the liver of tilapia was 25 µg/mℓ. Upon exposure of tilapia to 35 µg/mℓ cadmium, there was a significant increase ($P<0.01$) in MT levels in the exposed fishes to 51 µg/mℓ. Concentrations of cadmium above 35 µg/mℓ did not cause any further increase in MT concentration in the liver tissue. (Refer to Annexure A: Report 2B for details.)

3.3.1.2.4 Set-up and validation of an *in vitro* fish cell culture bioassay to study estrogenicity and cytotoxicity in water samples

It was decided to set up and validate a yeast-based steroid hormone receptor gene transcription assay, which is different from the Glaxo Wellcome yeast screen currently in use in most South African EDC laboratories.

It is well known that environmental chemicals may interact directly with steroid receptors and, in doing so, affect the expression of steroid-responsive genes. It is important to remember that when evaluating chemicals or environmental water for endocrine-disrupting effects, the reporter gene assays refer only to the receptor-mediated process and not necessarily to secondary alternate pathways. Two systems have been widely used for the receptor-mediated assays: (1) by using hormone-dependent cancer cells (E-screen), or (2) by reconstituting the hormone signal pathway in non-mammalian cells (which are estrogen free), for example, yeast cells (eukaryotic organism). The yeast receptor-based assay has several advantages in that it is very sensitive, selective and reproducible. It is also easy to manipulate and lacks interference by endogenous receptors, steroid hormones or metabolism. Another considerable advantage is that the yeast-based assay can be employed quickly and inexpensively to screen large numbers of environmental samples.

In principle, the human steroid receptors – human estrogen receptor (hER), in this case hERα, human androgen receptor (hAR) – were introduced into the yeast strain *Saccharomyces cerevisae.* The DNA sequences of hER and hAR were integrated into the main chromosome of the yeast. The yeast cells also contain expression plasmids, containing a hormone response element site in the promoter, fused to the reporter gene, *lac-z* (encoding for the enzyme β-galactosidase). The production of the human steroid receptors is dependent on a multiple copy plasmid constructed by the yeast-inducible copper promoter fused to the receptors. This recombinant yeast strain differs from the Glaxo Wellcome yeast screen (Routledge and Sumpter, 1996) in that the receptor (hER) is not integrated into the yeast genomic DNA. The mechanism involves transcribing and translation of the human steroid receptor in the presence of copper, which forms a ligand-receptor complex that will bind to the hormone response element, thereby controlling the expression of β-galactosidase production and secretion into the surrounding medium. Differential expression of the reporter gene reflects the level of hormone or mimic present. The concentration of the produced β-galactosidase can then be determined using chromogenic substrate and measuring the absorbance of the resulting end product (Routledge and Sumpter, 1996).

The goal of this project was to obtain, set up and validate the recombinant yeast screen for estrogenicity and androgenicity screening of water samples. The yeast transformants previously described, implemented and evaluated were used for this.

The results show that both assays were successfully set up and that β-galactosidase dose response curves were found to be similar to those reported by Gaido et al. (1997). The assays will be validated by known estrogenic EDCs. This yeast screen does not differentiate between agonists and antagonists, but reports on binding with the steroid receptor. It has been suggested that this is because the yeast does not have the appropriate repressor proteins necessary for antagonism (Gaido et al., 1997). (Refer to Annexure A: Report 2B for details.)

3.3.1.2.5 Investigation into the possibility of using Y-chromosomal DNA markers to study sex differentiation in tilapia fish as a tool for examining whether fish exposed to effluents and test chemicals would express phenotypic sexes different from their genetic sex

A literature study was conducted on DNA markers associated with sex differentiation in fish. Tools may be developed to examine natural populations in rivers and dams to establish whether phenotypic sex corresponds with genetic sex. This will assist managers to establish whether historical exposures indeed affect population sex ratios, with a potential impact on the survival of the population in the specific population.

The literature review was conducted, and insight was gained for future research into the role of genomics and EDC studies. It was realised that characterising and using DNA markers to study the effects of EDC on sex determination in tilapia fish would not be straightforward, but that the success of such research would allow real population studies of EDC effects on the sex ratio in natural populations. It was also clear that the role of RNA studies in understanding the effects of EDCs in changing gene expression profiles may become the fast bioscreens of the future, using microarray methodology. (Refer to Annexure A: Report 2C for details.)

3.3.1.3 Environmentek, CSIR

The development work done in the Environmentek laboratory at the CSIR was aimed mainly at verifying the recombinant yeast assays for hER (estrogen activity) and hAR (androgen activity). The same procedure is used for both activities, except that for estrogen activity, the measurement is done against 17β-estradiol, and for androgen activity, the measurement is done against testosterone. The laboratory also took part in (1) a ring test to determine the recovery of the extraction procedure and (2) a small pilot study to determine EDC activity on two field samples.

The ring test involved the extraction of spiked water samples, environmental waters and sediments and the assay of the extracts for estrogen and androgen activity using recombinant yeast screens.

3.3.1.3.1 Recombinant yeast assays

In preparation for the testing of samples, fresh cultures were obtained from Prof. Sumpter, Brunel University, UK. The medium was freshly prepared under stringent quality control procedures.

3.3.1.3.1.1 Determination of estrogen activity (hER screen)

The yeast test is based on the metabolism of yellow chlorophenol red-ß-D-galactopyranoside (CPRG) to a red product, which can be assessed spectrophotometrically. The colour reaction is due to the action of ß-galactosidase, which is encoded by the reporter gene *lac-z* that is carried by expression plasmids in a transfected yeast cell. The yeast cultures used for estrogen and androgen detection respectively contain the human estrogen receptor (hER) and

androgen receptor (hAR). Binding of a suitable substrate to these receptors thus induces expression of the *lac-z* gene, which leads to the activation of ß-galactosidase. (Refer to Annexure A: Report 3A for experimental details.) A typical curve obtained for 17ß-estradiol in the hER screen is presented in Figure 2.

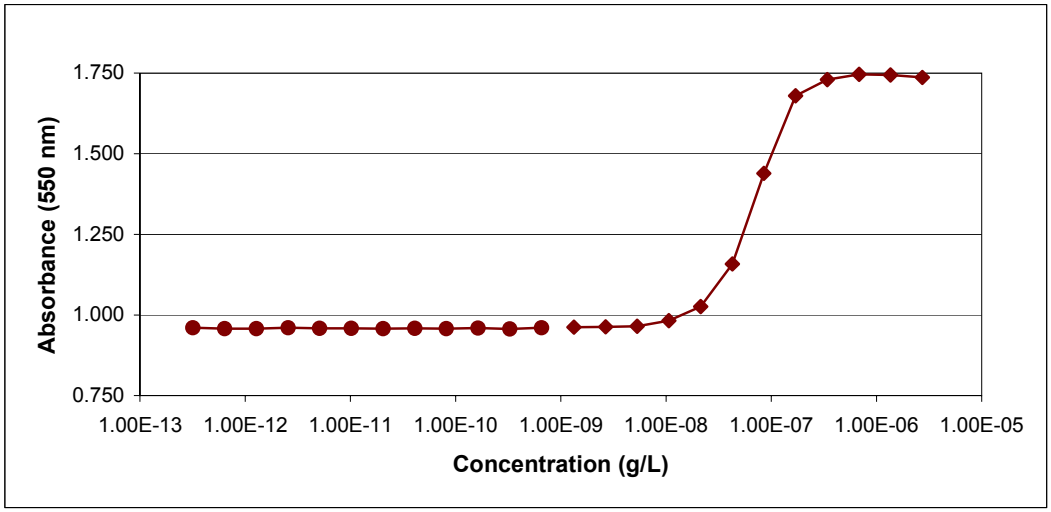


Figure 2: Estrogenic activity of 17ß-estradiol after three days' incubation

Extensive work has been done on different incubation periods as well as different extraction procedures in order to optimise the results of these assays. (Refer to Annexure A: Report 3A for full details on this work.)

3.3.1.3.1.2 Determination of androgen activity (hAR screen)

The test to determine androgen activity, which is similar to the estrogenic test, was successfully carried out. (Refer to Annexure A: Report 3A.)

3.3.1.3.1.3 Alkali-labile phosphate to determine EDC activity

The test was optimised with some difficulty (using kits off the market, changes in reagents, and other such methods). Details of the development are captured in a report. (Refer to Annexure A: Report 3A.) Table 2 shows the results obtained when 21-day-old zebrafish were exposed to 17ß-estradiol (100 and 1000 ng/ℓ) for approximately ten days. The results are reported in terms of protein and fish weight. The limited availability of snails meant that only limited tests could be conducted. Some of these results are captured in the report. (Refer to Annexure A: Report 3A.)

Table 2: Effect of 17ß-estradiol on vitellogenin in fish expressed in terms of alkali-labile phosphate

Exposure period (days)	Test concentration (ng/ℓ)	Alkali-labile phosphate	
		Calculated in mg/mg protein	Calculated in mg/mg fish
		% Induction	
10	1 000	**199**	**130**
14	100	**197**	**325**
10	100	**165**	**140**
10	10	**143**	**140**

Note: Results in bold indicate significant induction at P=95%

3.3.1.3.1.4 Gel electrophoresis

The gel electrophoresis technique was successfully established. A figure is included in the report (see Annexure A: Report 3A) to show the VTG bands (between the 100 and 150 kD molecular markers) when fish were exposed to 17ß-estradiol (100 ng/ℓ).

3.3.1.3.2 Conclusions and recommendations

The following conclusions were reached and recommendations made:

- The study showed that the water samples exhibited large estrogenic activity.
- Low androgenic activity was detected in the samples from confluence of Jukskei and Crocodile rivers (JC).
- The sediment extracts were toxic in the estrogen and androgen screen.
- It is possible that estrogenic/androgenic activity was masked by the toxicity exhibited by the sediment samples.
- Estrogenic activity was only detected in the cocktail (100 µg/ℓ) and the 200 and 300 µg/ℓ final extract concentrations.
- The cocktail and spiked samples were not androgenic.
- No clear conclusions could be reached on which of the extraction methods was best in terms of recovery, as most of the extracts did not exhibit estrogenic/androgenic activity.
- The advantage of XAD was that large volumes of water could be concentrated. The discs became clogged with algae and other material in the water, delaying the concentration process.
- Longer incubation periods increased the induction efficiency and potency of samples. It is recommended that, in future, absorbance is read after three and ten days' incubation in the hER screen.
- Firm recommendations on extraction and test methodologies will only be possible at the end of projects in the programme. Investigations are continuing.

3.3.2 Capacity building for chemical analysis and confirmation of activity

The methodology for the chemical analysis of EDCs is well established worldwide, but sophisticated instrumentation and well-trained analysts and scientists are required to conduct these analyses at the ultra trace levels needed in this field. No facilities exist in South Africa

for the analysis of certain EDC chemicals (including synthetic and natural hormones, dioxins and furans).

3.3.2.1 Analysis of natural and synthetic hormones

The Medical University of Southern Africa (Medunsa) was asked to conduct a literature survey on the occurrence of synthetic and natural hormones worldwide in order to create a facility for testing for these hormones in South Africa. They were also given support in conducting a small pilot study at selected sewage treatment works (STWs) to establish whether the levels of these hormones could be determined. (The most likely areas in which to find these substances are STWs.) The method developed by Medunsa was not appropriate for the determination of hormones at ultra-low levels.

Support was given to Du Buisson and Partners (AMPATH) to validate and verify a method from abroad for determining the levels of natural and synthetic hormones, as they already had the necessary infrastructure to conduct this analysis. The four most important endogenous estrogens are 17α-estradiol (17α-E_2), 17β-estradiol (17β-E_2), estrone (E_1) and estriol (E_3), while the most commonly used synthetic estrogen is ethinylestradiol (EE). (Refer to Annexure A: Report 4 for details of the literature study and method development.) The rationale for this selection of hormones is as follows:

- It was found that 17α- E_2 is not excreted by humans but by animals. Traces of this estrogen can thus be used to assess the contribution that animals make as a potential source of estrogenic contamination of the environment.
- 17β- E_2 is the most potent estrogen in humans, with three times the potency of E_1. It is a major hormone produced by ovaries and is readily oxidised to E_1. It is one of the most important and significant estrogens documented and analysed in the literature.
- E_1 was the first estrogen to be isolated, and is produced in the placenta in large quantities during pregnancy. It is excreted as the conjugate, estronesulphate and is more potent than E_3. E_1 is also documented as being an important environmental contaminant and should thus to be tested for.
- E_3 is the least potent – $1/60^{th}$ the potency of E_2 – but is one of the major estrogens found in human urine. E_3 is excreted as the conjugate estriol glucuronide and is excreted in high concentrations via the placenta during pregnancy. This estrogen is also an important environmental contaminant and should thus be tested for. EE is the most commonly used synthetic estrogen used in conjunction with a progesterone in oral contraceptive pills (OC). According to the literature, this estrogen has proved to be one of the most challenging to test for and analyse.

Estrogenic activity is shared by many steroidal and non-steroidal compounds. The ranking of these hormones for endogenous estrogenic potency is as follows: 17β- E_2>E1>E3. Each molecule is an 18-carbon steroid and contains a phenolic A ring and a β-hydroxyl group/ketone in position 17 of the D ring. The phenolic ring acts as a selective, high affinity binding point to the estrogen receptors, α and β.

EDCs, particularly steroidal estrogens, may be present at low concentrations (ng/ℓ to pg/ℓ) in both treated and untreated sewage effluent, and may present specific technical difficulties. These difficulties can often result in wide variations in measured concentration values and,

at worst, can be significantly biased with respect to the value of 'true' concentrations. The measurement of uncertainties is greatest with domestic sewage and tends to diminish with increasing quality of sewage treatment. Domestic sewage is a complex mixture containing materials in different phases, ranging from true aqueous solutions, colloidal suspensions, fine suspended solids and gross floating solids to rapidly settling solids. Some chemicals, such as those with low water solubility, tend to partition to a non-aqueous phase, either dissolving in fats and oils or being absorbed to the surface of colloidal and suspended solids. The measurement of steroidal estrogens is further complicated by uncertainties about the process of deconjugation.

Some analytical monitoring data are now emerging on concentrations of steroidal estrogens in domestic sewage. While the analysis of sewage is particularly complex, and studies often vary with regard to sampling strategies and analytical techniques, these studies have indicated reasonably consistent ranges of natural steroidal hormones. Reported concentrations range from E_3 being detected at the highest concentration (50–260 ng/ℓ), followed by E_1 (<0.5–50 ng/ℓ) to 17β-E_2 (<0.5–50 ng/ℓ). These quantities are consistent with the quantities of E_3 and E_1 present in urine produced during pregnancy, which are respectively about two and one orders of magnitude larger than that of 17β-E_2.

Sewage treatment plants receive both natural and synthetic EDCs from urban and industrial dischargers. They also undergo a variety of treatment processes of varying efficiency, such that many of the compounds are not removed efficiently and are ultimately discharged into surface water.

