THE ESTROGENICITY OF SEWAGE EFFLUENT ENTERING THE EERSTE-KUILS RIVER CATCHMENT SYSTEM

by

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EXECUTIVE SUMMARY

Background

Sewage effluents have a major impact on aquatic health. Studies done globally have shown that sewage effluents are major contributors of man-made chemical pollution in rivers.

Several man-made chemicals occurring in sewage effluents are known to interact with the development and functioning of endocrine systems in wildlife and humans (Colborn et al., 1993; Turner et al., 1997; Kime et al., 1999). These endocrine-disrupting contaminants (EDCs) or endocrine modulators act by either enhancing, or interfering, with the actions of natural hormones in the body. EDCs include natural hormones, synthetic hormones and hormone mimics. Treated sewage effluents are major sources of EDCs (Mitani et al., 2005; Gomes et al., 2003).

Due to the major shortage of water in the Western Cape, there is a great need to recycle water by either direct or indirect methods. The Western Cape is a winter rainfall area with very low/no rain in summer. The treated sewage effluent and natural surface water mixture found in the Kuils and Eerste Rivers is used directly for irrigation of agricultural areas. During summer most of the water in these rivers is treated sewage effluent. According to a recent report of the Cape Metropolitan Council (www.capetown.gov.co.za), Cape Town has 19 sewage treatment works (STWs), of which only 20% comply with the quality requirements specified in their permits issued by the Department of Water Affairs and Forestry (www.cndv.co.za). Problems encountered by most of these plants include inadequate sludge disposal, maintenance of the plants and bacteriological quality of the effluents (www.capetown.gov.co.za). To our knowledge the effluents from these sewage plants have never been tested for EDCs and the current study will attempt to generate data specifically for estrogenic EDCs in the sewage effluents from plants in the Western Cape.

The aim of this study was to analyse sewage effluents from plants in the Western Cape to assess their contribution to EDCs in river water.

Objectives

The objectives of this study were to:

1. Validate the locally produced UniVtg kit for the detection of tissue culture synthesised Xenopus laevis vitellogenin;
2. Assess the sewage extracts for cytotoxity;
3. Assess the estrogenicity of the sewage extracts using the vitellogenin induction by Xenopus liver cultures, the recombinant yeast screen and a commercially available ELISA for estrone, estradiol and estriol (E1&E2&E3 ELISA);
4. Compare the sensitivity, reproducibility and cost of the above three estrogen screening assays namely the Xenopus liver assay, the recombinant yeast screening assay, and the E1&E2&E3 ELISA.

Methods

Collection and extraction of water samples.

The control sample was collected near the origin of the Eerste River. The other samples assayed were collected at the outlets of the Stellenbosch, Bellville, Zandvliet and Macassar sewage treatment works (STWs). The treated sewage effluents from these sites are pumped into the Eerste-Kuils River catchment area. The water samples were collected just before the first rains and again after the first early winter rains. The water was collected in two liter glass bottles and transported immediately to the laboratory. The water samples were extracted immediately using C-18 column chromatography. The hydrophobic sample extracts were dried and stored at –20°C.

Xenopus Liver culture assay for estrogenicity

Extracts were assayed for estrogenicity using an *in vitro Xenopus laevis* liver culture assay. Livers were removed from animals, cut into 1 mm^3 pieces and placed into a culture plate. Extract reconstituted in tissue culture medium was added to the cultures

after which the cultures were incubated at 27°C for 6 days. At the end of the incubation period the culture medium was removed and assayed for the estrogen-induced protein vitellogenin using an ELISA. High vitellogenin in the medium is indicative of estrogens in the sample.

Whole blood culture assay for cytotoxicity

Human whole blood was cultured overnight in the presence of sample extract. The culture supernatants were collected and assayed for lactate dehydrogenase (LDH).

ELISA for steroid hormones

Estradiol, estriol, estrone and testosterone levels in the sewage extracts were analysed using commercially available ELISA kits according to the manufacturer's instruction manual.

Summary of major results

The cytotoxicity assay showed that none of the samples extracts prepared were toxic to cultured cells. When estrogenicity was monitored using the liver culture assay, only one sewage treatment work (STW) after the first rains came up positive. The ELISAs detected estradiol and estrone in all the sewage effluents. The sensitivity of the liver culture assay is 100 ng/l estradiol compared to 1 ng/l estradiol for the direct ELISA.

The cost of the ELISA (estradiol) is R270 per sample, while liver cultures (Vtg assay) cost R675 per sample. The time required to do the liver culture assay is 7 days, while it takes 2 days to complete 40 samples with the estradiol ELISA.

Conclusion

The ELISA for estradiol has a detection limit which is lower than the existing methods for screening estrogenicity. This method also requires less time to complete than existing methods. Twenty samples can be analysed at the same time using the ELISA, while no more than ten samples can be analyzed in a single run for the liver culture assay and yeast screen.

The concentrations of the major estrogenic compounds found in sewage effluent collected in the Western Cape, South Africa are similar to the levels found in Brittan (Desbrow et al., 1998), Italy (Baronti et al., 2000), Germany (Ternes et al., 1999a), Canada and Netherlands (Belfroid et al., 1999).

Recommendation

Due to the user friendliness of the ELISA methods for screening estrogens in environmental samples it is recommended that the method is included as an assay in the national toxicology programme. The inclusion of the testosterone ELISA as a screen for androgens present in environmental samples is also recommended as an assay in the national toxicology programme.

Proposal for archiving of data

The data generated are being stored on the UWC data storage system.

Knowledge dissemination

Articles

A manuscript has been submitted to Water SA under the working title: "Detection of selected steroids in treated sewage effluent using rapid ELISAs", April 2007.

Training

UWC: The laboratory and human resources potential have been developed for the training of students in environmental biotechnology in the Medical Bioscience Department, UWC.

City of Cape Town: Negotiations with the Water and Sanitation Department, City of Cape Town are currently being held to train City of Cape Town staff to analyse environmental water for estrogens. It is envisaged that the City of Cape Town will start a routine monitoring program for environmental estrogens in the near future. The analytical tools developed will enable the City Council to monitor the city's aquatic

environment and develop management plans to prevent pollution and adverse effects to human health.

Capacity Building

Degrees:

PhD:

1) Mr. C Swart: The development of biomarkers for endocrine disruptors.

BSc Honours:

1) Ms. Mavakla: Validation of the estriol ELISA for analyzing environmental samples
2) Ms. Geza: Validation of the estradiol ELISA for analyzing environmental samples
3) Ms. Wyngaard: Validation of the estrone ELISA for analyzing environmental samples
4) Ms Peter: The cytoxicity of environmental samples

BSc

16 third year B.Sc students did their group projects on ELISA detection of estrogens in environmental samples.

