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The implementation of a battery of *in vivo* and *in vitro* bioassays to assess river water for estrogenic endocrine disrupting chemicals

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ABSTRACT

Previous research has shown that accurate evaluation of environmental water samples for estrogenic activity requires a panel of *in vitro* and *in vivo* bioassays, which are based on different molecular and cellular action mechanisms. In the current study, a test battery containing four assays was used to analyze water from the Eerste River, South Africa for estrogenicity. Three sites were used for analysis, namely Jonkershoek (control site situated in the mountains at the origin of the Eerste River), sewage effluent from Stellenbosch sewage treatment works and Spier site (sampling site on the Eerste River downstream from Stellenbosch). Estrogenicity was determined using an estrone enzyme linked immuno sorbent assay (ELISA), estrogen induced proliferation of human breast cancer adenocarcinoma cells (MCF-7) also known as the E-SCREEN, estrogen induced suppression of estrogen receptor alpha protein expression (ER-α) in MCF-7 cells (ERα assay) and by monitoring estrogen induced vitellogenin (VTG) synthesis in juvenile *Oreochromis mossambicus* (VTG assay). Low concentrations of estrone (ranging between 1.4 and 2.2 ng/l) near the detection limit of the assay were detected in samples collected from Jonkershoek. Water from this site shows no estrogenicity in the E-SCREEN, ERα assay or VTG synthesis bioassay. The estrone concentrations in the sewage effluent extracts, as well as Spier site extracts, ranged between 14.7 and 19.4 ng/l. The assays using ERα induction by the MCF-7 cell line, MCF-7 proliferation and *in vivo* VTG synthesis by juvenile tilapia showed that these samples are estrogenic. The results obtained for the assays in the battery are comparable.

1. Introduction

The occurrence of estrogenic compounds in the environment is a well studied phenomenon. Most environmental estrogens are synthetic compounds produced as a result of industrial, domestic and agricultural activities (Kuiper et al., 1998). However, not all estrogenic compounds are man-made. Some environmental estrogens originate from natural sources (Kuiper et al., 1998; Giesy et al., 2002). Examples of these are the phyto- and the myco-estrogens. Several previous studies have focused on the development and implementation of assays to monitor environmental estrogens. Pollutants can act at several points on the endocrine/reproductive system to give estrogenic or anti-estrogenic effects (reviewed by Baker, 2001). Some pollutants mediate their action by directly binding to the estrogen receptor, while others modulate estrogen biosynthetic pathways resulting in either increased or decreased circulating estrogen concentrations (Sharpe and Irvine, 2004). Moreover, several pollutants demonstrate effects that are species, tissue- and cell-specific, as well as being influenced by metabolism (WHO/IPCS, 2002). Owing to the varied biological actions of estrogenic pollutants, current strategies for monitoring estrogens favor the use of a battery of tests, representative of several potential points on the estrogenic response pathways of different organisms, for monitoring (Reel et al., 1996; Shelby et al., 1996; Baker et al., 1999a, 1999b, 2000).

South Africa, like most countries in the world, faces ecological challenges due to endocrine disrupting chemicals (EDCs) found in the aquatic environment. The aim of the current study is to implement newly developed bioassays in a bio-monitoring battery of tests as a first tier screen for estrogenicity of a river. This battery consists of three *in vitro* and one *in vivo* screen for estrogenicity. Water samples were analyzed for the specific female hormone, estrone as the first *in vitro* assay of the battery (Swart and Pool, 2007). Estrone was chosen because municipal wastewater treatment plants rapidly convert 17 β-estradiol to estrone (Eun-Joung et al., 2007). Total estrogenicity of the water samples were determined using two more *in vitro* screens, namely a modified human breast cancer adenocarcinoma cell (MCF-7) proliferation assay (E-SCREEN) (Swart, 2009) and an MCF-7 estrogen receptor alpha (ERα) enzyme linked immuno sorbent assay (ELISA) (Swart and Pool, 2009a). The E-SCREEN assay measures estrogenicity by means of indirectly quantifying MCF-7