It should be noted that there have been no studies of these compounds in South African water systems, and no levels have thus been reported for South African waters, making this study not only important but also necessary. These levels and the removal efficiencies of the various sewage treatment works should be determined for South Africa, as steroidal estrogens could impact negatively on human health, thus making this study one of national significance.

It is hypothesised that the water environment of South Africa contains natural and synthetic estrogens, as well as other endocrine-disrupting substances, and should therefore test positive for estrogenicity biologically using the recombinant yeast cell bioassay, and that these hormones will be detected analytically using high performance liquid chromatography, mass spectrometry, mass spectrometry (LC-MS-MS), gas chromatography or mass spectrometry (GC-MS).

The yeast screen was successfully applied to determine hormone activity in water. The chemical analysis proved more difficult than anticipated. The detection limit for 17α-E_2, 17β-E_2, E_1 and EE was 0.36 ng/ℓ, and 0.3 ng/ℓ was achieved for E_1. The detection limit for E_3 was 1.2 ng/ℓ. The development of this method was to have continued during 2003. Limits of detection of 0.03 ng/ℓ may be necessary.

Unfortunately, the Medical University of Southern Africa (Medunsa) withdrew from this programme at the end of 2003.

AMPATH agreed to develop a methodology to determine the hormone content in water by GC-MS. (A report on this development is given in Annexure A: Report 6.)

A facility for the detection and determination of dioxins and furans would have to be created. This is a very expensive analysis, costing about US$4000 per sample to analyse.

3.3.2.1.1 Chemical analysis of individual EDCs

It was also deemed wise to support an investigation into the possibility of determining EDCs by gas chromatography utilising a time-of-flight mass spectrometer (GC-MSTOF). The advantages of this method are that more chemicals can be detected in a single run and that less sample preparation is required.

The CSIR (Biochemtek) conducted a comprehensive literature survey of the extraction of EDC compounds in water and sediment and their detection by means of GC-MSTOF. (Refer to Annexure A: Report 7.) Various extraction procedures were considered, such as liquid-liquid extraction (LLE), solid phase extraction (SPE), supercritical fluid extraction (SFE), solid phase micro extraction (SPME), microwave-assisted extraction (MAE) and open tubular trapping (OTT). Comparison was made between conventional GC-MS detection and GC-MSTOF detection, and various injection techniques were investigated. The methodologies are summarised in Table 3.

Table 3: Summary of methodologies

Parameter	Pesticides and PCBs	Alkyl phenols
Extraction methods		
Water	Automated SPE, online SPE/SPME, SPE, SPME, SFE, LLE (21), SBSE (2,13), OTT (2,11,18,19)	SPE (6)
Sediment	Soxhlet, SFE, ASE, MAE, LP (6,13,14), TDS (10,24)	
Injection	LVI (9,21), conventional (6,14,16,17) TDS (2,10,18,19,24)	Conventional (6)
Detection	GC-MS (quadrupole, ion trap, TOF), GC-MS-MS, GC-GC-TOFMS (5,9)	GC-MS, full scan mode and SIM (6)
LOD		
Water	TOF: 1-4pg injected mass (5), LLE: 5.5-20ng/ℓ (7), Online and off-line SPME: 0.8 ng/ℓ–2 µg/ℓ (21), Automated on-line GC systems: 0.01-0.1 µg/ℓ (9), SPE, GC-MS-MS: 2.26 ng/ℓ (14), SBSE: low ng/ℓ (2), OTT ng/ℓ–4 µg/ℓ (11,18,19)	0.1 µg/ℓ, 0.01 µg/ℓ
Sediment	Soxhlet: 0.6 ng/g (7) MAE: 2.1 ng/g Soxhlet 10-30 µg/kg (16,17) SFE: 40-80 µg/kg (16,17)	–

Note:

ASE	Accelerated solvent extraction	OTT	Open tubular trapping
GC	Gas chromatograph	PCBs	Polychlorinated biphenyls
GC-MS	Gas chromatograph mass spectrometry	SBSE	Stir bar sorbtive extraction

LLE	Liquid-liquid extraction	SFE	Super critical fluid extraction
LODs	Limits of detection	SIM	Single ion monitoring
LP	Liquid partitioning	SPE	Solid phase extraction
LVI	Large volume injection	SPME	Solid phase micro extraction
MAE	Microwave assisted extraction	TDS	Thermo desorber system
MS	Mass spectrometer	TOF	Time-of-flight

In selecting an analytical technique, it is important that parameters such as sensitivity, selectivity, repeatability, robustness and suitability to matrix be considered. It was proposed that the following methodology be used for the evaluation and verification of the chemical analysis of selected pesticides, alkylphenols and PCBs:

Extraction: Water by multi-residue SPE utilising C18 cartridges
Sediment by multi residue LP using various solvents and ultra sound and/or mechanical shaking
Injection: Conventional
Detection: GC-MSTOF using the LECO GC-MSTOF.

The final method was verified in terms of the following parameters:

- Accuracy
- Precision
- Calibration
- Limit of detection (LOD)
- Limit of quantification (LOQ)
- Uncertainty (expressed as a percentage relative standard deviation)
- Selectivity.

The results of the ring test conducted with this method are given in Table 4.

Table 4: Results of recovery tests

Compound	**Detection level of analysis**		
	0.025 µg/ℓ	**0.1 µg/ℓ %**	**1.0 ug/ℓ %**
p-Nonylphenol	ND	70	93
Atrazine	ND	114	116
1,1'-biphenyl, 2,2', 5,5' tetrachloro biphenyl	ND	90	102
Endosulfan I	ND	60	98
p,p'-DDE	ND	80	115
Dieldrin	ND	112	121

Note:
ND Not detected

According to Table 4, the lowest limit of detection (LOD) for this methodology is 0.1 µg/ℓ, which should be sufficient for EDC analysis of these compounds, with the exception of PCBs, which require an LOD of 0.003 µg/ℓ, and methoxychlor, which requires an LOD of 0.03 µg/ℓ. This problem may be overcome, however, by concentrating the sample before injection. According to the calculations based on the potency, the LODs for EDC determinations are given in Table 5.

Table 5: Lowest limits of detection calculated on potency of EDC compounds

Compound	Detection limit
17β-estradiol	0.03 ng/ℓ
Estriol	0.04 ng/ℓ
17α-Ethinylestradiol	0.03 ng/ℓ
Estrone	0.03 ng/ℓ
p-Nonylphenol	0.2 µg/ℓ
p-Octylphenol	0.05 µg/ℓ
BBP	3.0 µg/ℓ
DBP	3.0 µg/ℓ
DEHP	3.0 µg/ℓ
DDT	30 µg/ℓ
DDE	30 µg/ℓ
Methoxychlor	0.03 µg/ℓ
PCB	0.003 µg/ℓ

3.3.2.2 Analysis of heavy metals and minerals

3.3.2.2.1 University of Fort Hare

The University of Fort Hare was assisted in upgrading its facility in order to determine cadmium and mercury at trace levels. A detector for the determination of mercury was sponsored by the Water Research Commission. The major research during 2002 was the implementation of test methods for cadmium, mercury and zinc. The investigation of zinc was important because of its synergistic reaction with cadmium in the aquatic environment. Calibration curves were prepared, and detection limits determined in order to ensure the quality of the results and to determine whether the detection limits would meet the limits set for EDC analysis (Fatoki and Awofolu, 2003; Fatoki et al., 2004) The results of the recovery tests and the determination of detection limits are given in Tables 6 and 7.

Table 6: Recovery tests and detection limits for cadmium, mercury and zinc in water

Element	Detection limit ng/ℓ	% Recovery	% Recovery
		Open beaker extraction	Van Loon extraction
Cadmium	1	90.7±6.80	83.3±5.73
Zinc	6	90.5±0.29	88.2±3.09
Mercury	3	103.3±6.83	

Table 7: Recovery tests for cadmium and zinc in sediment

Element	% Recovery	% Recovery
	Aqua regia extraction	Perchloric acid digestion
Cadmium	66.0±3.27	92.67±2.49
Zinc	92.0±9.93	93.4±0.71

The results of the study of cadmium, zinc and mercury in rivers of the Eastern Cape Province are summarised in Table 8.

Table 8: Metal concentrations in selected sites in the Eastern Cape province of South Africa

Sample sites	Metal concentration (mg/ℓ)				
	Cadmium		Zinc		Mercury
	Water	Sediment	Water	Sediment	Water
Buffalo river	0.008–0.016	0.009–0.017	0.031–0.043	0.030–0.040	0.001–0.002
Sandile dam	0.007–0.009	na	0.045–0.120	na	ND–0.002
Keiskammahoek dam	0.001–0.009	0.008–0.058	0.009–0.092	0.029–0.033	0.0007–0.001
Tyume river	0.003–0.017	0.018–0.063	0.026–0.028	0.072–0.141	ND–0.003
Umtata river	0.001–0.007		0.019–0.196		ND–0.002

Note:
ND Not detected

The South African Water Quality Guidelines (DWAF, S.a.) indicate that the concentration of cadmium in fresh water sources should be below 0.005 mg/ℓ. Based on this guideline, the concentration of cadmium in the Buffalo River is exceptionally high. The sources of this contamination may be industrial effluent or agricultural run-off in the catchment area of the river.

The South African guideline for zinc in water is 3 mg/ℓ. The levels in all the sites investigated comply with this level.

The levels for mercury were well within the limits of the guidelines for South Africa in all the sites investigated.

3.3.2.2.2 Pretoria Technikon

The Pretoria Technikon (now the Tshwane University of Technology) was funded to carry out development work on the analysis of selected pesticides (DDT, DDE, DDD) in river water and sediment.

Various methods of extraction and clean-up were investigated and compared, including liquid-liquid extraction (LLE), solid phase extraction (SPE), activated charcoal (AC) and Soxhlet extraction (SE). Recovery tests were conducted on DDT, DDE and DDD, the results of which are given in Table 9.

Table 9: Percentage recoveries of DDD, DDE and DDT standards obtained from spiked water and sediment samples

Extraction method	% Recovery		
	2,4-'DDD 4,4'-DDD	2,4'-DDE 4,4'-DDE	2,4'-DDT 4,4'-DDT
Liquid-liquid (ws)	96 90	80 95	86 76
Solid phase (ws)	71 69	56 66	76 70
Activated charcoal	75 87	80 90	84 96
Soxhlet (ss)	85 80	95 90	65 91

Note:
ws Water sample
ss Sediment sample

Samples were taken from the Mvudi and Madandze rivers near Thohoyandou during June, August and October 2003 and analysed for the presence of the DDT family of pesticides. The results are given in Table 10.

Table 10: Total DDT (2,4'- and 4,4'-DDT) concentrations in the Mvudi and Madandze rivers (µg/ℓ)

Rivers	Extraction method	Collection dates (2003)		
		June	August	October
Mvudi (ws)	LLE	2.6	3.2	ND
Madandze (ws)	LLE	2.0	ND	ND
Mvudi (ws)	SPE	1.6	2.3	ND
Madandzi (ws)	SPE	1.2	ND	ND
Mvudi (ws)	AC	2.2	2.8	ND
Madandzi (ws)	AC	1.8	ND	ND
Mvudi (ss)	SE	3.6 (µg/kg)	5.2 (µg/kg)	ND
Manadzi (ss)	SE	1.8 (µg/kg)	ND	ND

Note:
ws Water sample
ss Sediment sample
ND Not detected (below detection limit)
LLE Liquid-liquid extraction
SPE Solid phase extraction
AC Activated charcoal extraction
SE Soxhlet extraction

The limits of detection are given in Table 11.

Table 11: Limits of detection

Compound	Limit of detection (µg/ℓ)
2,4'-DDD	0.52
4,4'-DDD	0.52
2,4'-DDE	0.55
4,4'-DDE	0.55
2,4'-DDT	0.40
4,4'-DDT	0.30

This study showed the presence of DDT in the two rivers studied. The levels were found to be marginally higher than the World Health Organisation (WHO) guidelines. Possible sources include small-scale agricultural activities around these rivers, DDT spraying programmes in the Thohoyandou area, sewage and waste dump sites around the rivers. The activated charcoal extraction technique used in this study compares favourably with the other extraction techniques. Considering the fact that charcoal is fairly cheap, it can be used in monitoring programmes involving DDT and its metabolites in surface water samples. However, it remains to be seen whether the activated charcoal extraction method used in this study can be extended to other persistent substances apart from DDT. Little is known about the levels of such substances in the chosen study area.

The absence of DDT metabolites (DDE and DDD) suggests recent low-level contamination of the rivers by DDT. However, since DDT spraying programmes have been reintroduced within the villages outside the town of Thohoyandou, more studies need to be conducted in these areas in order to obtain more data to indicate the overall surface water and sediment contamination by organochlorines in the northern region of Limpopo province. To enable tracking of the results of action taken to combat the release of toxic pollutants, environmental monitoring efforts should be intensified to include studies of biological effects.

In view of the ever-growing use of chemicals in South African society, it is vital to enable basic research on persistent pollutants to continue undiminished. Such research will go a long way towards contributing to the global debate and official measures to prevent the use and release of toxic and persistent substances. (For a full report on this project, refer to Annexure A: Report 8.)

3.3.2.2.3 University of the Western Cape

The chemical determination of hormones by GC-MS proved to be difficult and costly. An alternative method had to be developed for confirmation purposes. ELISA techniques are well known for their sensitivity and selectivity. It was decided to purchase ELISA kits from abroad for verifying the methods in South Africa. Only estriol and estradiol kits could be obtained; kits for estrone were unfortunately not available.

The aim of this study was to standardise ELISA assays for estradiol and estriol. The ELISA assays would then be used for the analysis of sewage effluents collected from sewage treatment plants in the Eerste-Kuils river water catchment area. (For details of the methodology, refer to Annexure A: Report 9.)

Water samples were analysed for estrogen using a Human Gesellschaft für Biochemica und Diagnostica Estradiol ELISA kit according to the manufacturer's instructions and a DSL Ultra-Sensitive Unconjugated Estriol EIA kit according to the manufacturer's instructions.

3.3.2.2.3.1 Results

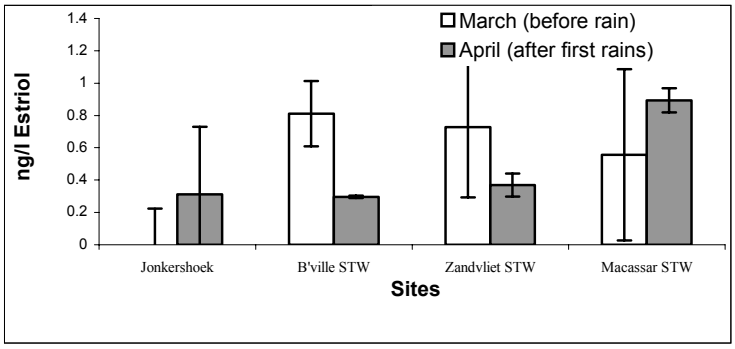


Figure 3: The detection of estriol in sewage samples before and after the first seasonal rains of 2005

Water samples were collected from several sewage plants in the Western Cape, South Africa. Samples were extracted on C18 columns. The extracts were assayed for estriol using the DSL Ultra-Sensitive Unconjugated Estriol EIA kit (Figure 3).