1) Ms. Hendricks
2) Ms. Neethling
3) Ms. Shoko
4) Ms. Bottoman
5) Ms. Erasmus
6) Mr. Kapofi
7) Ms. Mabope
8) Ms. Mesenya
9) Ms. Mashele
10) Ms. Matlala
11) Mr. Mavunda
12) Ms. Mtuze
13) Mr. Shabani-swedi
14) Ms. Dempers
15) Ms. Dick
16) Ms. Africa

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Steering committee members:

Mrs APM Moolman	WRC (Chairperson)
Mrs AEC Burger	EDC Program Manager
Prof MS Bornman	University of Pretoria
Prof CJ de Jager	University of Pretoria
Dr HH Du Preez	Rand Water
Mr SA Pieterse	Cape Metropolitan Council
Mrs JL Slabber	CSIR Environmentek
Ms C Van Ginkel	DWAF
Prof JH Van Wyk	University of Stellenbosch

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LIST OF ABBREVIATIONAS

DON	deoxynevalenol
E-KRCA	Eerste-Kuils River catchment area
ELISA	enzyme linked immunosorbent assay
HPLC	high pressured liquid chromatography
LDH	lactate dehydrogenase
MTT	mitochondrial dehydrogenases
NaCl	sodium chloride
NaOH	sodium hydroxide
PBS	phosphate buffered saline
RPMI 1640	Rosswell Park Memorial Institute medium 1640
SDS-PAGE	sodium dodecyl sulphate poly acrylamide gel electrophoresis
SPE	solid phase extraction
STW	sewage treatment work
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
Vtg	Vitellogenin
XL medium	*Xenopus laevis*
YES	yeast estrogen receptor assay

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1. BACKGROUND

Sewage effluents have a major impact on aquatic ecosystem health. This is compounded in areas such as the Western Cape (winter rainfall area) where rivers that would normally be dry in the summer are kept running during these months mainly due to sewage effluents being discharged into the river. Studies in Europe and Asia have indicated that sewage effluents are major contributors of man-made chemical (xenobiotic) pollution in rivers (Ma et al., 2004; Sikora et al., 2004).

Some of the chemicals found in effluents may cause acute or chronic toxicity in man and animals (Reemtsma et al., 2004). Tests for acute toxicity employ lethal dose assessment and involve an *in vivo* assay that directly measures the concentration of a particular toxic substance that is required to kill 50% of exposed animals. The concentration of a substance to kill 50% of exposed animals is often referred to as the LD50 of that substance. Due to ethical reasons LD50 assays are being replaced by *in vitro* cytotoxicity assays. Several cytotoxicity assay systems have been described in the literature. Currently two of these assays systems are used for routine cytotoxicity analysis. The cytotoxicity assays used routinely for toxicity assessments are: 1) the mitochondrial dehydrogenases assay (MTT) used as a biomarker of cellular activity and 2) the lactate dehydrogenase assay (LDH) which measures LDH leakage from the cell as a biomarker of cell breakage (Skaanild et al., 1989).

The MTT assay is based on the fact that all cells are metabolically active and require energy to repair cellular structures such as the cell membrane, the DNA, etc. In this assay a tretrazolium dye is added to the cells being tested. During metabolic processes in the mitochondrial the dye is converted to a coloured formazan which is then measured as an end-point for cellular metabolic activity. Substances that are toxic for the cell will decrease metabolic activity. For the MTT assay a decrease in the coloured formazan formation is thus used as an indicator of toxicity.

The LDH assay is based on the fact that the cell membrane acts as a barrier and the cell membrane prevents intracellular material from leaching into the extracellular area. When

cells are exposed to toxins normal cellular processes such as membrane repair cannot function properly and compounds normally found in the cytoplasm leaches into the extracellular medium due to breaks in the cell membrane. The LDH assay measures the leaching of LDH from cells into the culture medium. LDH is a cytoplasmic enzyme that can only be detected in the extracellular fluid upon leakage/breakage of the cell wall. Under normal conditions the LDH levels in the culture medium is low. However when cells are exposed to toxins there are breaks in the cell wall and cytoplasmic LDH leaches into the medium. The LDH level in the medium of cells exposed to toxins are thus high.
Some xenobiotics are known to interact with the development and functioning of endocrine systems in wildlife and humans (Coburn et al., 1993; Turner et al., 1997; Kime, 1999). These endocrine-disrupting contaminants (EDCs) or endocrine modulators act by either enhancing, or interfering, with the actions of natural hormones in the body. Many of these chemicals exhibit estrogen-like activity (Fry et al., 1981; Carey et al., 1995; Facemire et al., 1995; Palmer et al., 1995; Guilette et al., 1996, Tyler et al., 1998; Gronen et al., 1999; Kloas et al., 1999, Pickford et al., 1999). Environmental estrogens include natural estrogens, synthetic estrogens and estrogen mimics commonly produced by humans (sewage effluents), agricultural practices, such as fertilisers, fungicides, herbicides and pesticides, and industrial effluents, for example, paper, paint and plastic products (Ternes et al., 1999).

In a special U.S. EPA report and several other international workshop reports on EDCs, a tiered strategy for testing and monitoring such chemicals has been proposed (Tyler et al., 1998). This approach suggests starting-off with short-term *in vivo* and/or *in vitro* tests with endocrine end-points followed by more extensive *in vivo* testing with reproductive and developmental end-points. Third tier testing includes long-term whole life-cycle studies covering one or two generations.

Bioassays, mostly *in vitro* assays, have been developed for the first tier assessment of endocrine disruptor activity (e.g. estrogen-like activity). These assays may employ several end-points, including enzyme and gene induction, ligand binding, increased protein expression and cell proliferation and differentiation (Tyler et al., 1998;

Zacharewski, 1997; Gaido et al., 1997). Although *in vitro* assays offer several advantages, including cost effectiveness, rapid screening and reproducibility, these tests are mostly employed for screening purposes only. *In vitro* testing is regarded as complementary to, and not as substitute for *in vivo* testing on whole animals (Tyler et al., 1998).

One of the most widely used approaches for assessing estrogenic activity in non-mammalian oviparous species is the development of bioassays for detecting vitellogenin (Vtg) in the plasma of animals (Tyler et al., 1998; Tata et al., 1979; Sumpter et al., 1995). Vitellogenin is the yolk precursor synthesised by the liver in response to estrogenic stimulation in adult females (Gapp et al., 1979; Wallace et al., 1985). Developing oocytes endocytose Vtg from the bloodstream and convert it into yolk (Tata et al., 1979). Vtg production is normally restricted to adult females. Males and immatures also have the ability to produce Vtg, although at lower levels than adult females (Ho et al., 1981). Vtg can be detected using *in vitro* liver cultures (Shilling et al., 2000) or *in vivo* in the bloodstream when males and immature animals are exposed to exogenous estrogens or estrogen mimics (Whali et al., 1998). Most antibodies against Vtg have limited cross-reactivity with Vtg from other species. One study reported an antibody that reacted with several species (Heppel et al., 1995). Several monoclonal antibodies against *Xenopus laevis* Vtg were screened for cross-reactivity and only the one that is being reported on in this study shows cross-reactivity with different species.

Pilot studies done by us showed that the UniVtg can detect VTG from several species such as *Oreochromus mosambicus* (tilapia), *Xenopus laevis* (frog), *Crocodylus niloticus* (crocodile) and *Gallus domesticus* (chicken) by Western Blotting. This antibody can thus be a potentially useful tool for estrogenicity monitoring in a large number of species.

1.1. OBJECTIVES

The objectives of this study were to

- monitor the toxicity of effluents collected from the Eerste-Kuils River catchment area (E-KRCA) for cytotoxicity using the LDH assay.

- validate the UniVtg ELISA for *Xenopus laevis* liver culture synthesised Vtg.
- validate rapid competition ELISAs for monitoring environmental estradiol, estriol and estrone.
- assay sewage effluents collected from plants in the E-KRCA for estrogenicity using the *Xenopus laevis* liver culture assay, direct rapid ELISAs and the yeast assay for estrogens.