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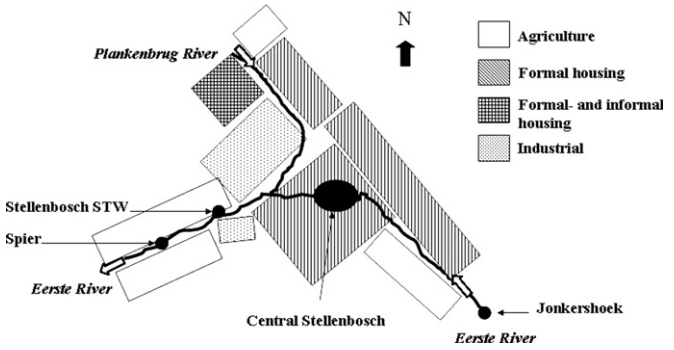

Fig. 1. Map of the Eerste River in Stellenbosch, South Africa. Water samples were collected at Jonkershoek, Stellenbosch sewage treatment works (STW) and Spier site. Water samples were collected in January during high summer as well as April after the first winter rains. Water samples were collected in 2007 and 2008 for analysis.

cellular proliferation or cell number (MCF-7 is an estrogen dependent breast cancer adenocarcinoma cell line). The MCF-7 estrogen receptor alpha ELISA measure ERα protein levels indicative of estrogen concentration in the cell's environment. Vitellogenin induction in juvenile tilapias was used as a short term exposure *in vivo* assay (Swart, 2009; Swart and Pool, 2009b). The river that was selected for the present study is the Eerste River, Stellenbosch, South Africa. Several studies have investigated this river system. A study conducted by Reinecke et al. (2003) found significant concentrations of Mn, Cu, Pb, Cd and Zn in both the water (ranging between 0.006 and 0.222 mg/l) and sediment (ranging between 0.43 and 184.7 mg/kg). The same study reported total coliforms concentrations of 1.2×10^5/100 ml as well as fecal coliforms concentrations of 1.5×10^4/100 ml from water that passed through Stellenbosch town center. In another study, all the water samples collected from the Eerste River showed inflammatory potential using interleukin 6 syntheses by whole blood cultures as a biomarker (Pool et al., 2000). Fig. 1 is a map of the river indicating the sites that were used for the present study. Sites were selected to include water not affected by human activity (Jonkershoek) as well as samples from sites severely affected by agricultural, industrial and domestic pollution.

2. Methods and materials

2.1. Study area

The origin of the Eerste River is in the Jonkershoek Valley of the Western Cape in South Africa. It flows through the Jonkershoek Forest Reserve, the Assegaaibosch Nature Reserve and several vineyards before reaching the town of Stellenbosch (33°56′10″S; 18°51′34″E). For the rest of its course the river is bordered by agricultural land, industrial areas, formal and informal housing (housing without sanitation) before reaching the sea on the False Bay coast (Fig. 1). Eerste River water samples were collected at Jonkershoek, Stellenbosch sewage treatment works (Stellenbosch STW) and near the Spier wine estate.

2.2. Water sampling

Water samples were collected in clean 1 l glass bottles. The bottles were cleaned by washing with soap, followed by rinsing with tap water. The bottles were then rinsed with high performance liquid chromatography (HPLC) grade water and finally it was rinsed with HPLC grade ethanol (Merck, Germany). The bottles were then allowed to dry up-side down in a dry rack. Samples were collected in January, at the peak of the dry summer season and at the beginning of the rain season in April. Water samples were taken to the laboratory and were processed immediately.

2.3. Water hydrophobic content extractions

The hydrophobic content of environmental water samples (1 l samples) were extracted on C18 SPE columns (Anatech) using a method previously described by Swart and Pool (2007). C18 columns were pre-washed with 4 ml of solvent mixture (40% hexane, 45% methanol and 15%, 2-propanol), followed by another wash with 4 ml of ethanol. The column was then washed with one column volume of reverse osmosis water after which the water sample was applied onto the column. The column was then air-dried. The bound hydrophobic substances were eluted with solvent mixture. The dried water extracts were reconstituted in 1 ml dimethyl sulfoxide (DMSO) to give a final volume 1/1000 times that of the original sample volume. The samples were stored at −20 °C until further use.

2.4. Estrone ELISA

The estrone concentrations of the water extracts were quantified as previously described by Swart and Pool (2007). In short, concentrated water extracts were diluted 1/10–1/10,000 using 0.1% (w/v) bovine serum albumin in 0.9% NaCl. The diluted (100 × concentrated) extracts were assayed directly on the estrone ELISA kit (cat number DB52051, IBL, Germany). In brief: microtitre 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/well), followed by the addition of working conjugate solution (100 µl/well). The contents of the wells were mixed by tapping the plate. The ELISA plate was then incubated for 1 h at room temperature, followed by washing the plate four times with wash buffer (300 µl/well). Tetramethylbenzidine (TMB) substrate was dispensed at 150 µl/well after which the plate was incubated for 15 min at room temperature. The reaction was stopped by the addition of stop solution (50 µl/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.