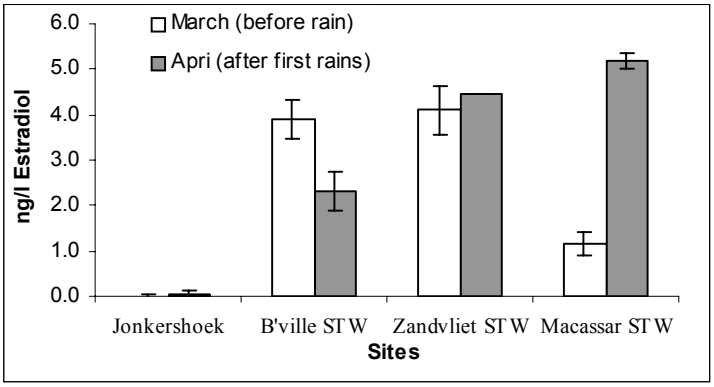


Figure 4: The detection of estradiol in sewage samples before and after the first seasonal rains of 2005

Water samples were collected from several sewage plants in the Western Cape, South Africa. Samples were extracted on C18 columns. The extracts were assayed for estradiol using the Human Gesellschaft für Biochemica und Diagnostica Estradiol ELISA kit (Figure 4). (Refer to Annexure A: Report 9 for details.)

The data show that estradiol and estriol were present in all the sewage samples. The levels detected for estradiol were below 6 ng/ℓ, while those for estriol were below 1 ng/mℓ. The data are in the same range found in other countries (Colborn et al., 1993; Turner and Sharpe, 1997; Kime, 1999).

3.3.3 Human resource capacity building

The analysis of EDC activity as well as the chemical analysis of EDCs requires a very high level of scientific skill. Most of the chemical analyses have to be conducted at ultra trace levels (ng/ℓ). It generally takes at least a year to train a competent graduate in this field.

Sophisticated instrumentation is required, which also requires the knowledge and experience of highly qualified scientists. It was outside the scope of this project to embark on a training exercise. Researchers were, however, encouraged to train and utilise previously disadvantaged individuals (PDIs) in their individual projects. During the period 2002–2003, 11 PDIs were actively involved in the projects as scientists, analysts and assistants. It was decided to conduct two workshops for postgraduate students during 2002–2003 in order to promote interest in this field of science and an awareness of what research in this field entails. The students attending these workshops were encouraged to further their studies in this field. The overview of the two workshops is as follows:

- A short course on environmental analysis was presented at the University of Stellenbosch on 11 December 2002, just after the ANALYTIKA congress. Overseas experts on chemical analysis at trace levels (Prof. J. Roerade from Sweden and Prof. P. Sandra from Germany), as well as local experts (Prof. E. Rohwer of the University of Pretoria, Mrs H. Meyer of the SABS and Mr R. Meinardt of the Agricultural Research Council), were invited to conduct the course. Unfortunately, Prof. Sandra could not attend for personal reasons. The topics addressed included:
  - Sampling in the environment
  - Novel analytical procedures
  - Quality assurance in the laboratory
  - Community involvement in research projects.

  Eighteen students attended the course, nine of whom were from previously disadvantaged communities.

- A workshop on EDCs and toxicants was held in Stellenbosch from 5–7 May 2003. Fifty-five participants registered for the course, 20 of whom (37%) were PDIs. Three separate workshop sessions took place, namely:
  - WRC/EDC programme review (refinement and overview)
  - Bioassay workshop
  - Chemprop workshop.

  The current status of EDC research and development was presented by the following three overseas experts:
  - Prof. Lou Guillette, University of Florida, USA
  - Prof. Gerrit Schuurman, Department of Chemical Ecotoxicology, Germany
  - Prof. Taisen Iguchi, Okazaki National Research Institute, Japan.

  These experts presented a series of lectures to students and representatives of organisations that are currently involved in EDC analysis and research.

  The focus of the bioassay workshop, presented by Dr Edmond Pool and Prof. Hannes van Wyk, was biochemical assays. Nineteen PDI students attended the workshop. The feedback was very positive, and a number of students indicated their intention to pursue postgraduate studies in this field.

3.4 Selection of laboratories

Criteria were set for laboratories that would be considered capable of conducting EDC analysis. The process of selecting laboratories is described in volume 1 of this report (WRC Report No. KV143/05). A summary of laboratories selected for chemical analysis is given in Table 12, and Table 13 reflects the laboratories conducting toxicity tests.

Table 12: Summary of laboratories conducting chemical analysis on EDCs

Laboratory	Hormones	Alkylphenols	PCBs	Pesticides and herbicides	PAHs	Heavy metals
SABS[1,2]		X	X	X	X	X
ARC (Plant Protection Research Institute)[2]				X		X
AMPATH[2]	X	X	X	X	X	X
CSIR[1] (Biochemtek)			X		X	
Pentech			X	X	X	
Rand Water[1]			X	X		X
Umgeni Water[1]			X	X		X
ERWAT[2]		X				X
University of Cape Town				X		
City Laboratory of Cape Town			X	X		
North-West University[2]			X	X	X	
Durban Institute of Technology			X	X		X
Waterlab[2]						X
University of Johannesburg (Zoology)[2]						X
Johannesburg Water[1]		X	X	X		X

Note:
[1] Laboratory is accredited
[2] Laboratory complies with good laboratory practice (GLP)

The analysis of volatile organic compounds (VOC) and semi-volatiles, as well as F, SO_4, NO_3, NO_2 and PO_4 is also undertaken by the University of the North-West.

Table 13: Summary of laboratories conducting toxicant tests

Laboratory	Number of qualified analysts	Routine capabilities	Matrix analysed	Acute toxicity	Chronic toxicity	Algal toxins
Rand Water[1]	6	X	W,S	X	X	X
Umgeni Water[1]	4	X	W,S,F	X	X	X
North-West University[2]	7		W,S,B	X		
Waterlab[2]	10	X	W,S	X	X	X
CSIR Biotoxicology	5		W,S,B	X	X	

Laboratory	Number of qualified analysts	Routine capabilities	Matrix analysed	Acute toxicity	Chronic toxicity	Algal toxins
Laboratory (Environmentek, CSIR)[2]						
University of Johannesburg (Zoology)	8	X	W,S,B	X	X	
Onderstepoort Veterinary Institute (Residue Laboratory)[1]	7		W,S,B			
Unilever Centre for Environmental Water Quality[2]	5	X	W	X	X	
Water and Environmental Technology, Sasol Technology R&D[2]	3		W,S	X	X	
Johannesburg Water[1]	25	X	W,S,F,D	X		

Note:
[1] Laboratory is accredited
[2] Laboratory complies with GLP
W Water
S Sediment
B Biological tissues
F Food
D Dairy

There is at present no facility in South Africa for the chemical determination of dioxins and furans, although the North West University offers a bioassay to determine dioxins, dibenzofurans and coplanar PCBs in extracts (sediment, soil, water and air). The procedure provides an integrated I-TEQ value, using the H4IIE cell line (semi-quantitative) and is subject to a quality control arrangement with the University of Michigan. (This assay is not available on a commercial basis, however.) These substances are among the most toxic substances known to man. The creation of a chemical facility to determine the presence of these compounds in South Africa should have high national priority.

A list of laboratories conducting EDC activity tests is given in Table 14.

Table 14: Laboratories conducting EDC activity tests

Laboratory	Type of test
University of Pretoria	E-screen, yeast screen, MVLN reporter analysis, Uterotrophic, Herschberger test Catfish VTG analysis – end 2003
CSIR (Environmentek)	Yeast screen, AIMS test, daphnia test, anti-androgen test, urease enzyme tests, mammalian cell cloning efficiency, Ames Salmonella mutagenicity tests, frog embryo teratogenicity test
University of Stellenbosch	Vitellogenin, ELISA, thyroid function *Xenopus* assays

Laboratory	Type of test
North-West University	Dioxin, dibenzofuran, PCB bioassay, MVLN cell line – 2003 MDA cell line anti-androgenic activity – 2004, EDC Xenopus testing
Department of Water Affairs and Forestry	*Daphnia pulex, Poecilia reticulate, Oreochromis mossambicus*
Department of Trade and Industry	Yeast screen to be developed

3.5 Selection of sites for limited surveillance study

Stakeholders selected four sites for a limited surveillance study during a workshop at Morgenson in May 2003. Although several sites had been proposed by the main researchers, it was difficult to determine the location of the so-called 'hotspots' in the country, but the following guidelines were considered sensible in selecting the sites:

- Sites where industrial pollution is known to have been detected
- Sites where considerable agricultural activity takes place
- Sites where activities such as malaria control take place
- Sites where effects on humans and wildlife have been noted
- Sites where research is already being done and from which additional data may therefore be available.

The following sites were proposed and evaluated:

- Western Cape area near Grabow/Elgin:
  Considerable agricultural activity takes place in this area. Large volumes of agricultural pesticides are utilised for insect, weed and fungal control. A vast number of different pesticides are used on a variety of crops. The University of Stellenbosch is involved in a research programme in this area in collaboration with a German group.
- Jozini dam in northern KwaZulu-Natal:
  Apart from the variety of agricultural crops that are planted in this area, it is also regularly sprayed with DDT and Deltamethrin for malaria control. Little industrial activity takes place in this area. The Agricultural Research Council (ARC) is conducting a research programme in the area.
- Rietvlei dam near Pretoria:
  Apart from agricultural activity, the inflow to this dam is also exposed to industrial and social pollution. The University of Pretoria and the CSIR (Environmentek) are involved in a research programme in this area.
- Jukskei river near Johannesburg:
  This river flows through areas that are severely polluted by industrial activity, social pollution and mining activity.
- Bloemfontein:
  The geographical position of this area is vastly different from the rest of the sites suggested. Agricultural activity ranges from maize and wheat crops to cattle and sheep husbandry. Industrial activity also takes place. The Central University of Technology, in collaboration with the University of Stellenbosch, is planning a study on the dams around Bloemfontein, where it is reported that EDC effects have been noticed in wildlife.
- Limpopo valley, Venda, Limpopo province:

The climate of this area ranges between subtropical and tropical, and large subtropical fruit crops are grown in the area. Pesticide control of these crops differs substantially from control methods used on deciduous fruit crops in the Western Cape and seed crops in the Free State. There is also evidence of industrial activity and malaria control. The University of Venda is already involved in research in this area.

- Eastern Cape (East London area):
  This site was suggested because it is situated in an industrial area with heavy mineral contamination. The University of Fort Hare is conducting research on the harbours and rivers of the area.

It should be understood that this study is only a starting point and that the sites suggested will by no means reflect the situation in the entire country.

After evaluation of the sites, the workshop participants selected the following sites:

- Makhatini flats
- Vaal River barrage
- Hartbeespoort dam
- Rietvlei dam.

3.6 Conducting the pilot study

A small pilot study was conducted with the aim of determining whether the systems were in place to embark on a larger survey. This study, as well as a ring test, was conducted under the auspices of the SABS and ARC. (For details of the protocol, refer to Annexure A: Report 5A.)

3.6.1 The ring test

This test was conducted to determine the recoveries of the extractions used in the bioassays, because concern was expressed that some of the EDC components might be lost during the extraction. At the same time, the 'cocktail' used for this exercise was distributed to the laboratories doing the chemical analysis for quality assurance purposes. The results of this study are given in Tables 15 and 16. Recoveries done at level 0.025 µg/ℓ gave very erratic results in all laboratories. This may be because the uncertainties at this level are too great. The conclusion may thus be drawn that these extraction procedures are only suitable for levels of 0.01 µg/ℓ and above.

Table 15: Results of recovery test done at the level of 1 µg/ℓ

	% Recovery at 1 µg/ℓ level			
	US-EH	**CSIR (Environmentek)**		**CSIR/SABS**
	C18	**XAD**	**Disc**	**C18**
p-Nonylphenol	90	60	65	80
Atrazine	70	57	65	80
PCB 52 (tetra)	50	56	50	100
α-Endosulfan	80	43	15	114
DDE	100	63	55	100
Dieldrin	120	63	95	100

Note:

Code	**Institution**	**Responsible person/institution**
US-EH	University of Stellenbosch	Ettienne Hurter
CSIR Environmentek.	CSIR Environmentek	Laetitia Slabbert
CSIR/SABS	CSIR Biochemtek	Now SABS

Table 16: Results of recovery test done at the level of 0.1 µg/ℓ

	%Recovery at 0.1 µg/ℓ level			
	US-EH	**CSIR (Environmentek)**		**CSIR/SABS**
	C18	**XAD**	**Disc**	**C18**
p-Nonylphenol	70	95	43	100
Atrazine	60	49	53	100
PCB 52 (tetra)	20	64	50	110
α-Endosulfan	60	28	15	100
DDE	50	40	58	130
Dieldrin	30	58	80	100

3.6.2 Analysis of field samples from the pilot study

The SABS took water and sediment samples at two sites (Jukskei river and Rietvlei dam), and sub-samples were sent to the laboratories involved in the chemical and bioassay study. EDC activity was determined, and chemical analysis was carried out. In the study plan, it was planned to do this sampling only in December 2003, but the study had to be repeated in March 2004 because one of the biochemical laboratories was not ready to conduct the analysis in December 2003. No positive results were obtained in this study.

The ARC conducted a small study on the Makhatini flats. The ARC had been involved in another research project in this area during 2000 and 2001. This area is undergoing tremendous agricultural development, particularly with regard to cotton and winter wheat production. The water and sediment samples taken in a once-off exercise in November 2002 did not yield positive results. The full range of pesticides given in the proposed list was tested for. The LODs for pesticides were 30 µg/kg for sediment and 0.5–1 µg/ℓ for water. (Refer to Annexure A: Report 5A for the sampling protocol and details of the study.) Although the results were negative in this instance, various insecticides were found in the water samples of the 2000–2001 study, ranging from 0.34 to 1.7 µg/ℓ, and in sediment, ranging from 0.02 to 180 µg/kg (Sereda and Meinardt, 2003).

The aim of the project was to detect commonly used agricultural insecticides as well as DDT and metabolites. The project did not focus on the compounds in the EDC project protocol. There are indications that EDC compounds may be present, but that their concentrations are below the limits of detection in the methodology used in the EDC project. It is imperative that analytical procedures be selected that will conform to the specifications for analysing EDC compounds at the necessary low levels.

The results of an earlier study conducted in the area indicated the presence of various insecticides in the water sources in the study area at concentrations ranging from 0.02 to 180 µg/kg for sediment and 0.34 and 1.7 µg/ℓ for water (Sereda and Meinhardt, 2003). These analyses were conducted by the Pesticide Analytical Laboratory at the ARC Plant Protection Research Institute (PPRI). Residue confirmations were conducted using the standard two-column method. In both the matrices analysed, the concentration levels were predominantly at the lower end of the concentration range, with most residues found being present at below 5 µg/kg for sediment and 0.5 µg/ℓ for water.

The results of this study prompted the decision to analyse the five samples collected for EDC analysis for DDT and the metabolites DDE and DDD using the same method as used in the described trials. The results of these analyses are given in Table 17.