2. METHODS

2.1 Collection of samples.

Water samples were collected from four sewage plants that releasing its effluent in the Eerste-Kuils River water catchment area in March 2005 at the end of the dry season and again in April 2005 after the first winter rains. The water was collected in clean 2,5 liter glass containers and immediately transported to the laboratory for extractions. Extractions were done within 24 hours after sample collection. Samples were stored at 4°C until extraction.

2.2 Description of sewage samples collected.

Sewage effluent was collected at four sewage plants namely Bellville, Zandvliet, Macassar and Stellenbosch. All the treatment plants are situated on the Eerste-Kuils River catchment area. Figure 2.1 is a map showing the location of the catchment area.

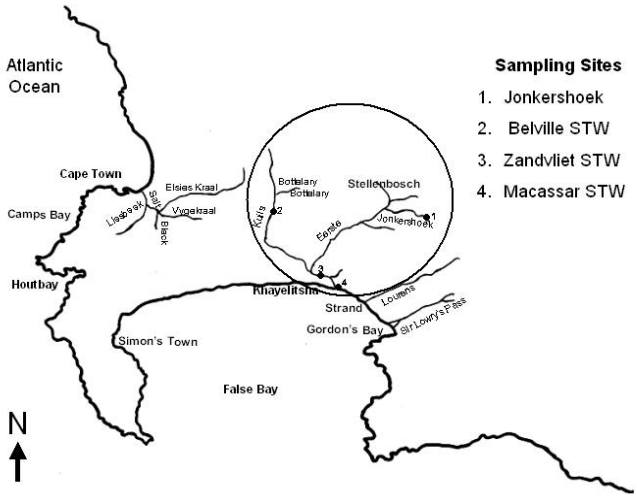

Figure 2.1: A map of the Western Cape, South Africa showing the major and minor catchments. The Eerste-Kuils River catchment is circled.

The Bellville sewage plant receives domestic and industrial waste while the other three plants receive mainly residential waste. Two sets of samples were collected. The first set was collected before the first rains and the second set was collected after the first rains.

The rainfall for March was less than 10 mm while the rainfall for April was more than 50 mm (Figure 2.2).

Control environmental water was collected at Witbrug in the Jonkershoek Nature Reserve. This site is near the origin of the Eerste River and upstream from all human activity. Table 2.1 is a summary of the samples collected.

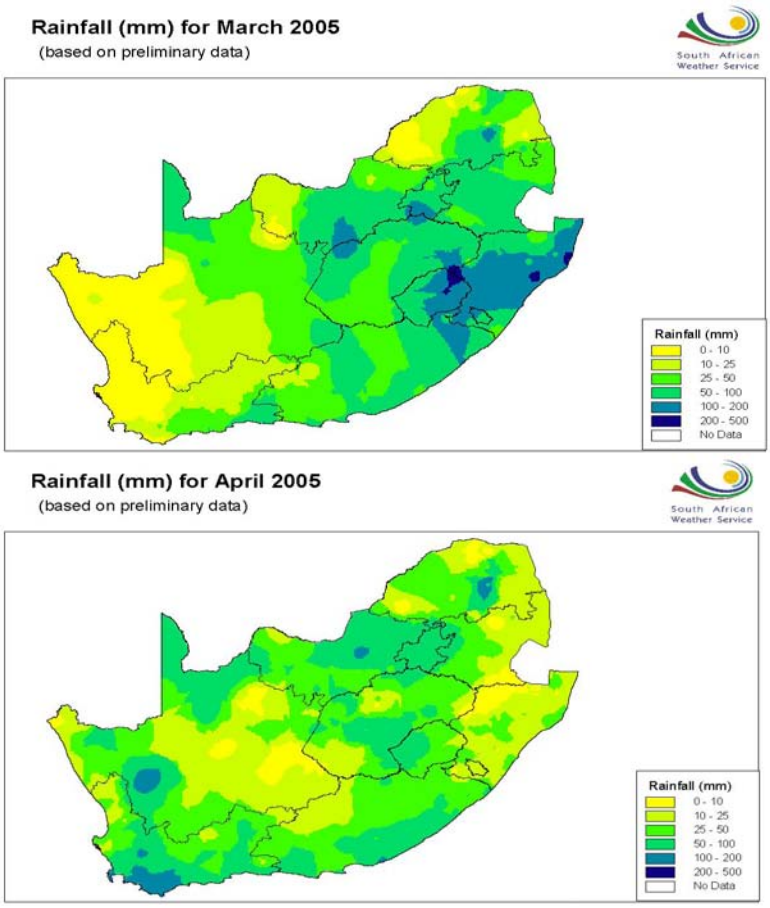

Figure 2.2: South African rainfall maps for March 2005 and April 2005.

Table 2.1: Summary of the samples collected for study.

Sample	Date	Site	Volume (l)	Extract (ml)
1	24-03-05	Jonkershoek control site	2	2
2	24-03-05	Stellenbosch STW	2	2
3	24-03-05	Bellville STW	2	2
4	24-03-05	Zandvliet STW	2	2
5	24-03-05	Macassar STW	2	2
6	29-04-05	Jonkershoek, control site	2	2
7	29-04-05	Swaaibrug, Stellenbosch	2	2
8	29-04-05	Bellville STW	2	2
9	29-04-05	Zandvliet STW	2	2
10	29-04-05	Macassar STW	2	2

2.3 Extraction of water samples.

Water samples were extracted on C18 SPE columns (Anatech) using our in-house extraction procedure. In brief: C18 columns were pre-washed with 4 ml of solvent mixture (40% hexane, 45% methanol and 15 %, 2-propanol), followed by another wash with 4 ml of ethanol. The column was then washed with one column volume of HPLC grade water after which the water sample was applied onto the column. The column was then air-dried with a vacuum pump until dry. The bound hydrophobic substances were eluted with solvent mixture. The eluate was dried under air and then reconstituted in 1/1000 th of the original sample volume with ethanol. The samples were stored at –20°C. To evaluate extraction efficiency of the extraction procedure, samples were spiked with known amounts of 17beta-Estradiol. The spiked samples were extracted as described above. The 17beta-Estradiol spike recovery (determined using the 17beta-Estradiol ELISA) was above 80% for all samples investigated.

2.4 Preparation of culture supernatants for the cytotoxicity assay.

Blood was collected from healthy males that were on no medication. All procedures were conducted under aseptic conditions. The blood was collected by venipuncture into heparinised vacutainer tubes (Beckton Dickinson, UK). The blood was diluted 1:9 with

RPMI 1640 tissue culture medium (Biowhittaker, USA). Extracts were transferred to triplicate wells of a 96 well culture tray (2 µl/well). Triplicate wells containing 2 µl ethanol were included as negative controls. For the positive control triplicate wells containing 2 µl of 1% m/v Tween 20 in ethanol was used (Tween is a detergent and solubilises the cell membrane to release the cytoplasmic material into the medium). The diluted blood was then added to the samples (200 µl/well). The cultures were incubated for 18 hours at 37°C. At the end of the culture period the culture supernatants were collected and assayed for LDH.

2.5 LDH assay on culture supernatants.

Culture supernatants were assayed for LDH using the Sigma colorimetric assay kit. The assay was done using our standard laboratory protocol. In brief: Culture supernatants were added to wells of a 96 well culture tray (5 µl/well). Kit substrate was then added to the wells (50 µl/well) and the plate was incubated at 37°C for 30 minutes. Kit colour reagent was then added to the wells (50 µl/well) after which the plate was incubated for 20 minutes at room temperature. The reaction was stopped by the addition of 50 µl/well of 2 M NaOH. The absorbance was then read at 450 nm. LDH standards were included on each plate. The standards were used to prepare a standard curve. LDH activities of the samples were read off the standard curve.

