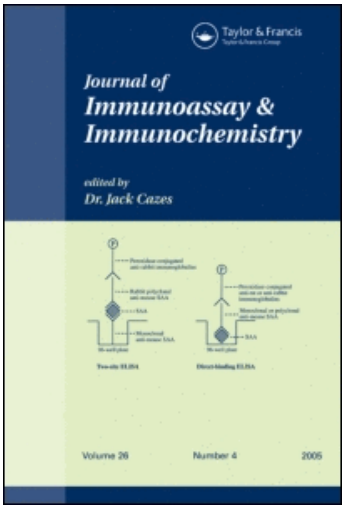

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Rapid Detection of Selected Steroid Hormones from Sewage Effluents using an ELISA in the Kuils River Water Catchment Area, South Africa

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Abstract: Steroid hormones are naturally synthesized by both humans and animals and are released into the environment. Significant levels of steroid hormones have been detected in sewage effluent around the world. The potential problem is that these hormones may interfere with the normal function of the endocrine systems, thus affecting reproduction and development in wildlife. Due to the major shortage of water in Western Cape, South Africa there is a great need to recycle water by either direct or indirect methods. The treated sewage effluent-natural surface water mixture found in the Kuils and Eerste Rivers is used directly for irrigation of agricultural areas. Sewage effluents were collected from four sites (Jonkershoek, Belville, Zandvliet, and Macassar) and subjected to C_{18} solid phase extraction. Commercially available rapid ELISA kits were validated for the quantification of estrogens in these sewage effluent samples. Analysis of estrone, estradiol, and estriol levels showed a significant difference between the control site (Jonkershoek) and sewage effluent from the three sewage treatment works. Steroid hormone concentrations detected in these sewage effluents were similar to reports from Brittan, Italy, Germany, Canada, and Netherlands.

Keywords: Steroid hormones, Estradiol, Estrone, Estriol, ELISA, Sewage

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INTRODUCTION

Steroid hormones are a group of biologically active compounds that are synthesized from cholesterol and have a cyclopentan-o-perhydrophenanthrene ring in common.[1] The steroids include progestogens, glucocorticoids, mineralocorticoids, androgens and estrogens.[2] Natural steroids are secreted by the adrenal cortex, testis, ovary, and placenta in both humans and other animals. Estrogens (estriol, estradiol, and estrone) are predominantly female hormones which are responsible for the maintenance of reproductive organs/tissue, breasts, skin, and brain. Androgens are predominantly male hormones and are responsible for tissue regeneration, especially the skin, muscle, and brain. Progesterone can be thought of as a hormonal balance, especially of estrogens. Glucocorticoids (cortisol) are produced by adrenal glands in response to stressors such as emotional upheaval, exercise, illness, surgery, or starvation.[1]

All humans and animals excrete hormones through their bodies, which can end up in the environment through sewage discharge or animal waste disposal.[3–5] Many of these hormones are peptides and are rapidly destroyed. However, steroid hormones are chemically very stable and are excreted in the free form or as conjugates. Steroid conjugates very readily biotransform to the free form.[5,6] Steroids have been detected in effluents from sewage treatment plants and surface water.[7–9] The potential problem of these natural steroids ending up in the environment is that they may interfere with the normal function of the endocrine systems, thus affecting reproduction and development in wildlife.[10] The steroids of major concern in the aquatic environment, due to their endocrine disrupting potential, are mainly estrogens.[1] However, little research has been performed on androgens in the aquatic environment and their potential endocrine disrupting properties. It has only been recently that androgens were found in treated sewage[11] and river water.[12]