Table 17: Residue levels of DDT and metabolites detected in samples analysed (µg/ℓ)

Sampling site	Matrix	DDE-o,p	DDE-p,p	DDD-o,p	DDD-p,p	DDT-o,p	DDT-p,p
Jozini dam	Water	ND	0.0015	ND	0.0042 I	ND	ND
Minor dam below rice fields	Water	ND	0.0040	0.0028	0.0064		0.0156 I
Balemhlanga swamp	Sediment	ND	ND	ND	ND	ND	ND
Minor dam below rice fields	Sediment	ND	0.329 ug/kg	0.053 ug/Kg	0.687 ug/kg	0.105 ug/kg	0.130 ug/kg
Mamfene drain	Sediment	ND	0.488 ug/kg	ND	0.154 ug/kg	0.080 ug/kg	ND

Note:
ND Not detected

These results indicate that at least DDT and some metabolites are present in the samples collected from the area, thus indicating a high pollution potential not only for DDT and its metabolites, but also for other potential compounds. It should be borne in mind that the concentrations determined were low. In most cases, the residues detected were below the LOD for GC-MS confirmation as set by the Test House Laboratory of the South African Bureau of Standards (SABS). This indicates that false negatives may be obtained when high LODs (30 µg/kg for sediments and 0.5–1 µg/ℓ for water) are used for EDC residue analysis.

It should be borne in mind that the limits of detection set are those for GC-MS confirmation. It may thus be necessary to consider older confirmation techniques, such as the two-column method, for this type of analysis, at least until such time as better sensitivity is possible using GC-MS technology.

3.6.2.1 Heavy metals

The results for the heavy metal analysis are given in Table 18.

Table 18: Heavy metals detected in samples analysed

Sampling site	Matrix	Heavy metal Concentration (µg/ℓ – water; µg/kg sediment)			
		Zinc	Cadmium	Mercury	Calcium
Jozini dam	Water	874.17	1.0801	0.9124	14944.77
Minor dam below rice fields	Water	548.51	1.0712	0.7117	1459.32
Balemhlanga swamp	Sediment	8.9795	0.1402	0.3318	14.97
Minor dam below rice fields	Sediment	7.1363	0.0624	0.4215	23.9
Mamfene drain	Sediment	8.945	0.1421	<0.001	14.36

The results of the analysis indicated the presence of zinc, cadmium and mercury in all the samples analysed, indicating the potential widespread contamination of the study area with these compounds. Calcium was also found in all the samples. The presence of these elements in the samples may not be surprising, but the potential for synergism between these elements and organic compounds to cause typical endocrine-disrupting effects is a matter of concern. Further monitoring work is required in the area to determine the presence of organic and heavy metals. These data should then be used as part of a health risks assessment. Furthermore, the source of the pollutants needs to be identified and suitable interventions developed to limit or eliminate these compounds from the water sources in the study area.

3.7 Conducting the main surveillance study

3.7.1 Study plan

The main study commenced in 2004 and was completed in April 2005. Stakeholders selected the following four sites during the workshop held at Morgenson in May 2003:

- Makhatini flats
- Vaal River barrage
- Hartbeespoort dam
- Rietvlei dam.

The four selected areas were divided into ten sampling areas, and samples of water and sediment were taken at each sampling site during each of the four seasons of the year. Samples were distributed to the different laboratories for activity testing and chemical analysis. All the samples were analysed for pesticides, hormones, industrial chemicals and heavy metals. Because of funding restrictions, the samples were combined for certain tests, such as hormone analysis (two samples were combined to form five sub-samples for analysis at AMPATH). For activity testing, five samples were combined to make two sub-samples. These were sent to the CSIR (Environmentek) and the University of Pretoria for *in vitro* testing and to the University of Stellenbosch for *in vivo* testing. Both water and sediment were analysed for heavy metals, industrial chemicals and pesticides. Hormone analysis was

conducted only on the water samples. (Refer to Annexure A: Report 5B for details). All the samples were taken, stored and transported in glass containers.

4. RESULTS OF LIMITED SURVEILLANCE STUDY

4.1 Activity tests

Activity was detected at all sites during the four sampling events. This may be due to the presence of estrone in the water. The following results were obtained by the CSIR (Environmentek).

4.1.1 Environmental samples

The results obtained with the yeast screen (hER and hAR) on four sets of environmental samples taken during the period November 2003 to November 2004 are shown in Tables 19 and Table 20.

Table 19: Estrogenic activity of environmental samples after 10 days' incubation

Water sample	Sampling date	Significant estrogenic activity[1] (Y/N)	Estrogenic potency (ng EEQ/ℓ)
MF	Nov 2003	Y (6)	0.67
	March 2004	Y (3)	<LOQ (0.18)
	Jul 2004	Y (4)	0.20
	Nov 2004	Y (8)	2.00
VB	Nov 2003	Y (6)	1.24
	March 2004	Y (4)	0.71
	Jul 2004	Y (5)	0.55
	Nov 2004	Y (7)	1.06
HD	Nov 2003	Y (5)	0.41
	March 2004	Y (3)	0.20
	Jul 2004	Y (5)	0.65
	Nov 2004	Y (5)	0.32
RD	Nov 2003	Y (5)	1.91
	March 2004	Y (5)	0.83
	Jul 2004	Y (6)	1.97
	Nov 2004	Y (8)	2.04

Note:
[1] Results in brackets: Number of absorbance values above the LOD
EEQ Estradiol equivalents
LOD Limit of detection (0.03-0.09 ng EEQ/ℓ)
LOQ Limit of quantification (0.05-0.19 ng EEQ/ℓ)
Q Value below LOQ given as estimate (in brackets)

Table 20: Androgenic activity of environmental samples after 2 to 3 days' incubation

Water sample	Sampling date	Significant androgenic activity (Y/N)	Androgenic potency (ng TEQ/ℓ)
MF	Nov 2003	N[1] (0)	<LOD
	March 2004	Y[1] (3)	15.05
	Jul 2004	N[1] (0)	<LOD
	Nov 2004	N[2] (4)	<LOD

Water sample	Sampling date	Significant androgenic activity (Y/N)	Androgenic potency (ng TEQ/ℓ)
VB	Nov 2003	N[1] (0)	<LOD
	March 2004	N[1] (1)	<LOQ (11.15)
	Jul 2004	N[1] (0)	<LOD
	Nov 2004	N[2] (2)	<LOD
HD	Nov 2003	N[1] (0)	<LOD
	March 2004	Y[1] (3)	22.02
	Jul 2004	N[1] (0)	<LOD
	Nov 2004	N[2] (5)	<LOD
RD	Nov 2003	N[1] (0)	<LOD
	March 2004	N[1] (1)	<LOQ (11.15)
	Jul 2004	N[1] (0)	<LOD
	Nov 2004	N[2] (5)	<LOD

Note:
[1] Results in brackets: Number of absorbance values above the LOD
[2] Significant cytotoxicity: Results in brackets indicate the number of absorbance values below the negative control-3SD
TEQ Testosterone equivalents
LOD Limit of detection (2.41-14.58 ng TEQ/ℓ)
LOQ Limit of quantification (3.72-29.53 ng TEQ/ℓ)
Values below LOQ given as estimates (in brackets)

The results obtained with the alkali-labile phosphate test on the samples collected during 2004 (two sets of samples) are shown in Table 21.

The results indicate significant induction in alkali-labile phosphate in the case of the VB (Vaal barrage) and HD (Hartbeespoort dam) water samples. The results also indicate that similar results were generally obtained when using protein and fish weight to express alkali-labile phosphate.

Table 21: Effect of water samples on vitellogenin in fish expressed in terms of alkali-labile phosphate

Water sample	Sampling date	pH	Dissolved Oxygen (mg/ℓ)	Exposure time (d)	% Lethality[2]	% Increase (expressed as)	
						mg phosphate/mg protein	mg phosphate/mg fish tissue
MF	July 2004	7.8	9.6	10	11	50	44
	Nov. 2004	7.0	6.7	14	13	-12	-11
VB	July 2004	7.8	10.1	10	7	**204**	**200**
	Nov. 2004	6.8	7.0	14	11	**20**	**18**
HD	July 2004	7.4	9.8	8[1]	>50	**204**	**220**
	Nov. 2004	7.4	7.0	14	21	**73**	**110**
RD	July 2004	7.3	10.2	10	15	48	41
	Nov. 2004	6.8	6.6	14	24	-9	**-24**

Note:
[1] Shorter exposure due to large lethality
[2] Control lethality usually <10%
- Reduction instead of increase
q Results in bold indicate significant increase/reduction (P=0.05)

Gel electrophoresis did not indicate vitellogenin bands in the case of environmental waters. This does not mean that estrogen activity was not present, but that the samples were too dilute to detect vitellogenin bands (or that the assay was not sufficiently sensitive).

4.1.2 Human whole blood cultures: cytotoxicity and inflammatory activity in water

4.1.2.1 Methods

4.1.2.1.1 Cytotoxicity: lLactate dehydrogenase activity (LDH)

The leaching of lactate dehydrogenase (LDH) from the erythrocytes is indicative of cell wall damage, and differential activity of LDH between samples therefore gives an indication of toxins affecting cells during an incubation period. Water samples (only two sets, as the other two gave unreliable results that could not be repeated) were tested for cytotoxic properties by incubation with whole blood cultures. (Refer to Annexure A: report 2A for details of the methodology.)

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

Water samples (three sets, as one set was contaminated and gave unreliable results that could not be repeated) 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. (Refer to Annexure A, report 2A for details of methodology.)

IL-6 was detected by a validated ELISA, described by Pool et al. (2000). All steps were performed at room temperature.

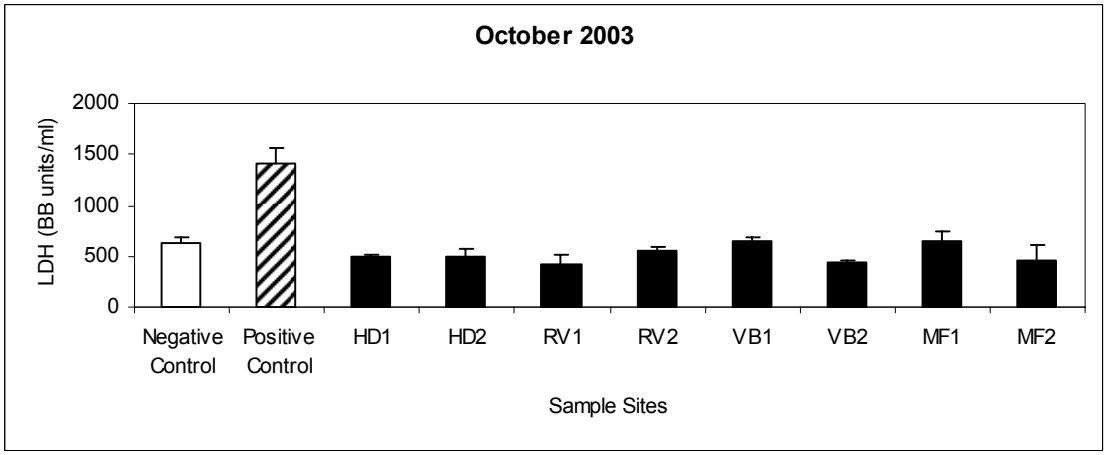
4.1.2.1.2.1 Statistics

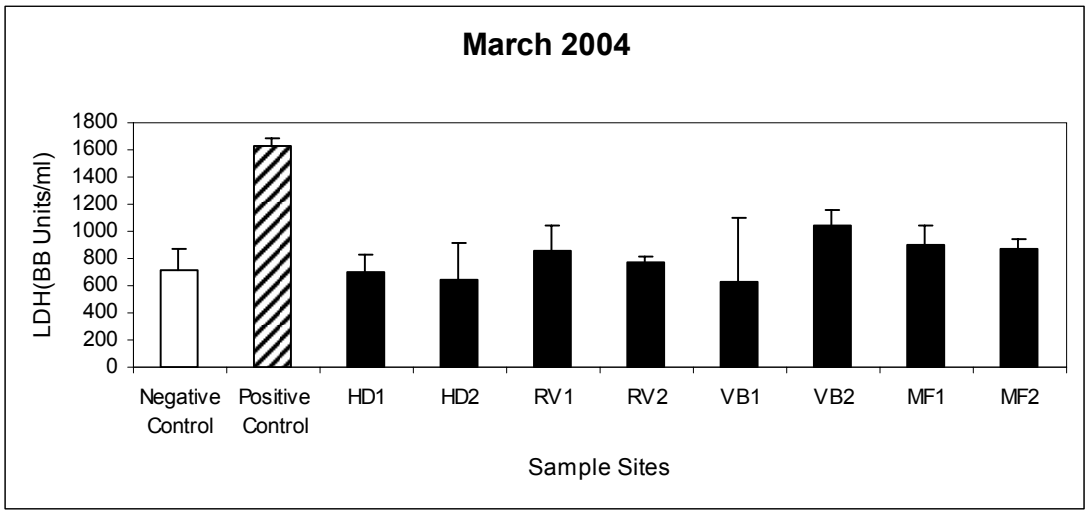
Analysis of variance (ANOVA) was used to test for significance between exposure groups and the negative water control. Significance was set at $P<0.05$.

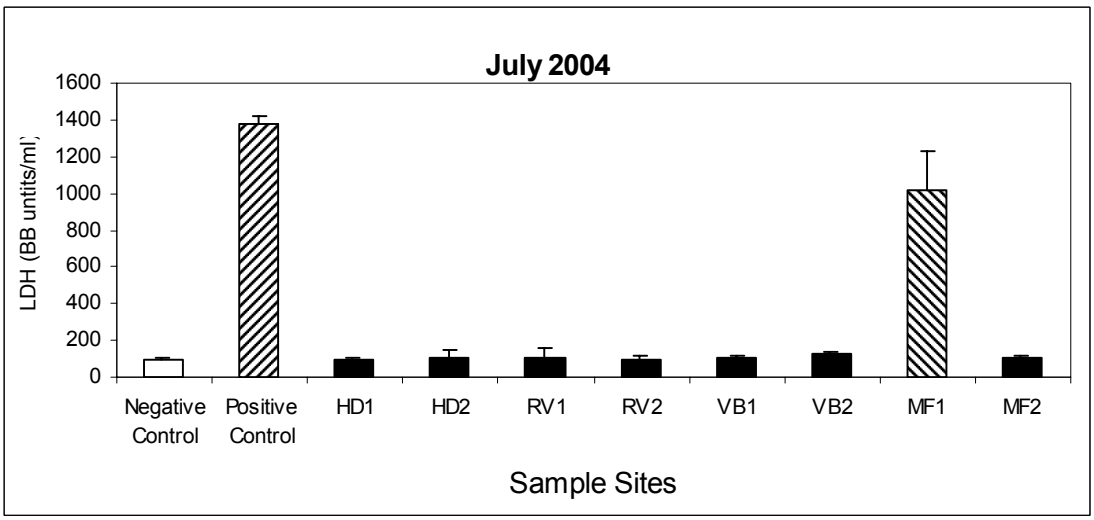
4.1.2.1.2.2 Results

4.1.2.1.2.2.1 Cytotoxicity: Lactate dehydrogenase activity (LDH)

The results are presented in the following graphs (Figure 5).