2.5. MCF-7 cell culture

MCF-7 cells are an estrogen responsive human breast cell carcinoma line. MCF-7 cells were maintained and harvested as previously described by Soto et al. (1992, 1995) with some changes. Seventy percent confluently growing MCF-7 cells were suspended in *full medium* (RPMI-1640 medium containing phenol red and L-glutamine and supplemented with 10% (v/v) fetal bovine serum (FBS) and 1/100 diluted antibiotic–antimycotic solution) to a concentration of 5×10^5 cells/ml. The cell suspensions were then dispensed at 200 µl/well in 96 well flat bottom tissue culture plates (Nunc, AEC-Amersham). Cells were allowed to attach for a minimum of 5 h before the medium was decanted and the wells rinsed twice with phosphate buffered saline (PBS) pre-heated to 37 °C. The wells were then filled with 200 µl *estrogen deprived medium* (RPMI-1640 modified without L-glutamine and phenol red and supplemented with 1/100 diluted antibiotic–antimycotic solution, 1/100 diluted glutamax and 1/50 diluted serum replacement). Cells were allowed to grow for 48 h in order to deplete estrogen levels. Medium was then replaced with estrogen deprived medium containing 1% v/v of the 17β-estradiol standard, environmental water extract or dimethyl sulfoxide (DMSO) vehicle control. Cells were cultured for another 48 h before any procedures were done.

2.6. MCF-7 proliferation assay (E-SCREEN)

The modified E-SCREEN assay was performed as previously described by us (Swart, 2009). Total cellular lactate dehydrogenase (LDH) activity was determined as an indicator of estrogen responsive total cell number or proliferation. Interpretation of LDH response from MCF-7 cells exposed to complex and dynamic environmental water extracts can be very difficult. Lactate dehydrogenase was measured using the instructions of a cytotoxicity detection kit supplied from Roche Applied Science. In short: cells were grown in a 96 well format as described in the previous section. In order to obtain total LDH levels, culture medium was removed from the exposed cells after the last 48 h incubation period. Estrogen deprived medium containing 2% (v/v) Triton X-100 to lyse the cells was then added at 200 µl/well. The plate was incubated on a shaker for 10 min at 300 rpm, after which the cell lysates were assayed for LDH. Lactate dehydrogenase reaction mixture was prepared according to the manufacturer's instructions immediately before use. To determine the LDH activity, 100 µl of LDH reaction mixture was added to 100 µl of 1:10 cell lysate preparation in an optically clear 96-well flat bottom plate. The absorbance was immediately measured at 492 nm to obtain a background reading. The plate was then incubated in the dark for 30 min at room temperature, where after the absorbance was again measured. Absorbance readings were corrected by subtracting the background reading for the specific sample.

2.7. MCF-7 ERα ELISA

The MCF-7 ERα ELISA determines estrogen receptor alpha protein levels synthesized by the MCF-7 cells in culture. The expression of this protein represents a bioindicator for estrogenicity because it shares an indirect correlation