Several studies in the United Kingdom have shown that wild fish exposed to treated sewage water exhibit reproductive abnormalities consistent with exposure to estrogen and estrogen mimics.[10,13–15] Toxicity identification revealed that natural and synthetic hormones excreted by humans, as well as some alkylphenolic industrial chemicals in sewage effluent, were responsible for the majority of estrogenic activity.[7,16] This problem is not confined to the United Kingdom. Studies in continental Europe, Japan, and North America confirmed that their sewage treated water also contains estrogenic chemicals,[17–19] and that these may be impacting a wide range of fish species.[20–22] The concentration of 17β-estradiol in sewage effluent range from 2.7–48 ng/L (United Kingdom),[7] $<$ limit of detection (LOD)-3 ng/L (Germany),[9] $<$LOD-64 ng/L (Canada),[9] 3.2–55 ng/L (Japan).[23]

In British sewage treatment works (STWs), the concentrations of estrone in the effluents vary widely, from 1.4 to 76 ng/L.[7] Estrone has

also been detected in sewage effluent in Italy (2.5–82.1 ng/L),[24] Germany (<LOD-70 ng/L),[9] Canada (<LOD-48 ng/L),[9] and The Netherlands (<0.4–47 ng/L).[25] Estriol was only recently reported in Italian STW influents and effluents (0.43–18 ng/L).[24] A survey of 139 polluted streams and rivers in the US found the following concentrations of steroids (in ng/L, maximum, median; LOD 5 ng/L): testosterone (214,111), estradiol (200, 160), estrone (112, 27), ethinylestradiol (831, 73), and estriol (51, 31).[26]

Estrogenic hormones in water matrix are usually quantified by techniques such as gas chromatography, mass spectrometry (GC–MS), GC–MS/MS, high performance liquid chromatography (HPLC), HPLC–MS, and HPLC–MS/MS.[27,28] These methods are reliable, but they have several potential drawbacks, such as expensive instrumentation, complex derivatization, extensive clean-up, and purification, and they also require a very high level technical expertise for operation. High cost and low throughput limit the use of these techniques. Thus, there is a strong need for rapid, simple, and cost-effective methods for quantitative analysis of estrogenic hormones, such as enzyme linked immunosorbent assay (ELISA). ELISA kits are commercially available for the quantification of estrogenic and androgenic hormones. Large numbers of samples can be analysed simultaneously and machines that do the readings are relatively cheap and also available in portable format that can be used in field studies.

Due to the major shortage of water in the Western Cape, South Africa, there is a great need to recycle water, either by direct or indirect methods. The treated sewage effluent-natural surface water mixture found in the Kuils and Eerste Rivers is used directly for irrigation of agricultural areas. The Western Cape has a high rainfall in winter with very low/no rain in summer. During summer, most of the water in these rivers is treated sewage effluent. According to a recent report of the Cape Metropolitan Council,[29] Cape Town, South Africa has 19 wastewater treatment works, of which only 20% comply with the quality requirements specified in their permits issued by the Department of Water Affairs and Forestry.[30] Problems encountered by most of these plants include inadequate sludge disposal, maintenance of the plants and bacteriological quality of the effluents.[29]

Three sewage treatment works were incorporated in this study for analysis of samples collected from respective sites, i.e., Belville, Zandvliet, and Macassar. Effluents from these plants enter the Eerste River-Kuils River system. Jonkershoek was used as a negative control, for it is situated near the origin of the Eerste Rivier, up in the Stellenbosch mountains. The aim of this study was, firstly, to validate rapid ELISAs for screening natural steroid hormones using commercially available ELISA test kits; secondly, to use the validated ELISAs to determine the level of estrogens in treated sewage effluent.

EXPERIMENTAL

Collection of Samples

Water samples were collected from three sewage plants on the E-KRCA in March 2005 at the end of the dry season and again in April 2005 after the first winter rains (Fig. 1). A control sample was collected at Jonkershoek. The control site is near the origin of the river and upstream from all human activity. 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.