2.6 The preparation of diluted extracts for liver cultures

RPMI 1640 culture medium containing streptomycin, fungizone, penicillin and gentamycin was prepared. The medium was diluted with sterile water (water : medium = 30 : 70). The diluted medium was used for *Xenopus laevis* liver cultures and is subsequently referred to as XL medium. A 100 µg/ml estradiol in ethanol was used as stock positive control and ethanol was used as negative control for the liver assays. The extracts and controls were diluted in XL medium (5 µl extract or control plus 495 µl XL medium). The diluted samples were used to stimulate the liver cultures.

2.7 Xenopus laevis liver cultures

The liver from a female *Xenopus laevis* frog was removed under aseptic conditions. The liver was then cut up into strips of 3 cm by 2 mm. The strips were immediately placed in XL medium. The strips were further cut up into 1 mm^3 pieces. The liver cubes were then transferred to the wells of a 96 well culture dish (1 cube per well). XL medium was then added to the wells (180 µl/well). Diluted extracts and standards were then added to the wells (20 µl/well). Four replicate wells of each extract and standard were prepared. The plates were then incubated at 30°C for 3 days after which the medium was replaced with fresh medium containing extract. The culture was again incubated for 3 days after which the medium was collected and assayed for Vtg.

2.8 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Plasma Samples

Laemmli SDS-Polyacrylamide gels were prepared (Laemmli, 1970). Vertical slab gels (1,5 mm thick) containing 7.5% acrylamide were used. Samples were prepared by mixing 2 µl of plasma with 21 µl PBS, 2 µl of 1% (m/v) bromophenol blue and 25 µl of 0.125 M Tris, pH 6.8 containing 4% (m/v) SDS, 20% (v/v) glycerol and 10% (v/v) mercaptoethanol. Samples (5 µl) were applied to the wells and electrophoresis was done at a constant current of 20 mA. Gels were stained with 0.125% (m/v) Coomassie Blue in 50% (v/v) methanol and 10% (v/v) acetic acid. A protein molecular mass marker (AEC-Amersham, South Africa) was included on each gel.

2.9 Transfer of proteins from gels to nitrocellulose

After electrophoresis the separated proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotechnology, Sweden) using the Towbin method (Towbin et al., 1979). In brief, after Laemmli electrophoresis the gel, a nitrocellulose sheet and two filter paper sheets (Whatman Ltd, England) were soaked in Towbin buffer. The gel, overlayed by a nitrocellulose sheet, was sandwiched between two filter paper sheets and then placed into the transfer apparatus (Hoefer mini VE, Amersham Pharmacia Biotech, Sweden). The proteins were transferred at 25 mA for 1 hour. After transfer the nitrocellulose sheet was air dried and then stored at 4°C.

2.10 Immunoblots for Vtg

Nitrocellulose sheets containing transferred protein were removed from the fridge and soaked in 0.9% NaCl (saline) for 5 minutes. The sheet was then blocked with 1% albumin (Sigma) in saline (blocking buffer) for 30 min at ambient temperature. UniVtg monoclonal antibody (Ecophysiology laboratory, University of Stellenbosch, South Africa) was added to the blocking buffer solution to give a final dilution of 1/10000 of the antibody concentrate. The sheet was then incubated at ambient temperature for 3 h. The sheet was then washed four times for 5 min each with saline containing 0.1% Tween-20 (Sigma), followed by the addition of sheep anti-mouse peroxidase complex (AEC-Amersham, South Africa) diluted to 1/4000 in saline containing 0.1% albumin. The sheet was incubated for 1 h at room temperature and then it was washed as before. Chromogenic substrate (TMB insoluble substrate, Boehringer-Mannheim, Germany) was added and the sheet was incubated at ambient temperature until visible bands develop. The sheet was immediately transferred to deionised water to stop the chromogenic reaction. The sheet was then dried and stored in the dark.

2.11 ELISA for Vtg

Assays were carried out in 96-well Nunc-Immuno MaxiSorp® plates (Nalge Nunc, Denmark). Plates were coated with 50 µl/well of the individual samples diluted 1/25 in saline by a 2-h incubation at ambient temperature. Samples containing known Vtg concentrations were included on all plates to construct a standard curve. The coating solution was aspirated and the plates were blocked with 200 µl/well blocking buffer for 30 min at ambient temperature. The plates were then washed four times with saline. UniVtg antibody diluted 1/10000 in blocking buffer was added at 50 µl/well and the plate was again incubated at ambient temperature for 2 h. The plate was then washed four times followed by the addition of 50 µl sheep anti-mouse peroxidase complex (AEC-Amersham, South Africa) diluted to 1/4000 in saline containing 0.1% albumin to each well. The plate was incubated for 60 min at room temperature and then it was washed four times. Substrate (TMB soluble substrate, Boehringer-Mannheim, Germany) was added at 50 µl/well and the plate was incubated at ambient temperature in the dark for 20 minutes. The chromogenic reaction was stopped by the addition of 25 µl/well of 0.5 M H_2SO_4. The

optical densities were read on a plate reader at 450 nm. A standard curve was constructed using the Microsoft Excel package and the concentrations of the samples were extrapolated from this curve.

2.12 ELISA for Estrone

Working conjugate solution was prepared by mixing 100 µl estrone-biotin and 100 µl avidin peroxidase conjugate and 9.8 ml assay buffer. The working conjugate were mixed and incubated at room temperature for at least 20 minutes prior to addition to the ELISA plate.

Concentrated (1000 x) water extracts were diluted 1/10 using 0.1% (w/v) bovine serum albumin in 0.9% NaCl. The diluted (100 x concentrated) extracts were assayed directly on the estrone ELISA kit (cat number DB52051, IBL, Germany) using the manufacturer's instruction manual. In brief: microtiter plate strips precoated with rabbit anti-estrone was removed from the strip holder and firmly fixed in the ELISA plate. All assays were done in duplicate. Samples and standards were transferred to the wells (25 µl per well), followed by the addition of working conjugate solution (100 µl per well). The contents of the wells were mixed by tapping the plate. The ELISA plate was then incubated for one hour at room temperature, followed by washing the plate four times with wash buffer (300 µl/well). TMB substrate was dispensed at 150 µl per well after which the plate was incubated for 15 minutes at room temperature. The reaction was stopped by the addition of stop solution (50 µl per well). The optic density (OD) was measured at 450 nm using a plate reader. A standard curve was drawn using the OD readings obtained for the standards and the concentrations for the samples were read off this curve.

2.13 ELISA for 17beta-Estradiol

Concentrated (1000 x) water extracts were diluted 1/10 using 0.1% (w/v) bovine serum albumin in 0.9% NaCl. The diluted (100 x concentrated) extracts were assayed directly on the estradiol ELISA kit (catalogue number RE52041, IBL, Germany) using the manufacturer's instruction manual. In brief: microtitre plate strips precoated with rabbit

anti-estradiol was removed from the strip holder and fixed firmly in the ELISA plate. All assays were done in duplicate. Samples and standards were transferred to the wells (25 µl/well). Estradiol – horseradish peroxidase conjugate was added to all the wells (200 µl/well). The solutions were mixed by gently tapping the plate, where after it was incubated for 120 minutes at room temperature. At the end of the incubation period the solutions in the wells were decanted after which the wells were washed three times with 300 µl/well of wash solution. Substrate was then dispensed at 100 µl per well after which the plate was incubated for 15 minutes at room temperature. The reaction was stropped by addition of stop solution (100 µl/well). The OD was determined at 450 nm using a plate reader. A standard curve was drawn using the reading obtained for the standards and the concentration of the samples was read off this curve.