Note: LDH activity is measured in Berger-Broida (BB) units/mℓ.

Figure 5: LDH leakage from blood cells in culture after exposure to extracted water samples from three selected water sample collections

LDH leakage was seen in only one sample from the Makhatini flats (MF2, panel C, ANOVA, $P<0.05$), and extraction removed the possible toxic effects in all the other samples.

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

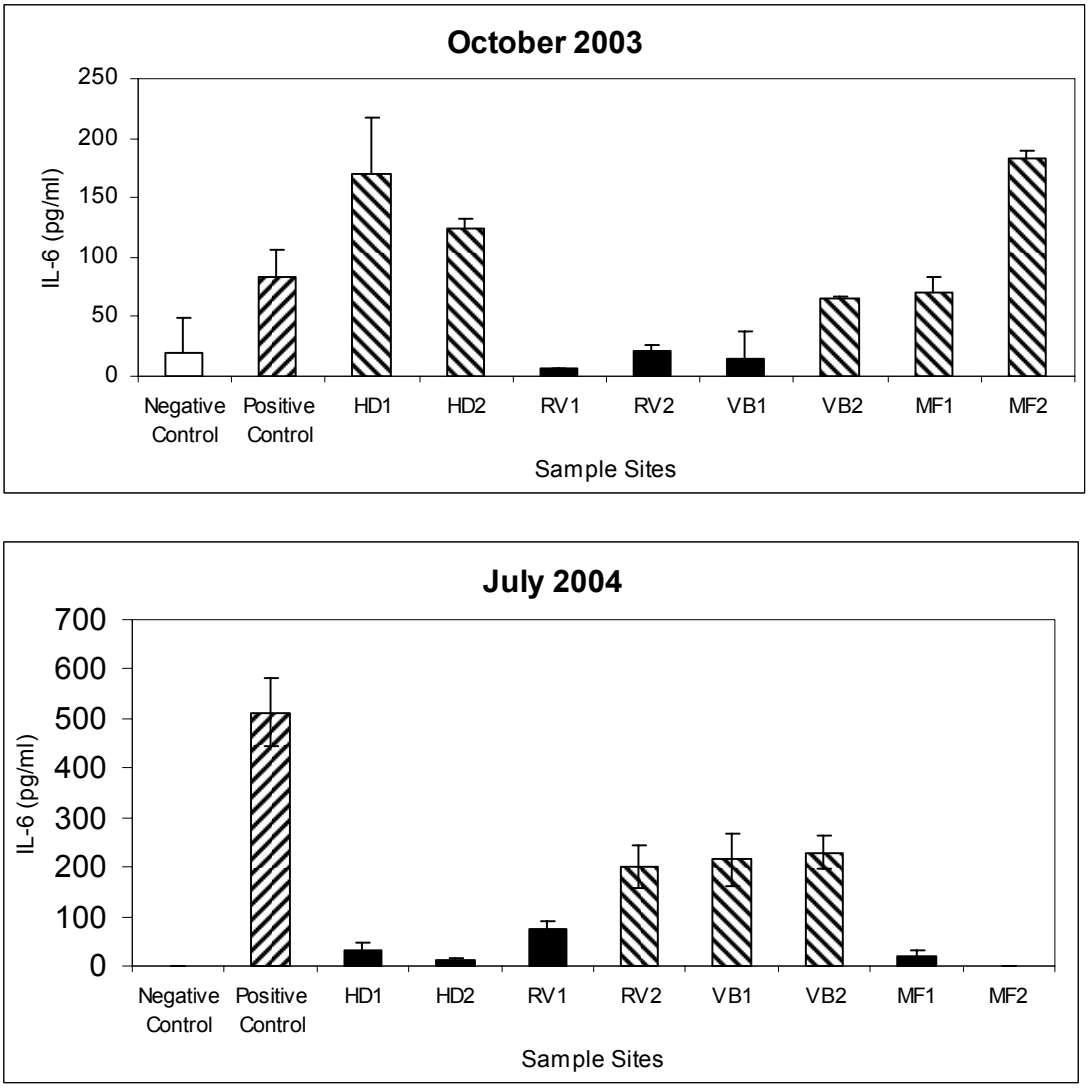


Figure 6: Inflammatory activity: Interleukin-6 (IL-6) response

Inter-site and seasonal variation in inflammatory activity measured in human whole blood cultures varied significantly ($P<0.05$) in raw (unextracted) water samples. Cross-hatched bars in Figure 6 indicate significantly increased activity (ANOVA, $P<0.05$) compared to the negative control.

4.1.2.1.2.3 Conclusion

The IL-6 response of the human whole blood cultures suggests that several of the sites were contaminated with pathogens or their breakdown products. Samples with IL-6 concentrations

between 100 and 200 pg/mℓ can be categorised as inflammatory and will pose a health risk to human users of the water (Pool et al., 2000; 2003). Cytoxic activity (LDH leakage from human blood cells) was mostly removed with C18 extraction, and *in vitro* bioassays should not have been affected by toxins in the samples, which would have created false negatives.

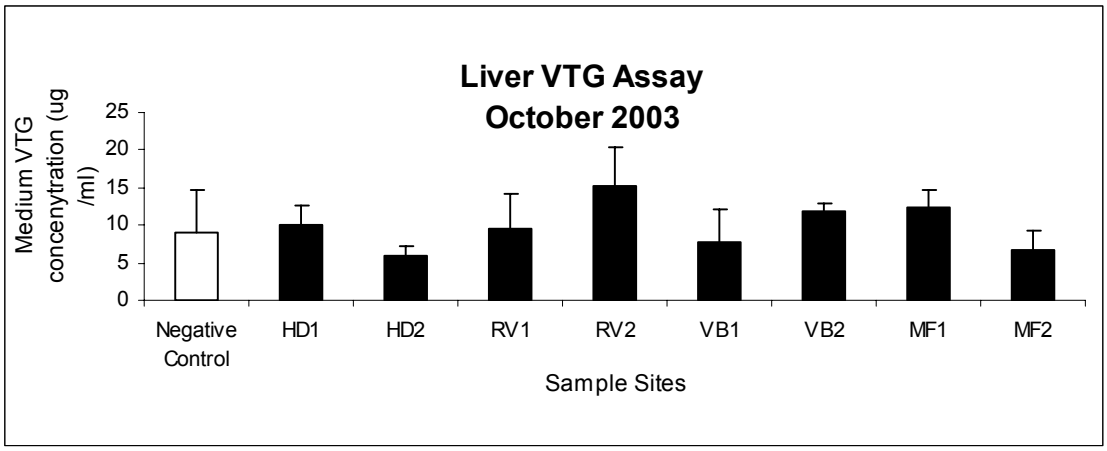
4.1.3 Frog liver VTG culture assay (estrogenicity)

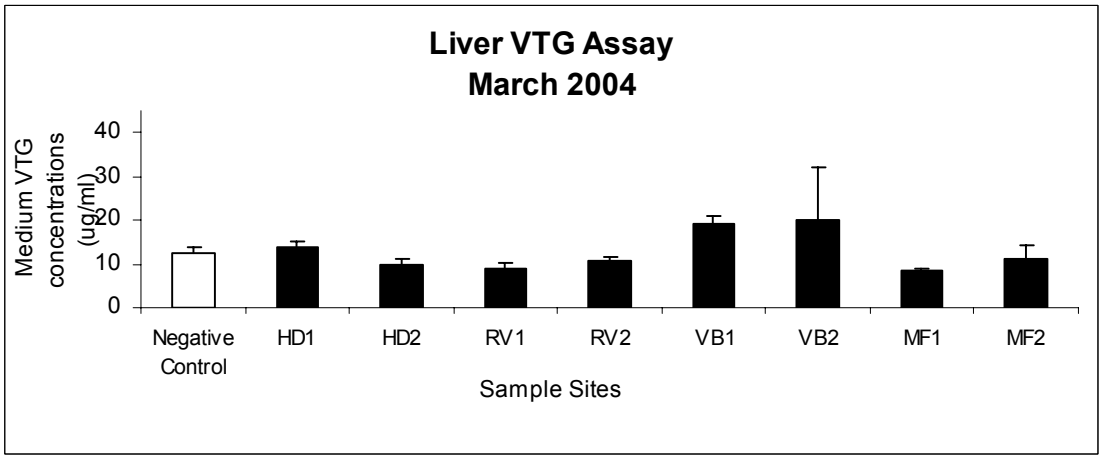
(Refer to Annexure A, report 2A for details of methodology)

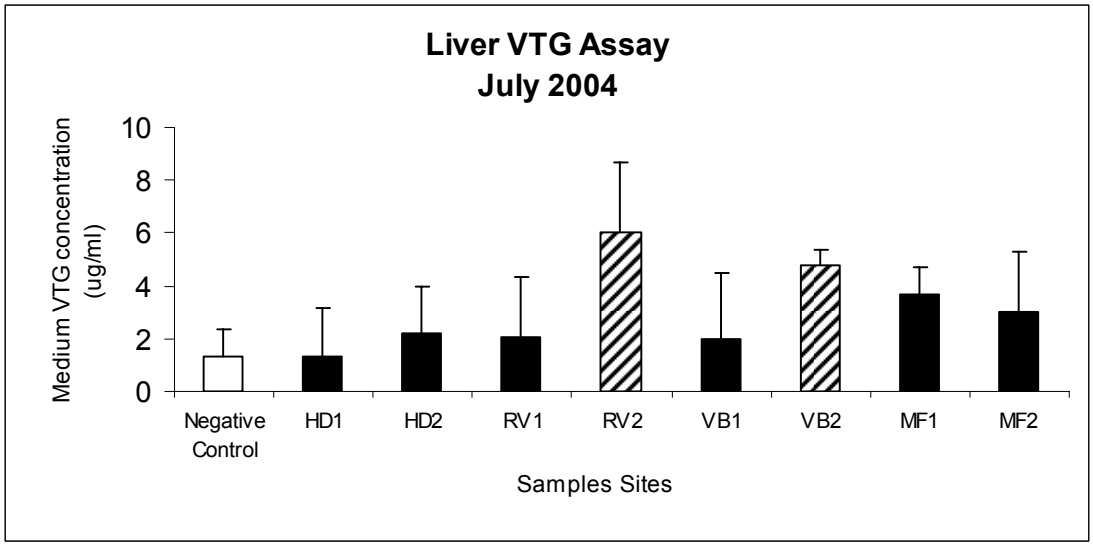
4.1.3.1 Results

The results are presented in the following graphs (Figure 7).

4.1.3.1.1 VTG concentration







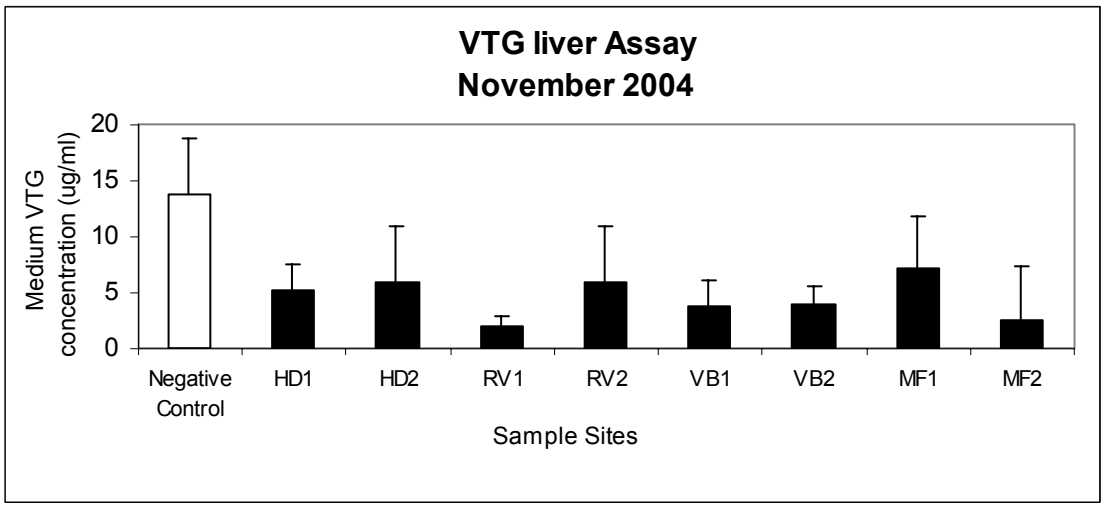


Figure 7: *Xenopus laevis* VTG liver assays: *in vitro X. laevis* liver cultures using C18 extracted water samples from collecting sites

Although the VTG production of the liver slices was varied, it was only in the July 2004 sample batch that estrogenic activity could be detected. Two samples (RV2 and VB1) resulted in increased VTG production in cultured liver slices. The high VTG production in the control sample was noteworthy.

4.1.3.2 Conclusion

The *Xenopus laevis* liver slice *in vitro* assay is a multi-cell tissue-culture assay and will give a result closer to *in vivo* than a single cell *in vitro* assay. It is clear that few of the samples tested were stimulating the liver slices to produce VTG, therefore reflecting estrogenic activity at this level in the collected water samples. The fact that the C18 extraction mostly removed cytotoxic activity from the water samples suggests that these results do not include many false negatives.