Extraction of Water Samples

Water samples were extracted on C_{18} SPE columns (Anatech) using our in-house extraction procedure. In brief: C_{18} 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

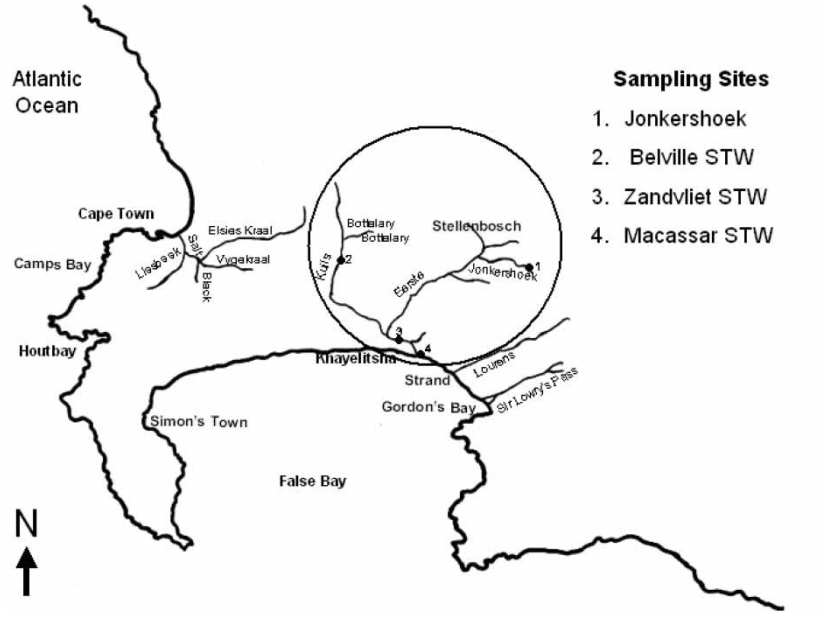

Figure 1. Water was collected from four sites along the Eerste- and Kuils River water catchment area. 1. Jonkershoek, 2. Belville STW effluent, 3. Zandvliet STW effluent, 4. Macassar STW effluent.

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 eluate was dried under air and then reconstituted to 1/1,000 th of the original sample volume with ethanol. The samples were stored at −20°C.

ELISA Reagents

All reagents required for the assays are supplied with the kits.

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 was mixed and incubated at room temperature for at least 20 minutes prior to addition to the ELISA plate.

Concentrated (1,000×) water extracts were diluted 1/10 using 0.1% (w/v) bovine serum albumin in 0.9% NaCl. The diluted (100 × concentrated) extracts were assayed directly with 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, were 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 optical density (OD) was measured at 450 nm using a plate reader. A standard curve was drawn using the OD readings obtained for the standards; the concentrations for the samples were read off this curve.

ELISA for 17β-Estradiol

Concentrated (1,000×) water extracts were diluted 1/10 using 0.1% (w/v) bovine serum albumin in 0.9% NaCl. The diluted (100 × concentrated) extracts were assayed directly with the estradiol ELISA kit (Cat. number RE52041, IBL, Germany) using the manufacturer's instruction manual. In brief: microtitre plate strips, precoated with rabbit anti-estradiol, were

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 of the wells (200 μL/well). The solutions were mixed by gently tapping the plate, whereafter 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; the concentrations of the samples were read off this curve.

ELISA for Estriol

Concentrated (1,000×) water extracts were diluted 1/10 using 0.1% (w/v) bovine serum albumin in 0.9% (w/v) NaCl. The diluted (100 × 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, were 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 readings obtained for the standards; the concentrations of the samples were read off this curve.

Validation of Assays

Kits were assayed for accuracy as follows: A dilution series of a sample containing high steroid hormone concentrations was prepared using 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 were 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.

RESULTS

Validation of Assays

Typical standard curve data for the estriol, estrone, and estradiol ELISAs are presented in Figure 2. 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 2,000 pg/mL, whereas the estradiol ELISA has a detection range between 25 and 2,000 pg/mL. Sensitivity (minimum detection limit) was determined by the supplier.