2.14 ELISA for Estriol

Concentrated (1000 x) water extracts were diluted 1/10 using 0.1% (w/v) bovine serum albumin in 0.9% (w/v) NaCl. The diluted (100 x concentrated) extracts were assayed directly on the estriol ELISA kit (cat number BM52011, IBL, Germany) using the manufacturer's instruction manual. In brief: microtitre plate strips precoated with rabbit anti-estriol was removed from the strip holder and fixed firmly in the ELISA plate. All assays were done in duplicate. Samples and standards were transferred to the wells (10 µl/well) followed by the addition of 100 µl estriol – horseradish peroxidase conjugate. The solutions were mixed by gently tapping the plate after which it was incubated for 1 hour at room temperature. The contents of the wells were decanted and the plates were washed four times with wash buffer (300 µl/well). TMB substrate was then added and the plates were incubated for 30 minutes at room temperature. The enzyme reaction was stopped by the addition of stop solution. The OD was determined at 450 nm using a plate reader. A standard curve was drawn using the reading obtained for the standards and the concentration of the samples was read off this curve.

2.15 Validation of assays

Kits were assayed for accuracy as follows: A dilution series of a sample containing high steroid hormone concentrations was prepared 0.1% (w/v) bovine serum albumin in 0.9%

(w/v) NaCl. The diluted samples were then assayed using the kit and the data obtained was plotted on the same graph as the standard curve to determine if the curves were parallel. Kit standard steroid hormones were also titrated with ethanol to determine the recovery of the ELISA assay on samples containing ethanol. The kits were assayed for intra-assay reproducibility by assaying replicates of the same sample on a single assay plate. The kits were also assayed for inter-assay reproducibility by assaying the same sample on several plates.

2.16 Yeast estrogen receptor assay (YES)

Transformed yeast cells, *Saccharomyces cerevisae* strain BJ3505 [MATα, pep4::HIS3, prb1-Δ1, 6R, his3-Δ200, lys2-801, trp1-Δ101, ura3-52 (can1)], was used as described by (Gaido et al., 1997). Transformed yeast cells were grown overnight at 30ºC with vigorous orbital shaking at 300 rpm in selective medium containing yeast nitrogen base without amino acids (6.7 g/L), plus dextrose (20 g/L), leucine (60 mg/L), and histidine (20 mg/L) for yeast transformed with the estrogen receptor or yeast nitrogen base without amino acids (6.7 g/L), plus dextrose (20 g/L), lycine (30 mg/L), uridine (40 mg/L), adenine (40 mg/L) and histidine (20 mg/L) for yeast transformed with the androgen receptor. Following the overnight culture, yeast cells were sub-cultured in fresh medium and allowed to grow until mid log-phase culture (OD_{600} approximately 1.0). For estrogen and androgen assays, yeast was diluted to an OD_{660} of 0.03 in growth media. Copper Sulfate is added to a final concentration of 0.05 mM. The diluted yeast were aliquoted into 50 ml screw cap polypropylene tubes (5 ml/tube) and doses of steroids added (creating a dilution series). The doses were added to constitute no more than 0.1% of the final volume. Yeast cells were exposed overnight (~ 16 hours) at 30ºC, with orbital shaking at 300 rpm. Following overnight incubation, samples were diluted to OD_{660} of 0.25 and aliquoted into a microtiter plate in volumes of 100 µl per well. Each sample was assayed in triplicate.

2.17 Beta galactosidase assay

A β-galactosidase assay kit was used (Pierce Biotechnology, Inc., Rockford, USA cat # 75768) to determine the β-galactosidase activity in the wells according to the

manufacturer's instructions for the non-stopped microplate assay. Absorbance measurements were taken at OD_{420nm} using a microtiter plate reader (PowerWaveX, Bio-Tek instruments, Inc.). β-Galactosidase activity is expressed as Vmax (OD_{420}/min) divided by cell density (OD_{590}).

3. RESULTS

3.1 Cytotoxicity of the samples.

Figure 3.1 is a standard curve for the LDH assay. This graph shows that the standard curve for LDH activity is linear with respect to concentration.

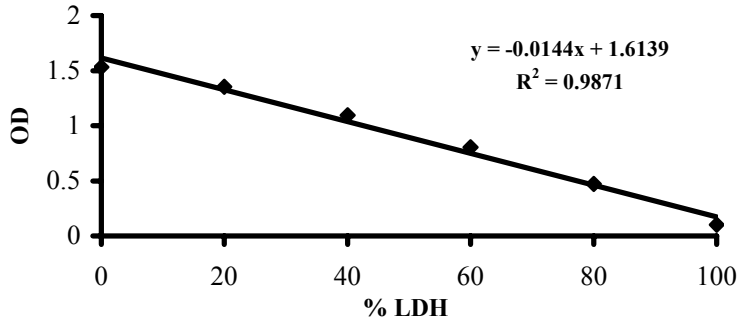

Figure 3.1: Standard curve for cytotoxicity using the Sigma LDH kit.

None of the samples collected differed significantly from the negative control. The positive control is a whole blood culture incubated with medium containing 0.1% Tween 20 to lyse the cells and release all the LDH. All the samples had cytotoxicity levels statistically significantly lower than the positive control. Figure 3.2 is a graph of the cytotoxicity of the samples.

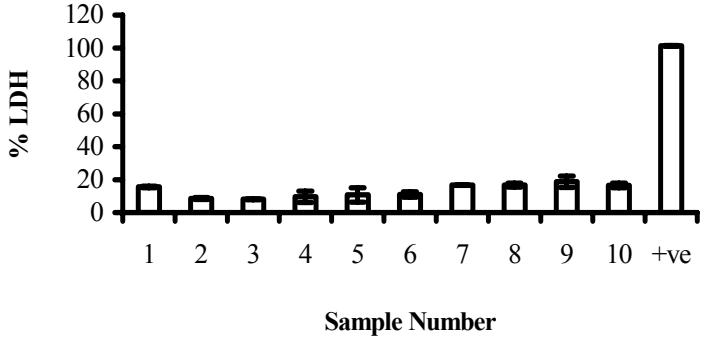

Figure 3.2: The cytotoxicity of the C18 extracts.

3.2 Validation of the Vtg ELISA

3.2.1 Specificity of the UniVtg antibody

Western blotting analysis of *Xenopus laevis* plasma samples shows that the UniVtg antibody detects a single major band of molecular weight 240 000 corresponding to the molecular weight of Vtg (Figure 3.3). The antibody detects a band of identical molecular weight in plasma from estrogen treated *Oreochromus mosambicus* (tilapia), *Crocodylus niloticus* (Nile crocodile) and *Gallus domesticus* (chicken). This band is only detected in plasma from estrogen treated males and is absent in males not treated with estrogen.

Figure 3.3: Western blot of plasma from males. The proteins in the plasma samples were separated using a 7% SDS polyacrylamide gel. The proteins were transferred to nitrocellulose and probed for Vtg using the UniVtg antibody. Lanes 1, 3, 5 and 7 is plasma from males not treated with *estrogen* while plasma from males injected with 1 mg 17β-estradiol per kg body mass are separated in lanes 2, 4, 6 and 8. Lanes 1 and 2 are *Oreochromus mosambicus* plasma, lanes 3 and 4 are *Xenopus laevis* plasma, lanes 5 and 6 are *Crocodylus niloticus* plasma and lanes 7 and 8 are *Gallus domesticus* (chicken) plasma.