4.1.4 Zebrafish blood VTG assay

4.1.4.1 Methods

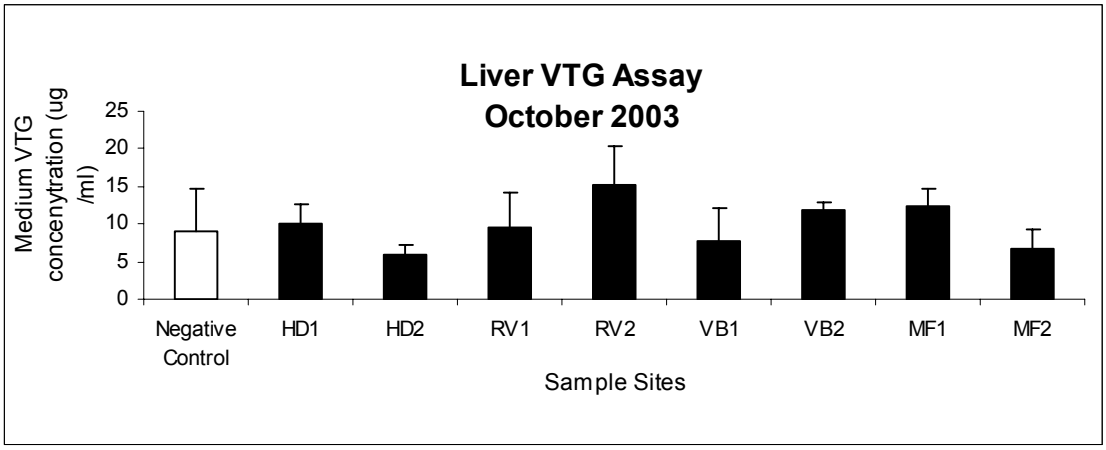
4.1.4.1.1 Zebrafish VTG ELISA

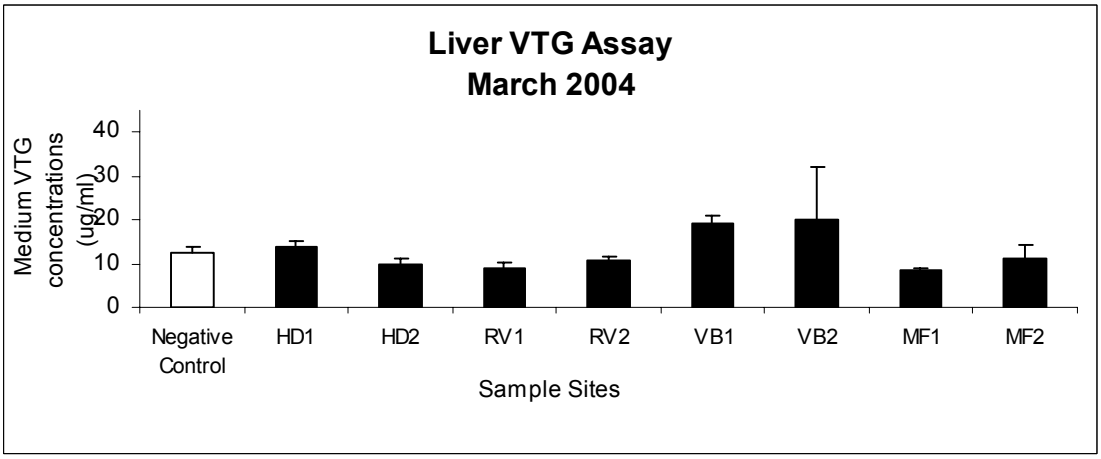
Adult male *Danio rerio* were obtained from a commercial supplier (Bromley's Fish and Pet Centres) and placed in laboratory water (reverse osmosis water containing 3.75 g/ℓ NaCl and 1.2 g/ℓ $NaHCO_3$). The fish were kept in aerated water, filtered through activated charcoal in a glass aquarium (15 ℓ) and subjected to a light:dark regime of 14:10 hours, with water temperature at 26°C (±1°C). The fish were acclimated to laboratory conditions for two days prior to an experiment. The zebrafish (15 fish per water sample) were then transferred to 3 ℓ glass aquaria, filled with 1.5 ℓ environmental waters received by courier. A negative control containing buffered laboratory water only and a positive control containing water spiked with 100 µg/ℓ 17β-estradiol were included with each run. After seven days of exposure, blood was collected from the tail vein into an anti-coagulate buffer solution (0.01% phenylmethylsulfenyl fluoride [PMSF] in saline). The blood samples were then centrifuged (at 8000 rpm) for five minutes, and the serum diluted with buffer for a total protein concentration of ~30 µg/mℓ (OD_{280} = 0.03). Fish specimens were transferred into Bouins' fixative for possible further histological studies.

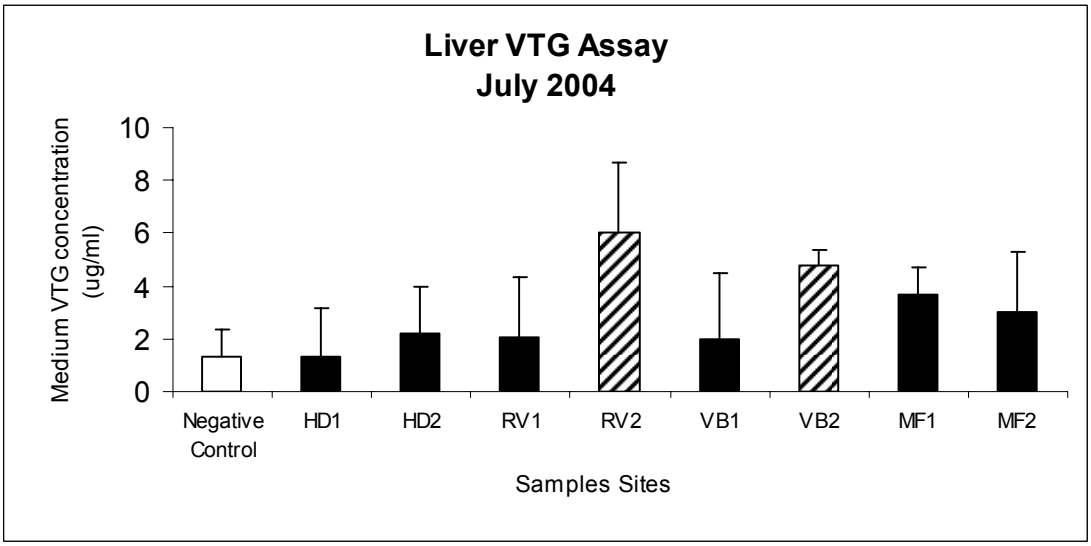
Refer to Annexure A, report 2A for details of the methodology for the zebrafish VTG ELISA.

4.1.4.2 Results

The results are presented in the following graphs (Figure 8).


Liver VTG Assay
October 2003
Medium VTG concenytration (ug /ml)
25
20
15
10
5
0
Negative Control
HD1
HD2
RV1
RV2
VB1
VB2
MF1
MF2
Sample Sites


Liver VTG Assay
March 2004
Medium VTG concentrations (ug/ml)
40
30
20
10
0
Negative Control
HD1
HD2
RV1
RV2
VB1
VB2
MF1
MF2
Sample Sites


Liver VTG Assay
July 2004
Medium VTG concentration (ug/ml)
10
8
6
4
2
0
Negative Control
HD1
HD2
RV1
RV2
VB1
VB2
MF1
MF2
Samples Sites

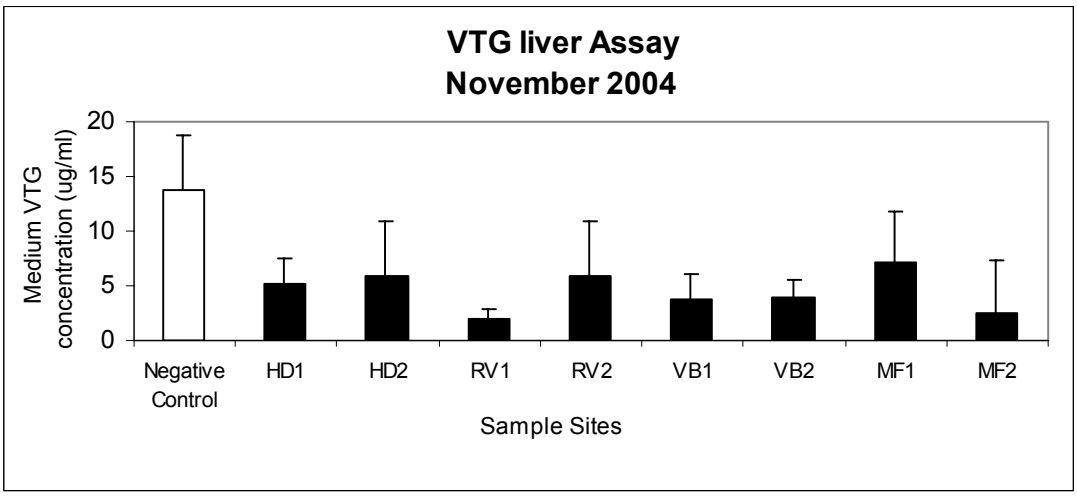


Figure 8: Zebrafish *in vivo* VTG assay

The results of this assay were varied, and selected water samples showed significant ($P<0.05$ versus the control) increased mean plasma VTG levels in the exposure groups. However, although care was taken to include only adult males in the exposure groups, females were included. The samples in all exposure groups comprised ten individuals. In one of the exposure groups (MF1, November 2004), all the fish died.

4.1.4.3 Conclusions

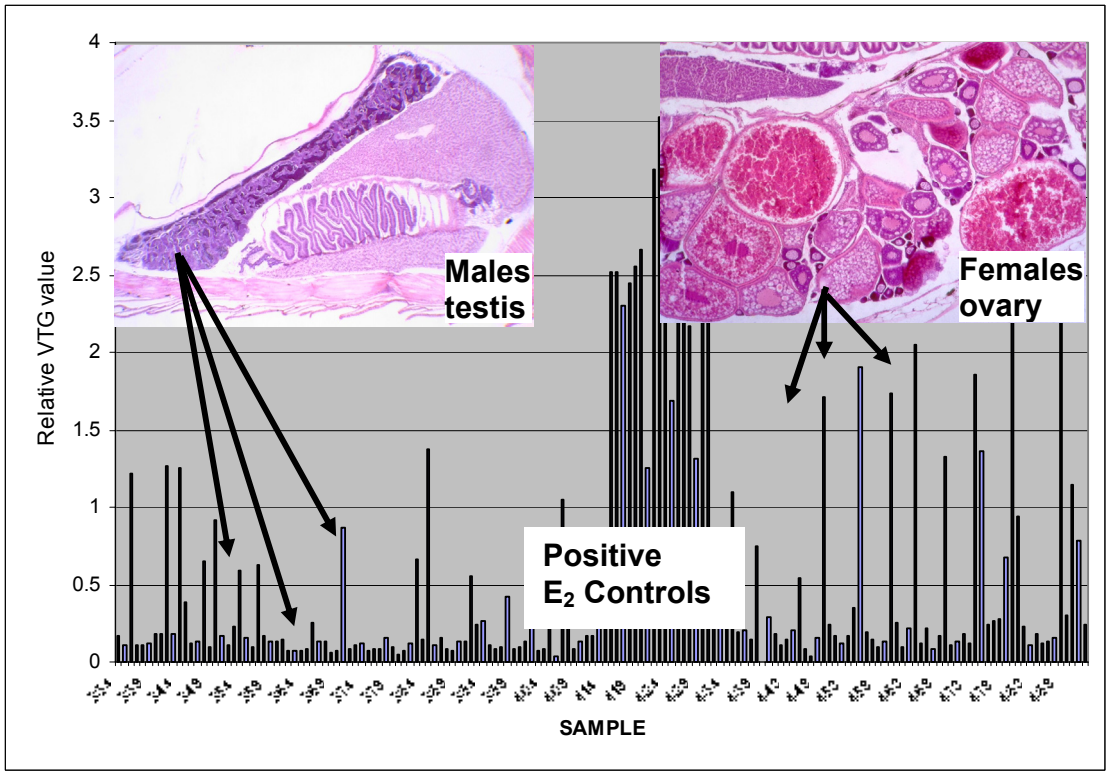


Figure 9: Individual variation in plasma VTG measured in exposed zebrafish

Histological evidence revealed that several of the increased VTG values were attributed to the fact that these individuals were adult females that had been misidentified as adult males. In the positive control group (dosed with estradiol), all individuals showed increased plasma VTG levels (Figure 9).

In most cases, the zebrafish survived exposure to the undiluted water samples. Increased mean plasma VTG levels were shown in several groups, but little pattern emerged, either seasonally or between sample sites. Large inter-group variation in individual plasma VTG levels was selectively investigated, and histological studies revealed that most of these could be attributed to the inclusion of females in the exposure samples. The fact that little sexual dimorphism is present to differentiate between males and females makes the use of adult zebrafish problematic. The implication is that all individuals with high plasma VTG levels need to be subjected to histological investigation to determine their sex, which is labour-intensive and expensive. This would also mean that much larger exposure groups are needed in order to accommodate the possible exclusion of females from the data set. It is therefore clear that the possibility of using juvenile zebrafish in *in vivo* exposure screening to eliminate the problem of misidentification of adult sex needs further investigation. Alternatively, models using other fish species in which clear sexual dimorphism exists need to be explored.

4.2 Chemical analysis

4.2.1 Hormone analysis

The hormone analysis was conducted by AMPATH. A summary of the average hormone content found at the different sites during the four sampling events is given in Table 22.

Table 22: Average hormone content found at four sites during four sampling events

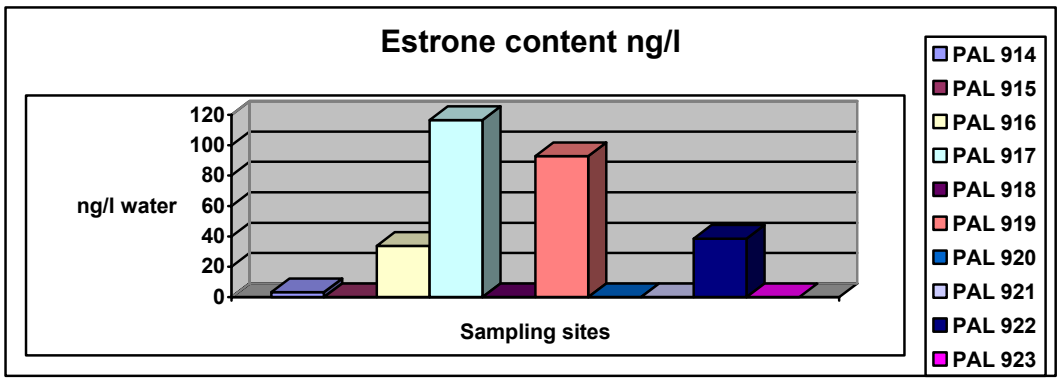
			Hormones ng/ℓ		
Event	**Date**	**Site**	**Estrone**	**Estriol**	**Ethinylestradiol**
1	November 2003	Makhatini flats	56.98	3.73	ND
		Vaal river barrage	73.2	6.73	ND
		Hartbeespoort dam	59.58	ND	ND
		Rietvlei dam	40.88	ND	ND
2	March 2004	Makhatini flats	19.1	61.1*	76.00*
		Vaal river barrage	25.78	13.52*	64.2*
		Hartbeespoort dam	22.05	12.80*	9.76*
		Rietvlei dam	24.68	48.8*	31.5*
3	July 2004	Makhatini flats	15.93	ND	ND
		Vaal river barrage	65.00	ND	ND
		Hartbeespoort dam	41.45	ND	ND
		Rietvlei dam	87.00	ND	ND
4	November 2004	Makhatini flats	267.03	ND	ND
		Vaal river barrage	43.83	ND	ND
		Hartbeespoort dam	44.06	ND	ND

			Hormones ng/ℓ		
Event	**Date**	**Site**	**Estrone**	**Estriol**	**Ethinylestradiol**
		Rietvlei dam	151.50	ND	ND

Note:
* Values recalculated and corrected

No 17β-estradiol was found in any of the samples. Ethinylestradiol was found only during the second sampling event (March 2004), when it was detected at all four sites (Range ND–380 ng/ℓ). It was significant that whenever ethinylestradiol was detected, estrone and/or estriol were detected in the same sample. Sampling for event 2 took place after the first rains had fallen after a very dry period. In contrast to the other events, the water taken during this event was murky and contained considerable particulate matter as well as plant material. Estrone was detected at all the sites and during all the sampling events. The estrone content varied quite extensively between sampling points as well as events. Figure 10 shows the variation in estrone at one site during the first sampling event. Figure 11 shows the average estrone content at the four different sites during the four sampling events. The estrone values found were significantly higher than values reported in water in Europe. In sewage treatment works in Canada, values of <10–250 ng/ℓ were reported, in Italy 28–100 ng/ℓ and in Germany 27–700 ng/ℓ. (Refer to Annexure A, Report 4 for details.)