with the concentration of estrogen in the culture medium which the MCF-7 cells are cultured in. The lower the ELISA OD, the lower the ERα protein levels and therefore the higher the estrogen concentration to which the MCF-7 cells are exposed to. The ERα bioassay was performed as previously described by us (Swart and Pool, 2009a). Following the culture of MCF-7 cells, medium was carefully removed from the wells and cells fixated to the tissue culture plate as previously described by Maggiolini et al. (2002). In short, 200 μl of 2% v/v paraformaldehyde in phosphate buffered saline (PBS) was dispensed in all the wells and the plate was then incubated for 45 min at room temperature. The paraformaldehyde solution was then replaced with 200 μl of 3% v/v H_2O_2 in methanol and incubated for another 45 min. Later fixation wells were washed twice with PBS. Protein adsorption sites in the well were then blocked with 3% w/v low fat milk powder in PBS for 1 h at room temperature with gently shaking. Anti-ERα was diluted 1/200 in saline containing 0.3% w/v milk powder and dispensed at 50 μl/well. The plate was then incubated for 2 h at room temperature. The wells were then washed 4 times with 200 μl PBS. Horseradish peroxidase conjugated anti-mouse immunoglobulin (AEC-Amersham International) was diluted 1/2500 with PBS containing 1% w/v human serum albumin and 0.01% v/v Tween and dispensed at 50 μl/well. Plates were incubated for another hour and the same wash procedure was followed. Tetramethylbenzidine soluble peroxidase substrate was heated to 37 °C and dispensed at 50 μl/well. Plates were incubated at room temperature for 30 min followed by the addition of 50 μl/well of stop solution (0.5 M H_2SO_4). The optical density was lastly determined at 450 nm. All ELISA readings were corrected for background. ELISA background controls are wells that receive all procedures except that the anti-ERα step is replaced by an incubation step with 0.3% (w/v) milk powder in saline. Results were normalized as ERα OD/mg cell protein which was calculated using the formula: ERα OD/mg cell protein=(OD experimental well—OD background control)/cellular protein (mg). Total cellular protein concentration was determined by replacing the cell culture supernatants of duplicate wells which is dedicated for the ERα ELISA with 100 μl of a 1 M NaOH solution, following the last 48 h culture incubation period. The 96 well plates were allowed to gently shake at room temperature for 30 min. A multi pipet was used to gently detach all cells after which the appropriate volume of cell lysate was transferred to clear 96 well plates. Total protein yield of the cell lysate was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard protein (Sigma).

In order to compare the results of multiple plates with each other, ELISA results of the experimental wells being analyzed were expressed as a percentage of the ERα OD/mg protein obtained for the 0.1 nM E2 exposure control used on all 96 well plates.

2.8. Juvenile tilapia in vivo bioassay for estrogenicity

The induction of vitellogenin by juvenile tilapias (*Oreochromis mossambicus*) was used as an *in vivo* bioassay for environmental estrogenicity. In order to determine estrogenicity, tilapias were exposed to estrogen controls or samples as previously described by us (Swart, 2009). Fourteen days post fertilization swim-up fry stage juveniles from a single breeding hatch of a single adult breeding pair (one female and one male) were used for each experiment. Exposure of fish was performed under semi-static conditions in 1 l glass bottles filled with 0.5 l of water. Samples for analysis or estrogen standards were prepared in analytical grade ethanol. Exposure of fish to analytical grade ethanol only served as a negative control. The final solvent concentration never exceeded 0.01% (v/v). A total of 25 juveniles per experimental parameter were exposed for 5 days. At the end of the exposure period, juveniles were sacrificed and protein was extracted from whole body homogenates. In short, fish were weighed, placed in protein extraction buffer (saline containing 0.01% (w/v) phenylmethylsulphonylfluoride [PMSF]) pre-cooled at 4 °C, at 10 ml buffer/g body mass. Samples were then sonicated (Omni-Ruptor 400; Omni International Inc.) at 40% power. Samples were sonicated for 15 s at 5 s bursts followed by 1 min incubation on ice. Cell debris was removed by centrifugation at 12,000*g* for 10 min at 4 °C. Cell pellets were discarded and the supernatants were aliquoted and stored at −80 °C until further use. Protein was measured according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard protein (Sigma).

2.9. Tilapia VTG ELISA

The tilapia vitellogenin ELISA was performed similar as previously described by us (Swart and Pool, 2009b). In this ELISA, an in-house VTG antibody was used to detect VTG. An in house VTG antibody was prepared by immunizing mice with vitellogenin isolated from estrogen exposed tilapia females. Nunc-Immuno Maxisorp® plates (Nalge Nunc, Denmark) were used for all ELISA assays. Plates were coated overnight at 4 °C with 50 μl/well of 1/2000 diluted anti-VTG antiserum in saline. At the end of the incubation period wells were decanted and washed four times with saline. Following the wash procedure, remaining adsorption sites were blocked by dispensing 0.2 ml of block solution (2% v/v human serum albumin [HSA] in saline) per well. The plate was then incubated for one hour at room temperature. The plate was washed as before where after samples or purified VTG standards (50 μl) as well as 50 μl biotinylated-VTG were added to each well. Plates were then incubated for three hours at room temperature. The plate was washed using the same procedure as earlier described. Avidin horse radish peroxidase (AV–HRP) was diluted 1/2000 with saline containing 1% (w/v) HSA and dispensed at 50 μl/well. Plates were incubated for 1 h at room temperature where after it was decanted and washed eight times with saline. Tetramethylbenzidine soluble substrate was heated to 37 °C and dispensed at 50 μl/well. Plates were incubated at room temperature for 20 min followed by the addition of 50 μl/well of stop solution (0.5 M H_2SO_4). The optical density was finally determined at 450 nm. A standard curve was drawn from the VTG standards. The VTG concentration of juvenile tilapia homogenates was calculated using the standard curve.