3.2.2 The detection of tissue culture synthesised Vtg using the UniVtg ELISA

Two-fold dilutions of isolated *Xenopus laevis* Vtg and a *Xenopus laevis* tissue culture supernatant with a known Vtg concentration were coated onto plates. The ELISA standard curves generated from the culture supernatant standard curve lies on top of the standard curve generated using the isolated Vtg (Figure 3.4).

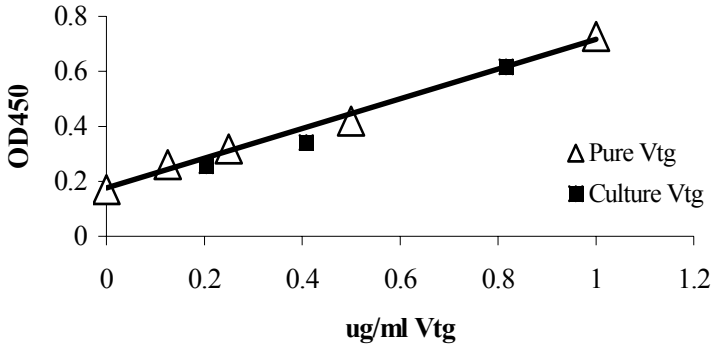

Figure 3.4: ELISA standard curves of purified *Xenopus* Vtg and *Xenopus laevis* liver culture Vtg.

ELISAs were also conducted to determine the inter- and intra-assay variability of the UniVtg ELISA for *Xenopus laevis* tissue culture Vtg. The intra-assay variability was 4.34% and the inter-assay variability was 8.9 %.

3.3 The effect of sewage extracts on Vtg synthesis by frog liver cultures.

Supernatants of liver cultures incubated in the presence of sewage extracts were assayed for Vtg using the UniVtg ELISA. Ten extracts were assayed. Figure 3.5 is a graphic representation of the results obtained from this experiment. Low levels of Vtg were detected in supernatants obtained from cultures incubated with the negative control (ethanol) and the samples. The positive control (1 ppm estrogen in ethanol) induced high levels of Vtg secretion into the medium. Only sample 8 corresponding to Bellville STW after the first seasonal rains had a Vtg level statistically higher than the control sample ($p < 0.05$).

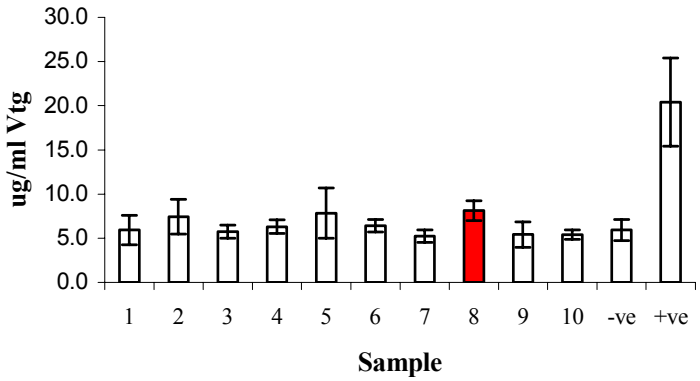

Figure 3.5: The effect of water extracts on Vtg synthesis by *Xenopus laevis* liver cultures. Liver cultures were incubated with extracts for 6 days as described in the methods section. The supernatants were then screened for Vtg synthesis using the UniVtg ELISA. Each point represents 4 replicate cultures.

The UniVtg ELISA can be used for the detection of tissue culture synthesized *Xenopus laevis* Vtg. By using the *Xenopus laevis* liver culture assay and the UniVtg ELISA it was shown that Bellville STW effluent after the April 2005 rains was estrogenic. None of the other samples tested activated Vtg synthesis by liver cultures. A possible explanation for the higher estrogenicity of the Bellville effluent compared to the other sewage plants might be the extent of industrial effluent entering the Bellville plant compared to the other sewage treatment plants. The other sewage treatment facilities receive mainly domestic discharge, while Bellville STW receives high concentrations of industrial discharge together with domestic effluents.

3.4 Validation of rapid ELISAs for the detection of selected steroids

3.4.1 Typical Standard Curves

Typical standard curve data for the estriol, estrone and estradiol ELISAs are presented in figure 3.6. The correlation coefficients for all three of the curves are between 0.972 and 0.994. The estriol ELISA has a detection range between 0.3 and 40 ng/ml. The estrone ELISA has range between 15 and 2000 pg/ml whereas the estradiol ELISA has a detection range between 25 and 2000 pg/ml. Sensitivity (minimum detection limit) was determined by the supplier.

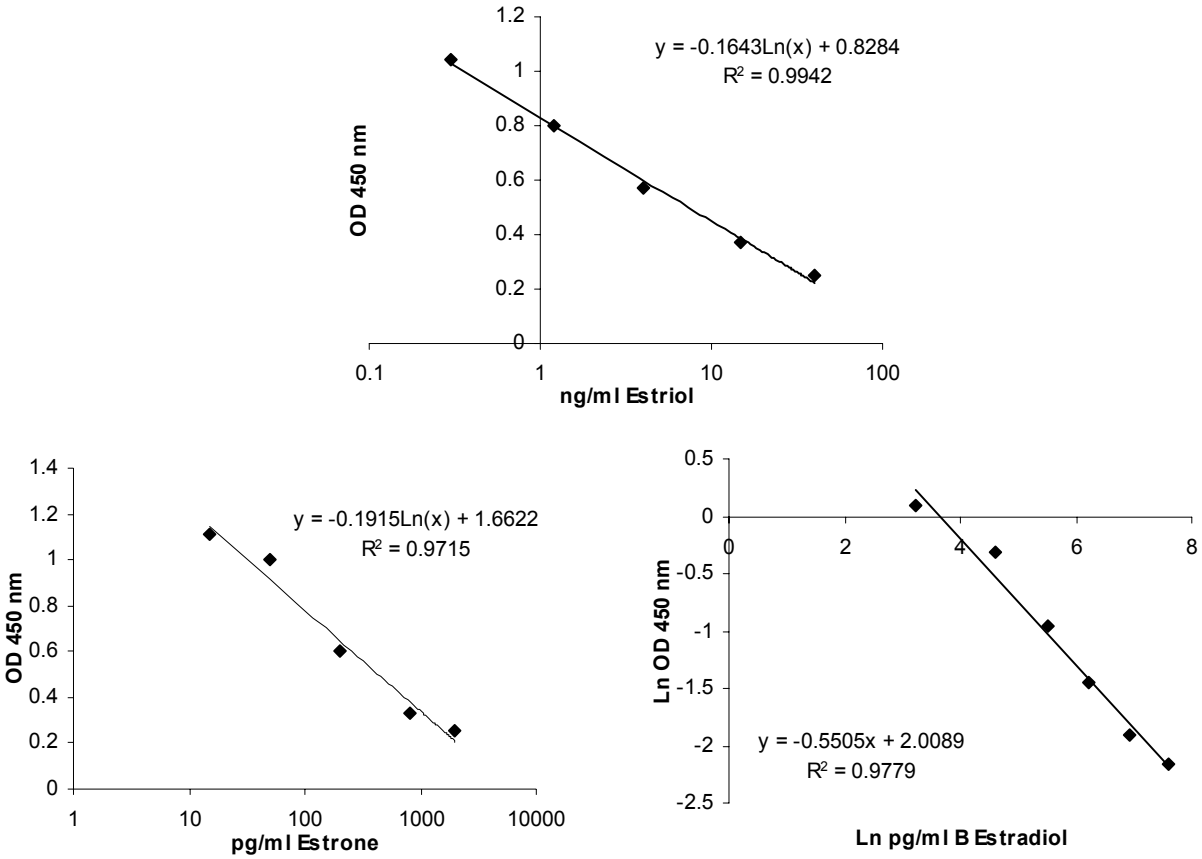

Figure 3.6: Standard curves for the ELISAs to determining estriol, estradiol and estrone concentrations in sewage effluent.