Estriol was found only during sampling event 1 (at the Makhatini flats and the Vaal river barrage) and event 2 (at all sites).



Note:
PAL 914 Balemthlanga natural outflow
PAL 915 Balemthlanga natural outflow
PAL916 Irrigation canal
PAL 917 Irrigation dam
PAL 918 2nd bridge
PAL 919 Balemthlanga (Block 6A)
PAL 920 Balemthlanga (Block 6A)
PAL 921 Balemthlanga (Block 6A)
PAL 922 Mapaya gardens (canal)
PAL 923 Pongolapoort dam

Figure 10: Estrone content of Makhatini flats during first sampling event (November 2003)

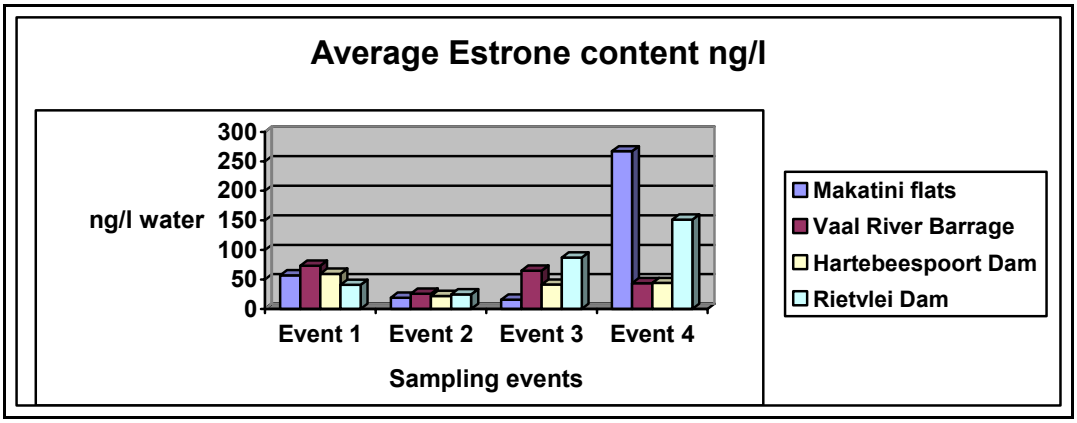


Figure 11: Average estrone content at four sites during four sampling events

No hormone analysis was done on sediment samples. The raw data produced may be obtained from AMPATH.

4.2.2 Pesticides

Pesticide analysis was conducted by the ARC (Plant Protection Research Institute) and SABS. EDCs may enter the water systems and the environment through agricultural and/or industrial activity. The majority of these compounds are toxic. Persistent organic pollutants (POPs) are also EDCs, and the toxic heavy metals (lead, mercury, arsenic and cadmium) also have endocrine disrupting properties. In addition, most of the compounds currently on the list of persistent toxic substances (PTS) are also EDCs. The United Nations Environmental Programme (UNEP) project on the Regionally Based Assessment of Persistent Toxic Substances in Sub-Saharan Africa has shown that there are very few data available on the occurrence of these compounds in Africa. This is also true for South Africa. Currently, no monitoring data are available for these compounds in the South African water system.

The pesticides targeted for analysis were:

- DDT-op and DDT-pp
- DDE-op and DDE-pp
- DDD-op and DDD-pp
- Dieldrin
- Aldrin
- Endrin
- Heptachlor, alpha-heptachlorepox, beta-heptachlorepox
- Lindane (gamma-BHC)
- Atrazine
- Simazine
- Terbuthylazine
- Methoxychlor
- Alpha-endosulfan, beta-endosulfan, endosulfan SO_4.

Only trace amounts of certain pesticides were detected in the water samples. The average amounts of detected pesticides in the different sites taken during the four sampling events are given in Table 23. Atrazine and terbutylazine are often used in grain-producing areas. Endosulfan is commonly used as an insecticide on various agricultural crops, and Lindane is often used in locust control.

The raw data may be obtained from the ARC.

Table 23: Average pesticide content at different sites during four sampling events

			Pesticides µg/ℓ						
Event	**Date**	**Site**	**Atrazine**	**Terbutyl-azine**	**DDT (total)**	**Lindane**	**α-Endo-sulfan**	**β-Endo-sulfan**	**Endosulfan SO_4**
1	**Nov. 2003**	**MF**	ND	ND	0.02	0.01	0.55	0.12	0.01
		VRB	0.78	0.48	ND	0.02	0.31	0.10	ND
		HD	3.76	ND	ND	0.03	0.18	0.04	ND
		RD	ND	ND	ND	0.01	0.55	0.17	ND
2	**March 2004**	**MF**	ND	ND	0.01	ND	ND	ND	ND
		VRB	ND	0.33	ND	0.01	ND	ND	ND
		HD	ND	ND	ND	ND	0.06	0.02	ND
		RD	ND	ND	ND	ND	0.16	0.05	ND
3	**July 2004**	**MF**	ND	ND	ND	ND	ND	ND	ND
		VRB	ND	ND	ND	ND	ND	ND	ND
		HD	ND	ND	ND	ND	ND	ND	ND
		RD	ND	ND	ND	ND	ND	ND	ND
4	**Nov. 2004**	**MF**	ND	ND	ND	ND	ND	ND	ND
		VRB	ND	ND	ND	ND	ND	ND	ND
		HD	ND	ND	ND	ND	ND	ND	ND
		RD	ND	ND	ND	ND	0.22	0.06	ND

Note:
MF Makhatini flats
VRB Vaal river barrage
HD Hartbeespoort dam
RD Rietvlei dam

4.2.3 Industrial chemicals

The analysis was done at the CSIR (Biochemtek) and SABS. Many industrial compounds that are not regarded as toxic have EDC properties, and many of these are utilised in household and personal care products. Some are used as plasticisers. The PCBs and dioxins are also listed as POPs. They have long half-life times and are persistent in the environment.

The following industrial chemicals with EDC properties were selected for analysis:

Alkylphenols: Octylphenol (OP) and *p*-nonylphenol (PNP)

Phthalates: Dibutylphthalate (DBP)
Di(2-ethyl hexyl) phthalate (DEHP)
PCBs: Eight key representative congeners of environmentally found PCBs

The average industrial compound content in water is given in Table 24, and the average industrial chemical content in sediment is given in Table 25. Most of these compounds are lipophyllic and will therefore only be present in water when attached to solid particles.

The environmental risk limits (ERLs) for DBP and DEHP in water are 10 and 0.19 µg/ℓ respectively, for sediment with 10% organic material and 0.7 and 1 mg/kg wet weight respectively. These levels are derived using data on (eco)toxicology and environmental chemistry. Survival, growth and reproduction were used as endpoints (Van Wezel et al., 2000).

Table 24: The average industrial compound content in water (µg/ℓ)

			Industrial chemicals in water (µg/ℓ)				
Event	**Date**	**Site**	**PCB total**	**PNP**	**ONP**	**DBP**	**DEHP**
1	**Nov. 2003**	MF	0.03	0.12	<0.08	<3.9	<l.6
		VRB	ND	ND	ND	<3.9	ND
		HD	ND	<0.14	ND	<3.9	ND
		RD	ND	0.65	<0.08	ND	<1.6
2	**March 2004**	MF	ND	<0.14	ND	ND	ND
		VRB	ND	ND	ND	<3.9	ND
		HD	ND	ND	ND	ND	ND
		RD	ND	<0.14	<0.08	<3.9	ND
3	**July 2004**	MF	ND	ND	ND	ND	ND
		VRB	ND	ND	ND	ND	ND
		HD	ND	ND	ND	1.97	ND
		RD	ND	0.41	ND	ND	ND
4	**Nov. 2004**	MF	ND	ND	ND	2.0	ND
		VRB	ND	ND	ND	ND	ND
		HD	ND	ND	ND	2.0	ND
		RD	ND	ND	ND	ND	ND

Note:
MF Makhatini flats
VRB Vaal river barrage
HD Hartbeespoort bam
RD Rietvlei dam
PCB Polychlorinated biphenyls
PNP Para-nonylphenol
ONP Ortho-nonylphenol
DBP Dibutylphthalate (DBP)
DEHP Di(2-ethyl hexyl) phthalate

Table 25: Average industrial chemical content in sediment (µg/kg)

			SEDIMENT				
Event	**Date**	**Site**	**PCB total**	**PNP**	**ONP**	**DBP**	**DEHP**
1	**Nov. 2003**	MF	ND	16.37	ND	ND	102.61
		VRB	ND	7.14	ND	ND	17.50
		HD	0.4	4.04	ND	5.27	32.75
		RD	ND	33.81	3.87	9.04	34,50
2	**March 2004**	MF	ND	1.20	ND	6.25	67.00
		VRB	ND	2.69	ND	7.70	19.02
		HD	0.4	3.94	ND	10.83	41.00
		RD	ND	35.88	2.40	7.16	22.68
3	**July 2004**	MF	ND	ND	ND	ND	ND
		VRB	ND	ND	ND	ND	ND
		HD	ND	ND	ND	ND	ND
		RD	ND	0.97	0.07	ND	ND
4	**November 2004**	MF	ND	7.40	ND	38.0	388.9
		VRB	ND	80.20	ND	38.25	80.40
		HD	0.70	53.25	ND	36.33	76.20
		RD	ND	97.30	1.89	47.00	83.57

Note:

MF	Makhatini flats
VRB	Vaal river barrage
HD	Hartbeespoort bam
RD	Rietvlei dam
PCB	Polychlorinated biphenyls
PNP	Para-nonylphenol
ONP	Ortho-nonylphenol
DBP	Dibutylphthalate (DBP)
DEHP	Di(2-ethyl hexyl) phthalate

The raw data may be obtained from the SABS and CSIR.

4.2.4 Heavy metals and minerals

Heavy metal analysis was conducted by the ARC (Institute for Groundwater and Climate). Heavy metals such as lead (Pb), mercury (Hg) and cadmium (Cd) have EDC properties. Minerals such as calcium (Ca) and zinc (Zn) have a synergistic effect with other EDCs and either enhance or diminish the effect of other EDCs. The average mineral content in water samples taken at the four sites is given in Table 26, and the mineral content in sediment samples is given in Table 27.

Table 26: Average mineral content in water

			Mineral content in water µg/ℓ						
Event	**Date**	**Site**	**As**	**Pb**	**Cd**	**Hg**	**Ca**	**Zn**	**Se**
1	**Nov. 2003**	**MF**	10.36	4.37	1.84	2.53	38.68	112.14	33.28
		VRB	4.10	3.85	2.55	1.69	57.98	543.44	11.08
		HD	5.86	0.63	3.04	2.12	24.01	26.70	20.90
		RD	2.61	1.18	2.11	1.74	33.91	32.81	6.63
2	**March 2004**	**MF**	62.84	7.48	1.83	11.80	42.29	152.29	70.90
		VRB	31.31	7.09	2.81	8.90	51.65	2255.93	37.92
		HD	6.34	11.23	0.77	1.74	19.66	143.75	5.42
		RD	6.94	16.74	0.72	1.01	31.54	218.45	3.15
3	**July 2004**	**MF**	18.30	9.19	1.87	6.79	31.94	501.58	25.87
		VRB	12.87	4.42	2.03	6.16	62.31	178.96	24.81
		HD	11.88	5.18	2.03	6.57	31.76	120.46	28.82
		RD	12.36	5.82	3.72	7.02	33.43	421.94	22.75
4	**Nov. 2004**	**MF**	3.80	2.45	0.19	0.79	43.74	9.82	11.46
		VRB	0.26	2.18	0.30	1.11	49.19	9.08	4.24
		HD	0.41	22.16	ND	0.92	28.85	84.02	9.87
		RD	0.32	1.22	ND	0.58	41.27	1.43	2.62

Note:
MF Makhatini flats
VRB Vaal river barrage
HD Hartbeespoort dam
RD Rietvlei dam

Table 27: Mineral content in sediment samples taken at the four sites

			Mineral content in sediment µg/kg						
Event	**Date**	**Site**	**As**	**Pb**	**Cd**	**Hg**	**Ca**	**Zn**	**Se**
1	**Nov. 2003**	**MF**	1.38	6.08	ND	0.12	10545.0	50.34	0.09
		VRB	0.82	7.96	1.19	0.05	3302.8	112.39	1.50
		HD	6.65	11.62	0.50	0.03	17453.3	131.52	1.79
		RD	5.47	22.55	0.58	0.29	7675.7	83.89	0.64
2	**March 2004**	**MF**	27.18	3.74	2.07	9.51	12422.5	268.15	28.30
		VRB	30.21	10.72	3.93	9.16	3359.0	1869.3	22.52
		HD	3.22	5.66	0.57	0.98	36148.6	134.92	2.32
		RD	6.02	16.91	1.13	1.05	15573.8	118.61	3.13
3	**July 2004**	**MF**	1.29	5.72	0.19	0.98	11322	54.28	0.26
		VRB	0.75	7.84	0.24	0.56	2474.1	98.02	0.11
		HD	3.06	25.14	0.38	0.99	29454	107.66	0.39
		RD	3.27	19.41	0.36	1.10	4763.3	70.99	0.56
4	**Nov. 2004**	**MF**	20.41	2.15	<0.01	0.11	14309	69.70	72.93

			Mineral content in sediment µg/kg						
Event	**Date**	**Site**	**As**	**Pb**	**Cd**	**Hg**	**Ca**	**Zn**	**Se**
		VRB	13.10	3.80	<0.01	0.14	3263.1	102.51	41.47
		HD	14.35	5.69	<0.01	0.07	24903	87.00	34.16
		RD	22.15	10.55	<0.01	0.13	10975	78.43	57.43

Note:
MF Mhakatini flats
VRB Vaal river barrage
HD Hartbeespoort dam
RD Rietvlei dam

The South African guidelines indicate that the concentration of cadmium in fresh water sources should be below 0.005 mg/ℓ. The South African guideline for zinc in water is 3 mg/ℓ (Fatoki et al., 2004; Fatoki and Awofolu, 2003*)*. Selenium (Se) is presented as a pro-oxidant when in inorganic form. When this value is high, it may have the effect of lowering copper (Cu) status, which may have adverse effects on health and on reproductive and immune systems. It may also affect the thyroid function by limiting iodine activation. The target water quality range (TWQR) for lead is 1–10 µg/ℓ, but levels of 5–10 µg/ℓ are of concern in the interests of human health. Inorganic mercury has limited adverse effects on humans, but mercury in organic form has severe effects. Animals such as fish and poultry may ingest the inorganic form, which may then be converted to organic form and ingested by humans through this pathway of exposure (Meyer, 2005).

4.3 Fate of hormones during water purification

WRC project K5/1555: An investigation into the occurrence of steroidal hormones (estrogens) in sewage effluent using biological/biochemical and chemical techniques.
WRC K5/1469/2 project: EDCs (sewage effluents) (Note: K5/1469 replaced K5/1402 in the second year).