Paralalism 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 (Fig. 3). Parallelism between the standard curve of the

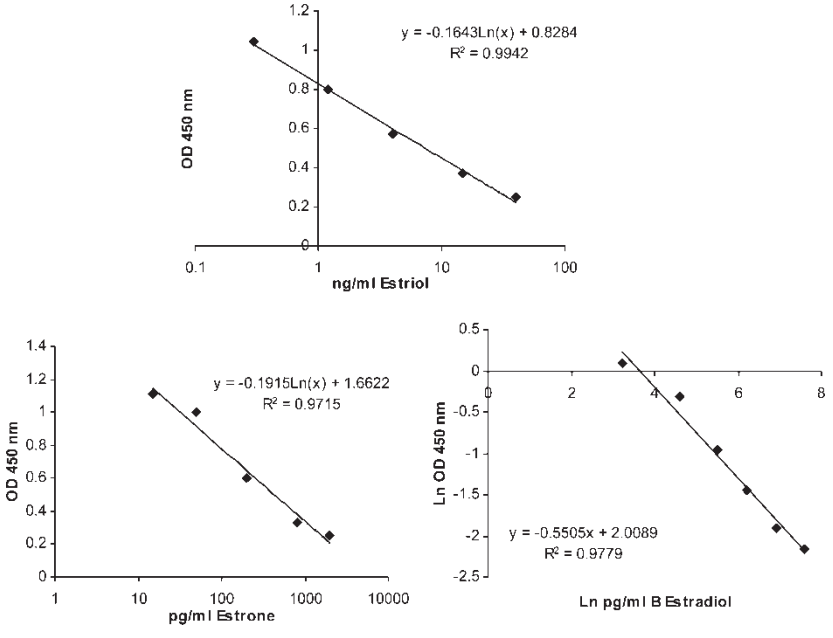

Figure 2. Standard curves for the ELISAs to determine estriol, estradiol, and estrone concentrations in sewage effluent.

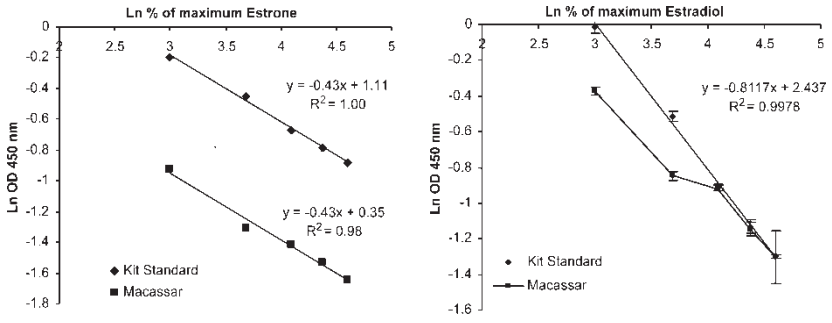

Figure 3. Parallelism between kit standard and sewage effluent sample.

estriol ELISA kit and the Macassar sewage effluent after the first rains could not be established because estriol concentrations in the C_{18} extraction were already near the detection limit of 0.3 ng/mL.

Sewage effluent was subjected to C_{18} solid phase extractions and, finally, dissolved in analytical grade ethanol as described in the Experimental section. 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 $\pm$ 7.1% at 10% ethanol, whereas 87.7 $\pm$ 2.4% could be established at 20% ethanol (Table 1). There were no significant differences between the percentage recoveries ($P = 0.125$). Ethanol had no effect on the estrone ELISA ($P = 0.576$) with recoveries of 105.3 $\pm$ 7.2% at 10% ethanol and 102.1 $\pm$ 6.5% at 20% ethanol (Table 2). The estradiol ELISA had a recovery of 88.3 $\pm$ 3.9% at 10% ethanol and 98.6 $\pm$ 14.5% at 20% ethanol (Table 3). Ethanol, again, had no significant influence on the recovery of the estradiol ELISA ($P = 0.346$).