2.10. Statistical analysis

The results were analyzed by using single factor analysis of variance (ANOVA) and pairwise multiple comparison procedure (Student–Newman–Keuls method). A pairwise multiple comparison procedure (Tukey's HSD) was used to indicate significant different groups ($P<0.001$).

3. Results

In the present study, water from three sites of the Eerste River, namely Jonkershoek, Stellenbosch STW and Spier site, were analyzed for estrogenicity using a battery of biological assays as described in the introduction section. Water samples were collected in 2006 and 2007 during January (January: high summer) and April (April: after first winter rains). Table 1 shows the total rainfall and average temperature data obtained from the South African weather office (www.weathersa.co.za) for the area at the sample collection times.

3.1. Jonkershoek analyzed for estrogenicity

Table 2 shows the results obtained for assessing water samples collected from Jonkershoek for estrogenicity using the battery of assays. The concentration of estrone in the control site was very low, near the detection limit of the assay and was statistically similar ($P>0.05$) to the assay negative control. Similar to the results obtained for estrone, none of the samples collected at Jonkershoek was estrogenic when analyzed with the MCF-7 total LDH assay or the MCF-7 ERα assay because test assay values obtained with the latter two assays were statistically similar ($P>0.05$) to their respective assay negative controls. Exposure of juvenile tilapias to water extracts collected during April 2006 and 2007 resulted in statistically similar ($P>0.05$) VTG concentrations compared to the control fish. However, exposure of juveniles to water extracts collected during January 2006 and 2007 showed significantly ($P<0.001$) higher VTG levels in comparison with the assay negative control.

3.2. Stellenbosch STWs analyzed for estrogenicity

Estrone concentrations were significantly higher ($P<0.001$) compared to the assay negative control ranging between 14.756 ± 0.519 and 19.421 ± 0.066 ng/l for water samples collected during

Table 1
Rainfall and temperature recorded. Total rainfall and temperature recorded for Boland area (Stellenbosch) during January and April for both 2006 and 2007, respectively.

	2006		2007	
	January	April	January	April
Total rainfall (mm)	0	41	0	88
Average maximum temperature (°C)	32.08	26.18	32.88	26.04
Average minimum temperature (°C)	18.09	13.25	18.22	12.9

Table 2
Jonkershoek analyzed for estrogenicity using the battery of assays. Water samples were collected from Jonkershoek in January (high summer) as well as April (after first rains) during 2006 and 2007. Water samples were analyzed for estrone, estrogen dependent proliferation of MCF-7 cells using lactate dehydrogenase (LDH) as biomarker, estrogen dependent reduction of estrogen receptor alpha (ERα) expression by MCF-7 cells as well as estrogen dependent induction of vitellogenin (VTG) in Tilapia juveniles. Samples significantly higher ($P<0.001$) than the −ve control are indicated with **.

Jonkershoek					
Assay	−ve	2006		2007	
		January	April	January	April
Estrone ($n=4$) (ng/l)	1.823 ± 0.320	1.430. ± 0.492	2.234 ± 0.433	1.659 ± 0.398	1.809 ± 0.457
MCF-7 total LDH ($n=8$) (% of −ve)	100.000 ± 2.879	101.021 ± 6.065	96.086 ± 5.892	106.109 ± 7.3123	106.049 ± 8.275
MCF-7 Erα ($n=8$) (OD/mg protein, % of −ve)	100.000 ± 7.385	98.636 ± 14.265	97.39 ± 6.204	94.814 ± 12.604	91.727 ± 11.535
Tilapia VTG ($n=8$) (μg/mg total protein)	6.786 ± 1.447	11.506 ± 1.649**	9.811 ± 1.825	13.066 ± 1.195**	9.114 ± 1.092

Table 3
Stellenbosch sewage treatment works (STWs) analyzed for estrogenicity using the battery of assays. Water samples were collected from Stellenbosch STW in January (high summer) as well as April (after first rains) during 2006 and 2007. Water samples were analyzed for estrone, estrogen dependent proliferation of MCF-7 cells using lactate dehydrogenase (LDH) as biomarker, estrogen dependent reduction of estrogen receptor alpha (ERα) expression by MCF-7 cells as well as estrogen dependent induction of vitellogenin (VTG) in Tilapia juveniles. Samples significantly higher ($P<0.001$) than the −ve control are indicated with **. Assay values printed in italics indicate significant higher values ($P<0.001$) of a specific assay compared between the January and April samples analyzed for a specific year.