4.3.1 Sampling

Three sampling runs were carried out on treated sewage effluent before chlorination (Treatment Plant H – end of August 2004; Treatment Plant D – beginning of October 2004, Treatment Plant K – beginning of December 2004). All three plants are situated on the East Rand in the vicinity of Kempton Park:

- Plant H treats domestic and industrial discharges. This effluent is expected to contain natural and synthetic estrogens as well as estrogen-mimicking chemicals.
- Plant D receives mainly domestic effluent. Most of the estrogens in the effluent are expected to be natural.
- Plant K receives domestic effluent, which is expected to contain natural and synthetic estrogens.

All the facilities use an activated sludge process, which involves the oxidation of carbon, nitrogen and phosphorous compounds in the waste. The incoming waste is mixed with sludge, which is kept in suspension in a reactor fitted with aerators. Twenty-four hour composite samples were taken on five consecutive days, including weekends. Information on the sewage treatment facilities is provided in Table 28.

Table 28: Information on the sewage treatment facilities

Sewage treatment facility	Sample	Sampling date	Inflow (Mℓ/day)[2]	pH	COD (mg/ℓ)	NH_3 (mg/ℓ)
H	H1	20/08/2004	36.95	7.4	52	25.4
	H2	21/08/2004	30.74	not available		
	H3	22/08/2004[1]	31.96			
	H4	24/08/2004	36.02	7.5	51	22.8
	H5	25/08/2004	38.87	7.4	44	20.8
D	D1	07/10/2004	6.77	5.8	41	8.3
	D2	08/10/2004	5.23	5.5	31	4.7
	D3	09/10/2004	5.83	not available		
	D4	10/10/2004	6.52 (4)			
	D5	11/10/2004	6.21 (4)	5.5	28	4.5
K	K1	10/12/2004	0.53 (5)	6.4	14	5.2
	K2	11/12/2004	0.47	not available		
	K3	12/12/2004	0.58			
	K4	13/12/2004	0.53 (2)			
	K5	14/12/2004	0.62 (60)			

Note:
[1] Sample of 23/08/2004 broken; sampling therefore continued for six days
[2] Rainfall (mℓ/day) in brackets

4.3.2 Tests

The following assays/analyses were carried out:

- CSIR laboratory: hER screen
- University of Stellenbosch: vitellogenin on male fish exposed to the samples
- AMPATH: chemical analyses

Sample preparation and test protocols are described in previous reports (refer to volume 1, WRC Report KV143/05).

4.3.2.1 Yeast screen

The yeast test was run for three, seven and ten days. As in the case of the environmental samples, the best absorbance difference was obtained after ten days' exposure. Absorbance was also measured at various wavelength configurations (540 and 600 nm; 540 and 620 nm; 550 and 600 nm; and 550 and 620 nm). The lowest EC_{50}s (17-ß estradiol and effluent extracts) were obtained for the 550 and 600 nm configuration (in other words, the wavelengths that had been used all along). The second best results were obtained for the 550 and 620 nm wavelengths. Results are reported for the 550 and 600 nm combination, using the ten-day absorbance values. EC_{50}s were determined using non-linear regression analysis (Graphpad Prism).

4.3.2.2 Vitellogenin determination

Zebrafish were used for the *in vivo* exposure studies. Ten to fifteen fish were placed in a 1.5 ℓ sample. Fish were exposed for eight days at 27ºC (±2ºC) to a light:dark regime of 14:10 hours. A negative control of buffered laboratory RO (reverse osmosis) water was included with each sample set. Exposure water was partially replaced after four days. Dead fish were removed daily and fixed in Bouins fixative or 4% buffered formalin. Following exposure, blood was collected individually from the tail vein into an anti-coagulate buffer solution (0.01% PMSF in PBS) on ice and prepared as previously described. An in-house zebrafish VTG ELISA method (Pool and Van Wyk [S.a.]) was used to determine plasma VTG concentrations in the fish. The protocol is detailed elsewhere. After bleeding, the individual fish were fixed, as described, for the histological examination to sex the fish. The method is described in previous reports (refer to volume 1, WRC Report KV143/05). Females were removed from the samples for statistical analyses. However, all females were grouped together and included in the positive control graph and analyses. One-way ANOVA and Holm-Sidak multiple comparison tests were used for detecting differences between the fish in the negative control group and sewage samples. The level of significance was set at P=0.05. If the data set violated assumptions for parametric statistics, the Mann-Whitney ANOVA of Ranks and Dunn's multiple comparison test were used. SigmaStat 3 (SPSS) was used for all the statistical analyses.

4.3.2.3 Chemical analyses

The samples were analysed for α- and ß-estradiol, estriol, 17-α ethynylestradiol and estrone.

4.3.3 Results

4.3.3.1 Yeast screen

EC_{50}s obtained during the study are shown in Table 29.

Table 29: 17-ß estradiol EC_{50} values obtained during the study

Sample	EC_{50} (ng/ℓ)
H1-H4	16.4
H5	13.1
D1-D4	17.6
D5	16.4
K1-K4	9.99
K5	8.41

All the treated sewage samples exhibited estrogenic activity (Table 30). The curves were similar to those for 17-ß estradiol.

Table 30: Estrogenic activity detected with the yeast test after ten days' incubation

Sample	Significant estrogenic activity[1] (Y/N)	EC_{50} (ng/ℓ estradiol equivalents)	RIE[2]	RP
H1	Y	0.169	0.96	98
H2	Y	0.164	1.03	100
H3	Y	0.165	0.98	99
H4	Y	0.163	0.99	101
H5	Y	0.159	0.95	102
D1	Y	0.164	0.89	107
D2	Y	0.158	0.95	111
D3	Y	0.165	0.92	107
D4	Y	0.174	0.99	101
D5	Y	0.153	0.92	107
K1	Y	0.092	0.93	108
K2	Y	0.090	0.98	111
K3	Y	0.091	0.98	110
K4	Y	0.093	1.00	107
K5	Y	0.082	0.91	102

Note:
[1] ≥3 points above mean blank+3SDs
[2] Induction efficiency of 17ß-estradiol = 1.0
RIE relative induction efficiency (test abs/estradiol abs)
RP relative potency (estradiol EC_{50}/test EC_{50})

4.3.3.2 Vitellogenin

Male fish exposed to the sewage effluent samples did not show vitellogenin (VTG) induction. The histological examination indicated that the fish that showed increased VTG levels were all females. (Because of the problem of sexing adult fish, female fish were included in the exposure groups by error.)

4.3.3.3 Chemical analyses

Table 31 shows that the only hormone detected in the sewage effluent samples was estrone. During the first three days of sampling at Sewage Treatment Facility H, estrone levels were high. In most instances, estrone was absent from the samples collected from Sewage Treatment Facility D. This effluent contained mostly natural hormones.

Table 31: Hormone content of sewage effluent samples

Sample	α-Estradiol	ß-Estradiol	Estriol	17-α Ethynyl-estradiol	Estrone
	ng/ℓ				
H1	ND	ND	ND	ND	230.1
H2	ND	ND	ND	ND	225.5
H3	ND	ND	ND	ND	120.1
H4	ND	ND	ND	ND	15.7
H5	ND	ND	ND	ND	70.1
D1	ND	ND	ND	ND	1.2
D2	ND	ND	ND	ND	0
D3	ND	ND	ND	ND	0
D4	ND	ND	ND	ND	0
D5	ND	ND	ND	ND	0

Sample	α-Estradiol	ß-Estradiol	Estriol	17-α Ethynyl-estradiol	Estrone
	ng/ℓ				
K1	ND	ND	ND	ND	16.7
K2	ND	ND	ND	ND	9.4
K3	ND	ND	ND	ND	3.5
K4	ND	ND	ND	ND	5.5
K5	ND	ND	ND	ND	16.8

Note:
ND Not detected
Shadowed rows indicate weekends

5. CONCLUSIONS

1. EDC activity was detected at all sites, and chemical analysis confirmed the presence of EDCs. In related studies, EDC effects were noticed in animals (fish, birds and eland). There are indications that EDCs may also have had an effect on humans. The study was done at selected sites where EDC contamination was suspected, and the results may therefore not reflect the general situation in the country.
2. Some of the bioassays were not sensitive enough to be used in the study of EDCs in environmental waters. More research in this field is needed.
3. South Africa has the capacity to conduct studies such as these but lacks the capacity to conduct studies where large numbers of samples need to be analysed on a routine basis. Capacity and human resources were built up at organisations. Unfortunately, some of the organisations have subsequently closed their facilities, and most of the postgraduates in the field have left for better job opportunities in other areas.
4. There is an urgent need for a comprehensive surveillance programme in the country. Without the data produced in such a study, the risk to the human population and to wildlife cannot be determined.
5. The water resources (rivers and dams) are very dynamic, and 'spot' sampling may give confusing results. Ideally, water resources should be monitored 24 hours a day.
6. The results obtained during the pilot study and the main study were credible and satisfactory.

6. RECOMMENDATIONS

1. The proposed list of chemicals should be updated at least annually in order to remain abreast of with world trends.
2. The list containing the capacity and capability of laboratories conducting chemical analyses and bioassays should be updated annually.
3. Ongoing research is needed to develop bioassays as well as to conduct chemical analysis at ultra trace level.
4. Quality assurance in analytical facilities needs to be addressed, especially at tertiary education institutions. Human resource capacity has to be built in all participating laboratories in order to handle large numbers of samples.
5. A facility for the analysis of dioxins and furans needs to be established as a matter of urgency.
6. Water laboratories and state laboratories should become involved in the study.
7. A new approach to capacity building is needed. Postgraduate students need jobs at reasonable salaries to keep them in this field of research.
8. In order to assist decision-makers, a human health risk assessment model for EDC exposure should be developed, as the classic risk assessment model is inappropriate.
9. An ongoing surveillance programme should be put in place to ensure the safety of water resources for human consumption.
10. Water sampling should be done on a continuous basis (24 hours a day), and methods and sampling devices need to be developed to achieve this.

7. REFERENCES

BERGSTROM R, ADAMI HO, MOHNER M, ZATONSKI W, STROM H, EKBOM A, TRETLI S, TEPPO L, AKRE O and HAKULINEN T (1996) Increase in testicular cancer incidence in six European countries: A birth cohort phenomenon. *J. Natl. Cancer Inst.* **88** (11) 727-733.

COLBORN T, VOM SAAL FS and SOTO AM (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **101** (5) 378-384.

COMPRENDO CREDO (2004) Cluster workshop on Ecological Relevance of Chemically-induced Endocrine disruption in Wildlife, University of Exeter, United Kingdom, 5-7 July, 2004.

DWAF [S.a.] *South African Water Quality Guidelines, Domestic Use,* vol. 1, 2nd edition. Pretoria: Department of Water Affairs and Forestry.

FATOKI OS and AWOFOLU OR (2003) Levels of Cd, Hg, and Zn in some surface waters from the Eastern Cape province. *Water SA* **29** (4) 375-381.

FATOKI OS, AWOFOLU OR and GENTHE B (2004) Cadmium in the Umtata River and associated health impact of on the rural communities who are primary users of water from the river. *Water SA* **30** (4) 507-513.

GAIDO K, LEONARD L, LOVELL S, GOULD J, BABAI D, PORTIER C and MCDONNELL D. (1997) Evaluation of chemicals with endocrine modulating activity in a Yeast-based steroid hormone receptor gene transcription assay. *Toxicology and Applied Pharmacology* **143** 205-212.

GERHARD I and RUNNEBAUM B (1992) Environmental pollutants and fertility disorders. Heavy metals and minerals. *Geburtshilfe Frauenheilkd* **52** (7) 383-396.

GRAY LE, OSTBY J and COOPER R (1999) The estrogenic and antiandrogenic pesticide methoxychlor altersthe reproductive tract and behaviour without affecting pituiry size or LH and prolactin secretion in male rats. *Toxicology and Industrial Health* **15** 223-237

GUILLETTE LJ, GROSS TS, GROSS DA, ROONEY AA and PERCIVAL HF (1995) Gonadal steriodogenesis *in vitro* from juvenile alligators obtained from contaminated or control lakes. *Environ. Health Perspect.* **103** (Supplement 4) 31-36.

JOHNSTONE, R, COURT G, FESSER A, BRADLEY D, OLIFANT L and MACNEIL J (1996) Long-term trends and sources of organochlorine contamination in Canadian tundra peregrine falcons, *Falco peregrinus tundrius. Environ. Polut.* **93** 109-120.

KIME DE (1999) A strategy for assessing the effects of xenobiotics on fish reproduction. *Sci. Total Environ.* **225** (1-2) 3-11.

MØLLER H and WEIDNER IS (1999) Epidemiology of cryptochidism and hypospadias. *Epidemiology,* **10** (4) 364-369.

MØLLER, H (1998) Trends in sex-ratio, testicular cancer and male reproductive hazards: Are they connected? *APMIS* **106** 232 – 238.

POOL EJ, VAN WYK, JH and LESLIE AJ (2000) Inflammatory activity as an indicator of water quality: the use of human whole blood cultures. *Journal of Immunoassay,* **21** 387-399.

ROUTLEDGE EJ and SUMPTER JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem.* **15** 241-248.

SEREDA BL and MEINARDT HR (2003) Insecticide contamination of the water environment in malaria endemic areas of KwaZulu-Natal (South Africa). WRC Report No. 1119/1/03 ISBN No. 1-86845-928-4.

TILSON HA (1998) The concern for developmental neurotoxicology: Is it justified and what is being done about it? *Environ. Health Perspect.* **103** 147-151.

TOPPARI J, LARSEN JC, CHRISTIANSE P, GIWERMAN A, GRANDJEAN P, GUILLETTE LJJR, JEGAU B, JENSEN TK, JOUANNET P, KEIDING TV, LEFFERS H, MCLACHLAN JA, MEYER O, MŰLLER J, RAJPERE-DE-MEYTS E, SCHEIKE T, SHARPE R, SUMPTER J and SKAKKEBAEK NE (1996) Male reproductive health and environmental xenoestrogens. *Male Reproductive Health and Environmental Xenoestrogens* **104** (Supplement 4).

TURNER KJ and SHARPE RM (1997) Environmental oestrogens – present understanding. *Rev. Reprod.* **2** (2) 69-73.

VAN WEZEL, AP, VAN VLAADINGEN P, POSTHUMUS R, CROMMENTUIJN GH and SIJM DTHM (2000) Environmental risk limits for two phthalates, with special emphasis on endocrine disruptive properties. *Ecotoxicology and Environmental Safety* **46** (3) 305-321.

VOS, JG, DYBING E, GREIM H, LADEVOGED O, LAMBRE C, TARAZONA JV, BRANDT I and VETHAAK AD (2000) Health effects of endocrine disrupting-chemicals on wildlife with special reference to the European situation, *Crit. Revs. Toxicol.* **30** (1) 71-133.

WHO (2002) *Global Assessment of the State of the Science of Endocrine Disrupters.* WHO/PCS/EDC/02.2.

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