3.4.2 Parallelism between ELISA kit standards and environmental sample extracts

Parallelism of ELISA kits standard curves and environmental samples was established using Macassar sewage effluent after the first rains as an environmental sample. Curves produced by assaying environmental samples at various dilutions showed parallelism with the standard curve produced for estrone and estradiol (Figure 3.7). Parallelism between the standard curve of the estriol ELISA kit and the Macassar sewage effluent after the first rains could not be establish because estriol concentrations in the C18 extraction were already near the detection limit of 0.3 ng/ml.

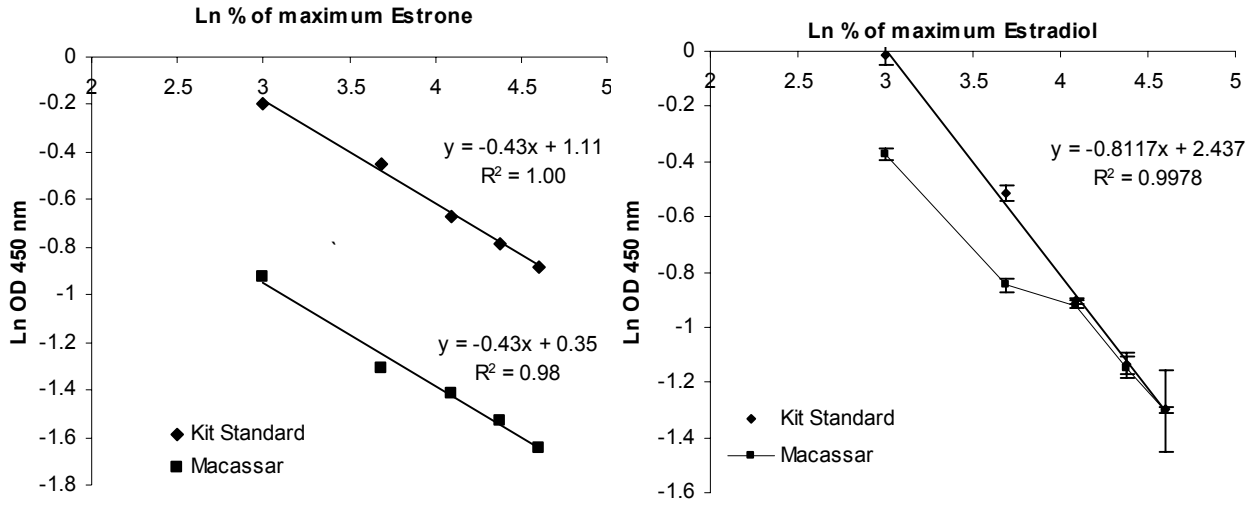

Figure 3.7: Parallelism between kit standard and sewage effluent sample.

3.4.3 Hormone ELISAs repeatability

Sewage effluent was subjected to C18 solid phase extractions and finally dissolved in analytical grade ethanol as described in the section, *methods and materials.* The supplier optimized the ELISA kits for the analysis of the specific steroid hormone in human serum. The effect of ethanol on the recovery of the ELISA assay was analyzed by assaying 10% of the kit's maximum standard steroid hormone at 0%, 10% and 20% ethanol. The estriol ELISA assay had a recovery of 98.3 ± 7.1% at 10% ethanol, whereas 87.7 ± 2.4% could be established at 20% ethanol (Table 3.1). There were no significant difference between the percentage recoveries (P = 0.125). Ethanol had no effect on the estrone ELISA (P = 0.576) with recoveries of 105.3 ± 7.2% at 10% ethanol and 102.1 ± 6.5% at 20% ethanol (Table 3.2). The estradiol ELISA had a recovery of 88.3 ± 3.9% at 10% ethanol and 98.6 ± 14.5% at 20% ethanol (Table 3.3). Ethanol again had no significant influence on the recovery of the estradiol ELISA (P = 0.346).

Table 3.1: The effect of ethanol on recovery and intra assay variation as well as the inter assay variation at 10% ethanol on the estriol ELISA kit.

n	% Ethanol	% Recovery	% Intra assay variation	% Inter assay variation
3	0	100.6 ± 9.2	2.5 ± 0.2	
3	10	98.3 ± 7.1	1.9 ± 0.1	5.6 ± 0.3
3	20	87.7 ± 2.4	0.6 ± 0.02	

Table 3.2: The effect of ethanol on recovery and intra assay variation as well as the inter assay variation at 10% ethanol on the estrone ELISA kit

n	% Ethanol	% Recovery	% Intra assay variation	% Inter assay variation
3	0	100 ± 3.3	3.3 ± 0.1	
3	10	105.3 ± 7.2	6.9 ± 0.5	8.2 ± 0.7
3	20	102.1 ± 6.5	6.4 ± 0.4	

Table 3.3: The effect of ethanol on recovery and intra assay variation as well as the inter assay variation at 10% ethanol on the estradiol ELISA kit

n	% Ethanol	% Recovery	% Intra assay variation	% Inter assay variation
3	0	100.5 ± 8.7	8.4 ± 0.7	
3	10	88.3 ± 3.9	4.5 ± 0.2	3.9 ± 0.1
3	20	98.6 ± 14.5	13.9 ± 2.1	

Intra assay variation was less than 2.5% at 0, 10 and 20% ethanol for the estriol ELISA, whereas inter assay variation was 5.6 ± 0.3% at 10% ethanol (Table 3.1). Intra assay variation was less than 7% at 0, 10 and 20% ethanol, whereas intra assay variation was

8.2 ± 0.7% at 10% ethanol for the estrone ELISA (Table 3.2). The estradiol assay had an intra assay variation of less than 8% at 0 and 10% ethanol. Intra assay variation is 13.9 ± 2.1% at 20% ethanol. Inter assay variation is 3.9 ± 0.1% at 10% ethanol (Table 3.3).

3.5 The detection of estriol in sewage effluent

Very low concentrations of estriol were detected in sewage effluent from all the STWs. All the samples tested had levels lower than 1.1 ng/l of estriol (Figure 3.8). These values were near to the lower detection limit of the ELISA kit. Samples collected in April and March, at all the STWs, show significant higher levels of estriol (P = <0.001) in comparison with the control site, except for Macassar in March.