Stellenbosch STW					
Assay	−ve	2006		2007	
		January	April	January	April
Estrone ($n=4$) (ng/l)	1.823 ± 0.320	*17.814 ± 0.215***	16.513 ± 0.431**	*19.421 ± 0.066***	14.756 ± 0.519**
MCF-7 total LDH ($n=8$) (% of –ve)	100.000 ± 2.879	108.794 ± 2.439	*130.163 ± 1.800***	*103.016 ± 6.399*	69.875 ± 5.984**
MCF-7 ERα ($n=8$) (OD/mg protein, % of −ve)	100.000 ± 7.385	87.447 ± 6.630	*61.946 ± 3.756***	33.300 ± 1.576**	28.265 ± 1.283**
Tilapia VTG ($n=8$) (μg/mg total protein)	6.786 ± 1.447	7.752 ± 1.753	*12.894 ± 1.720***	*17.167 ± 2.108***	10.636 ± 1.299**

January and April of 2006 and 2007 at Stellenbosch STWs (Table 3). The estrone concentration was significantly higher ($P<0.001$) in water samples collected during January 2006 compared to April 2006. Water samples collected in January 2007 contained significantly higher ($P<0.001$) estrone concentrations compared to April 2007.

Stellenbosch STWs extract collected in April 2006 was the only sample that tested positive with the MCF-7 proliferation assay. MCF-7 cells exposed to water extracts from this sampling date at Stellenbosch STWs resulted in a 1.3-fold increase ($P<0.001$) in total LDH compared to the assay negative control. No significant differences ($P>0.05$) in the total LDH levels could be detected comparing MCF-7 cells exposed to water extracts collected during January 2006 and 2007. The total LDH levels of MCF-7 cells exposed to water extracts collected in April 2007 were significantly ($P<0.001$) lower compared to that of the assay control. This may be an indication of cytotoxicity. MCF-7 cells exposed to water extracts from April 2006 resulted in significantly higher ($P<0.001$) total LDH levels compared to January 2006.

Stellenbosch STW water extract collected in January 2006 was the only sample that resulted in a statistically similar ($P>0.05$) ERα expression value compared to the assay negative control. Sewage treatment works water extracts collected in April 2006, January and April 2007 all resulted in significantly lower ($P<0.001$) ERα expression values compared to the assay negative control. ERα expression values were significantly lower ($P<0.001$) for MCF-7 cells exposed to water extracts collected during April 2006 compared to January 2006, whereas no significant differences ($P>0.05$) could be detected comparing ERα expression values of MCF-7 cells exposed to water extracts collected during January and April 2007.

Tilapia juveniles exposed to Stellenbosch STWs water extracts collected in January 2006 synthesized statistically similar ($P>0.05$) VTG concentrations compared to control fish. Exposure of tilapias to Stellenbosch STW water extracts from all the other sampling dates resulted in significantly higher ($P<0.001$) VTG concentrations. Juveniles exposed to water extracts collected during April 2007 resulted in significantly higher ($P<0.001$) VTG concentrations compared to January 2006.

3.3. Spier site analyzed for estrogenicity

Table 4 displays the results obtained for assessing water samples collected at Spier site for estrogenicity using the battery of assays. The results obtained among the test assays were similar for all the assays when water samples collected at Spier site were analyzed. All the test assays showed significantly different ($P<0.001$) values for each of the sampling dates being analyzed compared to their respective assay negative controls. Estrone concentrations ranged between 10.316 ± 0.570 and 22.989 ± 0.078 ng/l for water samples collected in January and April during both 2006 and 2007. Water samples collected in January 2006 contained significant higher ($P<0.001$) estrone concentrations compared to April 2006, whereas water samples collected in April 2007 contained significant higher estrone concentrations compared to January 2007.