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 $\pm$ 0.3% at 10% ethanol (Table 1). Intra-assay variation was less than 7% at 0, 10, and 20% ethanol, whereas intra-assay variation was 8.2 $\pm$ 0.7% at 10% ethanol for the

Table 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 $\pm$ 9.2	2.5 $\pm$ 0.2	
3	10	98.3 $\pm$ 7.1	1.9 $\pm$ 0.1	5.6 $\pm$ 0.3
3	20	87.7 $\pm$ 2.4	0.6 $\pm$ 0.02	

Table 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	

estrone ELISA (Table 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).

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 (Fig. 4). These values were near to the lower detection limit of the ELISA kit. Samples collected in April and March, at all the STWs, show significantly higher levels of estriol ($P = <0.001$) in comparison with the control site, except for Macassar in March.

Detection of Estrone in Sewage Effluent

Less than 0.2 ng/L estrone were detected in the Jonkershoek samples (Fig. 5). The levels of estrone were significantly 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 collected from Belville 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 it was statistically insignificant ($P = >0.001$).

Table 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	

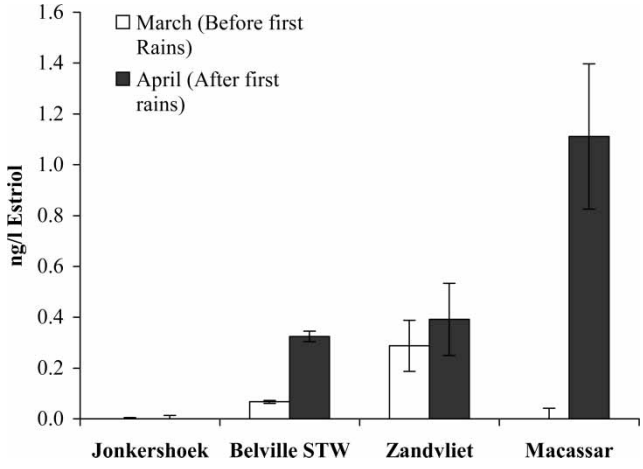

Figure 4. Water samples were collected from sites along the Kuils and Eerste Rivers in March and April. The samples were extracted using C_{18} chromatography and assayed using the estriol ELISA kit.

Detection of Estradiol in Sewage Effluent

Very low levels of estradiol were detected in samples obtained from the control site in Jonkershoek (Fig. 6). There were significantly 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.

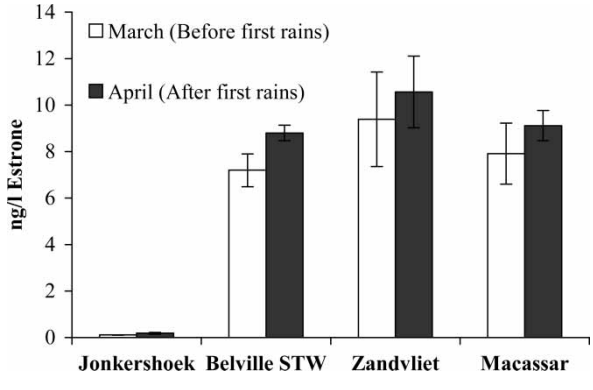

Figure 5. Water samples were collected from sites along the Kuils and Eerste Rivers in March and April. The samples were extracted using C_{18} chromatography and the assayed using the estrone ELISA kit.