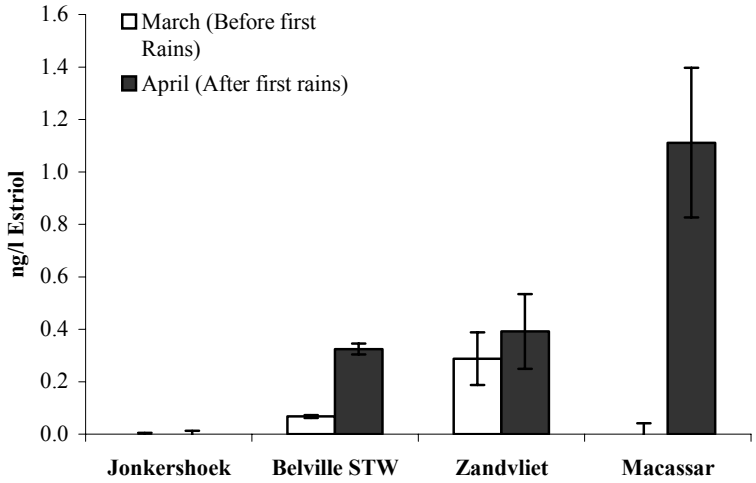

Figure 3.8: Water samples were collected from sites along the Kuils and Eerste Rivers in March and April. The samples were extracted using C18 chromatography and the assayed using the estriol ELISA kit

3.6 The detection of estrone in sewage effluent

Less than 0.2 ng/l estrone were detected in the Jonkershoek samples (Figure 3.9). The levels of estrone was significant higher in the sewage effluents in comparison with the control site (P = <0.001). Zandvliet had the highest level of estrone in both March and April, 9.4 and 10.6 ng/l respectively. Effluent collect from Bellville in March had the lowest level of estrone (7.2 ng/l). Generally samples taken in April have higher levels of estrone in comparison with March, although statistically insignificant (P = >0.001).

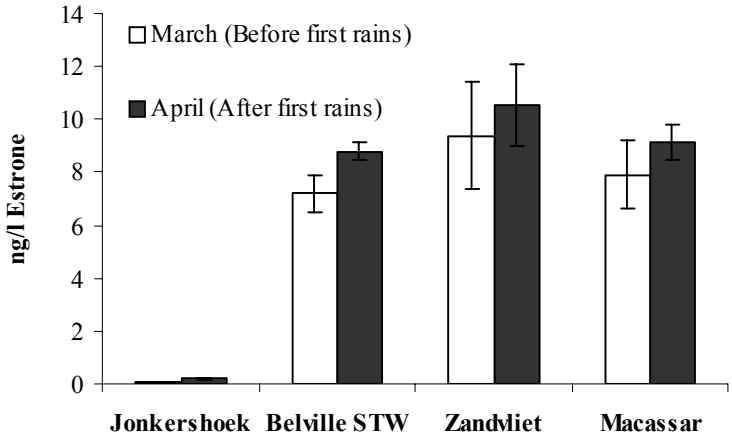

Figure 3.9: Water samples were collected from sites along the Kuils and Eerste Rivers in March and April. The samples were extracted using C18 chromatography and the assayed using the estrone ELISA kit

3.7 The detection of estradiol in sewage effluent

Very low levels of estradiol were detected in samples obtained from the control site in Jonkershoek (Figure 3.10). There were significant higher levels of estradiol detected from the sewage effluents in comparison with the control site (P = <0.001). Estradiol levels ranged between 0.8 ng/l (Macassar, April) and 4.7 ng/l (Zandvliet, April). At both Zandvliet and Macassar estradiol levels were higher in April than in March.

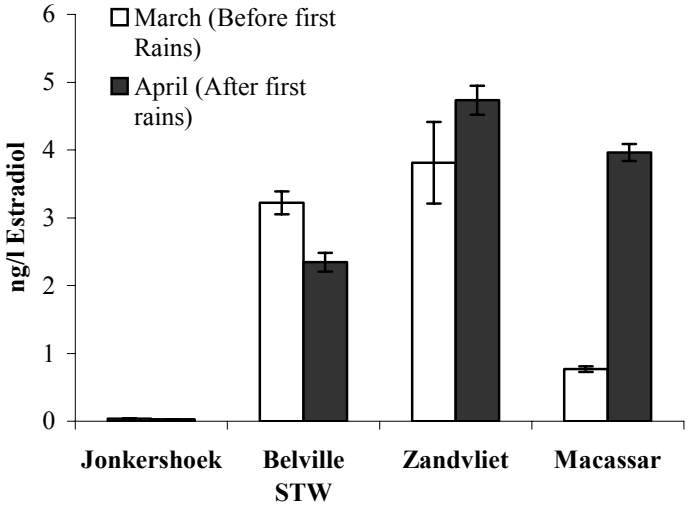

Figure 3.10: Water samples were collected from sites along the Kuils and Eerste Rivers in March and April. The samples were extracted using C18 chromatography and the assayed using the estradiol ELISA kit.

4. DISCUSSION AND CONCLUSIONS

During a WRC workshop held in Pretoria in May 2004 methods for screening environmental estrogens were evaluated with respect to specific criteria such as sensitivity, reproducibility, time required for analysis, user-friendliness and cost. The present report is an evaluation of an additional assay that can be used for the rapid screening of environmental estrogens. The criteria that were used for the original evaluation of estrogen screening assays were sensitivity, repeatability, robustness, time of analysis, optimum batch size, ease of use and the training level required by the analyst. A cost analysis of the assays was also performed. For cost analysis the total time of each analytical procedure was broken down into labour time (time spent by analyst on the bench to handle the samples) and non-labour time (time that samples are incubating etc.). To calculate the total cost per sample run, the labour cost (R300 per hour) plus non-labour cost (R150 per hour) was added. Due to the high cost of single sample runs, the average cost per sample in an optimum batch was calculated. Tables 4.1 and 4.2 are an updated version of the cost report for estrogen assays that were given in WRC report KV 143/05, 2005.

Table 4.1: Evaluation of bio-assays

Bio-assay	Sensi-tivity	Repeat-ability	Robust-ness	Total assay time	Batch size	Volume required	Analyst training	Critical factor
Yeast screen	1.3 ng/l	Good	Good	10-14 days	8	4 litres	B.Tek/Sc 2 yrs exp	Yeast cell line
Xenopus liver assay	100 ng/l	Good	Good	7 days	8	200 ml	B.Tek/Sc 2 yrs exp	Xenopus
Estrone ELISA	0.1 ng/l	Good	Good	2 days	20	100 ml	B.Tek/Sc 2 yrs exp	Kit
Estradiol ELISA	0.25 ng/l	Good	Good	2 days	20	100 ml	B.Tek/Sc 2 yrs exp	Kit

Table 4.2: Time and cost analysis of bio-assays (cost of chemicals excluded)

Bio-assay	Labour time	Non-labour time	Labour cost	Non-labour cost	Total cost per batch	Cost per sample when done in optimum batch
Yeast screen	16 hrs	40 hrs	R4 800	R6 000	R10 800	R1 350
Xenopus liver assay	8 hrs	20 hrs	R2 400	R3 000	R5 400	R675
Estrone ELISA	16 hrs	4 hrs	R4 800	R600	R5 400	R270
Estradiol ELISA	16 hrs	4 hrs	R4 800	R600	R5 400	R270

At present three laboratories in South Africa are running yeast screens for estrogens on a routine basis (CSIR, University of Pretoria and University of Stellenbosch). The estrone and estradiol ELISA can be done at two laboratories (University of the Western Cape and University of Stellenbosch).

With respect to ELISAs for estrogenic compounds, the UWC laboratory has already completed validations for chlorpirifos, Zearalenone and deoxynevalenol (DON). It is envisaged that the number of ELISA tests evaluated will be increased in the near future (dependent on availability of funds) to include atrazine, ethinylestradiol, PCBs, Dioxins, cyclodienes and DDT. Another compound of importance in water quality assessment for which a validated ELISA is available at UWC is microcystin-LR. It is recommended that the WRC support the efforts to build capacity in ELISA technology in South Africa.

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