MCF-7 cells exposed to water extracts collected from Spier site during 2006 and 2007 resulted in a significant increase in total LDH (ranging between 15.196% and 29.851%) compared to the assay negative control. No significant differences could be observed ($P>0.05$) comparing total LDH levels of MCF-7 cells exposed to water extracts of January and April 2006. The latter is also true comparing total LDH levels of MCF-7 cells exposed to water extracts collected during January and April 2007.

Exposure of MCF-7 cells to water extracts from Spier site also resulted in a significant ($P<0.001$) decrease in ERα expression levels ranging between 51.504 ± 3.078% and 11.564 ± 4.721%

Table 4
Spier site analyzed for estrogenicity using the battery of assays. Water samples were collected from Spier site in January (high summer) as well as April (after first rains) during 2006 and 2007. Water samples were analyzed for estrone, estrogen dependent proliferation of MCF-7 cells using lactate dehydrogenase (LDH) as biomarker, estrogen dependent reduction of estrogen receptor alpha (ERα) expression by MCF-7 cells as well as estrogen dependent induction of vitellogenin (VTG) in Tilapia juveniles. Samples significantly higher ($P<0.001$) than the −ve control are indicated with **. Assay values printed in italics indicate significant higher values ($P<0.001$) of a specific assay compared between the January and April samples analyzed for a specific year.

Spier site					
Assay	−ve	2006		2007	
		January	April	January	April
Estrone ($n=4$) (ng/l)	1.823 ± 0.320	*16.050 ± 0.280***	10.316 ± 0.570**	12.077 ± 0.065**	*22.989 ± 0.078***
MCF-7 total LDH ($n=8$) (% of −ve)	100.000 ± 2.879	115.196 ± 8.377**	115.840 ± 3.214**	122.970 ± 4.366**	129.851 ± 5.418**
MCF-7 Erα ($n=8$) (OD/mg protein, % of −ve)	100.000 ± 7.385	51.504 ± 3.078**	*11.564 ± 4.721***	28.394 ± 3.217**	35.701 ± 5.844**
Tilapia VTG ($n=8$) (μg/mg total protein)	6.786 ± 1.447	10.119 ± 2.014**	*12.988 ± 1.894***	13.076 ± 1.884**	13.958 ± 1.563**

compared to the assay negative control. MCF-7 cells exposed to water extracts from Spier site, April 2006 resulted in significantly ($P<0.001$) lower ERα expression levels compared to January 2006. No significant differences ($P>0.05$) in ERα expression levels could be observed for the January 2007 and April 2007 water extracts.

Tilapia juveniles exposed to water extracts from Spier site collected during January and April 2006 and 2007 synthesized significantly higher ($P<0.001$) VTG compared to control fish. Vitellogenin concentrations ranged between 12.988 ± 2.014 and 13.958 ± 1.563 μg/mg. Fish exposed to water extracts collected during April 2006 induced significantly ($P<0.001$) higher VTG concentrations compared to water collected during January 2006. No significant differences in VTG concentrations were observed for tilapia juveniles exposed to water extracts collected during January and April 2007.

4. Discussion

Earlier studies have indicated that seasonal variation in river water quality and EDCs concentration do occur (Ouyanga et al., 2006; Eun-Joung et al., 2007). Climatic data from the Stellenbosch area for 2006 and 2007 (Table 1) show that there are major difference in the total rainfall and average temperature between January and April. The run rates as well as quality of river water from the Eerste River has previously been reported to differ between January and April (Ngwenya, 2006).

During January the river contains very little or no water and high concentrations of sewage and industrial effluents. During the winter the concentration of sewage and industrial effluents are highly diluted because of the high rainfall. Due to this, water was collected during high summer (January) and after the first winter rains (April) to analyze for estrogenicity using a battery of assays. The Eerste River was chosen to implement this battery of assays, because several previous studies have investigated contaminants in this river (Pool et al., 2000; Reinecke et al., 2003). The Eerste River also provides an ideal study area because of various degrees of domestic, agricultural and industrial activities along the river. Water samples were firstly analyzed for the specific female hormone, estrone. Total estrogenicity of the water samples were determined using two more *in vitro* screens namely a modified MCF-7 proliferation assay (E-SCREEN) and an MCF-7 ERα protein determination ELISA. Vitellogenin induction in juvenile tilapias was used as a short term exposure *in vivo* assay.

Analysis of the water samples collected at Jonkershoek was negative for the estrone ELISA, MCF-7 LDH assay as well as the MCF-7 Rα assay. Each of the above mentioned assays produced assay results statistically similar ($P>0.05$) compared to the respective assay negative control. The VTG ELISA suggests that samples collected from Jonkershoek January 2006 and 2007 were estrogenic. However, none of the other test assay could confirm this result. Moreover, significant VTG induction was only observed for fish exposed to water samples collected during January, which is high summer when water flow is restricted and fungal growth was observed in the water. No human, agricultural or industrial activity occurs at Jonkershoek. Therefore, a possible explanation for the induction of VTG in juvenile tilapias exposed to water samples collected during January 2006 and 2007 may be that the water was contaminated with estrogenic mycotoxins produced by fungi or even phytoestrogens. Estrogenic mycotoxins produced by *Fusarium* species have previously been detected in river water and sediment samples (Wu et al., 1990). It is also well known from previous studies that phytoestrogens do have the ability to disrupt endocrine disruption (Kuiper et al., 1998; Giesy et al., 2002). However, the sensitivity of juvenile tilapias towards phytoestrogens exposure and estrogenic mycotoxins in comparison with other *in vitro* assays for estrogenicity for instance the E-SCREEN assay has not been investigated before.

Analysis of the sewage treatment works' effluent entering the Eerste River for estrogenicity resulted in very consistent data using the battery of assays. All the assays except for the estrone ELISA suggested that water collected during January 2006 are not estrogenic. In contrast, all the assays suggested that water samples collected from Stellenbosch STWs during April 2006 are estrogenic. Similar, all the assays except for the MCF-7 LDH assay also suggests that water samples collected during January 2007 and April 2007 are estrogenic. The estrone concentrations detected in water samples collected from Stellenbosch STWs are similar to the lower range of estrone concentrations detected in sewage effluent from Britain (Desbrow et al., 1998), Italy (Baronti et al., 2000), Germany (Ternes et al., 1999a), Canada and Netherlands (Belfroid et al., 1999). No apparent trend could be detected, comparing the estrogenicity of water samples collected during January 2006 and April 2006 for 2006 and 2007, respectively. MCF-7 LDH results for April 2007 were significantly lower compared to the control. This data suggest that the specific water extract is cytotoxic. Cytotoxicity has been reported as a major drawback of the MCF-7 proliferation assay before (Reel et al., 1996). MCF-7 data should be interpreted in parallel with other *in vitro assays*. Cytotoxicity should not influence the outcome of the MCF-7 ERα assay because ERα levels are expressed as a percentage of total cellular protein.

The Spier collection site is on the Eerste River, downstream from Stellenbosch and water at this point in the Eerste River contains pollutants from formal and informal housing development, agricultural and industrial activities (Fig. 1). All of the assays used to assess the estrogenicity of the water samples

collected at all four sampling dates during 2006 and 2007 suggest that the water was estrogenic. Water samples analyzed with the estrone ELISA, MCF-7 LDH assay, MCF-7 ERα assay and the juvenile tilapia VTG assay resulted in significantly different ($P < 0.001$) assay results compared to the respective assay negative control, suggesting that the samples are estrogenic. The estrone concentrations detected in water samples collected from Spier site are similar to the estrone concentrations detected in river water samples from previous studies. A study conducted by Xiao et al. (2001) on the river Thames in England reported estrone concentrations ranging from 0.2 to 17 ng/l. Similar to the analysis of Stellenbosch STWs, no apparent trend could be noticed when comparing the estrogenicity of samples collected during January and April for 2006 and 2007, respectively.

The use of a combination of assays dependent on different modes of action is required to confidently analyze water samples as a first tier screen for estrogenicity. It is evident from the present study that the different assays that were used can be successfully employed as a battery of assays to screen environmental water samples for estrogenicity. The results obtained from this battery of assays should be interpreted as a first tier screen for estrogenicity and not monitoring of the Eerste River for estrogenicity. Samples that tested positive should be further investigated using second and third tier screens with routine sampling in order to monitor the Eerste River for estrogenicity.

To our knowledge this is the first study that has evaluated the estrogenicity of a river in South Africa and more specifically Stellenbosch, using a battery of assays consisting of both *in vitro* and *in vivo* screens. Inefficient sewage treatment works, industrial as well as agricultural activities, are polluting our natural environment. The development and implementation of both *in vitro* and *in vivo* screens for estrogenicity, which are applicable to the Southern African context (i.e. rapid assays that are affordable and uses indigenous species) is of cardinal importance to monitor our environment.

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