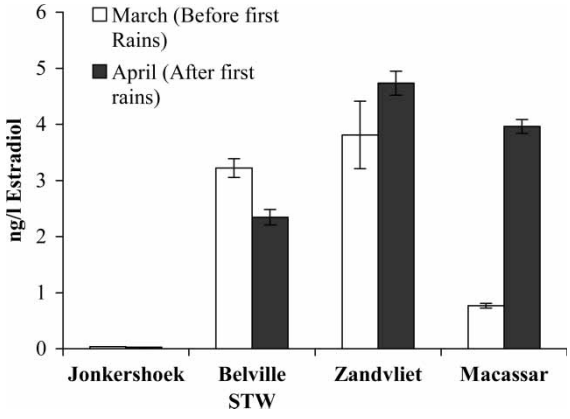

Figure 6. Water samples were collected from sites along the Kuils and Eerste Rivers in March and April. The samples were extracted using C_{18} chromatography and the assayed using the estradiol ELISA kit.

DISCUSSION

As stated in the introduction, the purpose of this work was to validate commercially available rapid ELISA kits for the quantification of estrogens in sewage effluent. One of the major potential obstacles to overcome was the fact that these ELISA kits are optimized for the quantification of estrogens in blood serum. Sewage effluent samples subjected to C_{18} solid phase chromatographic extraction is ultimately reconstituted in ethanol before assayed using the ELISA kits in this study. Recovery analysis showed that ethanol had no significant effect on the sensitivity of the ELISA kits towards the specific steroid hormone assayed (Tables 1, 2, and 3). One-way analysis of variance (ANOVA) on the hormone recovery of the ELISA kits showed that no significant difference exists between samples containing 0, 10, and 20% ethanol for the estriol, estrone, or estradiol kits. Moreover, intra- and inter-assayed variability was less than 5.6%, 8.2%, and 4.5% for the estriol, estrone, and estradiol ELISA kits, respectively, at 10% ethanol in the sample (Tables 1, 2, and 3). These data illustrate that these ELISA kits are highly repetitive, with minimal inter- and intra-assay ELISA kit interference. The validation of these ELISA kits used to determine steroid hormone levels in sewage effluent is further supported by good parallelism between dilution curves of the kit standards and sewage effluent samples (Fig. 3).

Analysis of estrone, estradiol, and estriol levels showed a significant difference between the control site (Jonkershoek) and sewage effluent from three STWs (Belville, Zandvliet, and Macassar) (Figs. 4, 5, and 6). The control site contained very low or less than the lowest observable level of estrone, estradiol, and estriol. This was to be expected because this sample site is situated in the mountains, near the origin of the Eerste River and is

not exposed to any human activity except for occasional hikers. Estriol, estrone, and estradiol concentrations were higher in April than in March, although this is not statistically significant. April samples were taken shortly after the first heavy winter rains. A possible explanation may be that the sewage plants were not able to handle the increased influent volumes of water. Therefore, some overflowing or contamination of sewage effluent with raw sewage water may have occurred.

Estriol had the lowest levels, i.e., less than 1.1 ng/L, of the three steroid hormones analyzed in this study. A study previously conducted by Baronti et al. (in 2000) showed the same trend, where estriol was detected at much lower levels than estradiol and estrone. Zandvliet showed the highest levels of both estrone and estradiol. Estrone levels were between 7.2 and 10.6 ng/L, whereas estradiol levels range between 0.8 and 4.7 ng/L. These values are similar to the lower range of estrone and estradiol concentrations detected in sewage effluent from Brittain,[7] Italy,[24] Germany,[9] Canada, and The Netherlands.[25] Although the steroid hormone levels in our study may seem low, it is still quite concerning due to the fact that this water is used for agricultural and horticultural irrigation, consumed by farm animals, farm workers, as well as people from informal settlements along river banks. Concentrations as low as 1 ng/L of 17β-estradiol (natural estrogen) led to induction of vitellogenin in male trout.[31] It was also observed that ova formed in the testis of Japanese medaka at concentrations as low as 4 ng/L 17β-estradiol.[32] Furthermore, alfalfa plants irrigated with sewage effluent containing steroid hormones display elevated levels of phytoestrogens.[33]

To our knowledge, this is the first study conducted in South Africa to demonstrate the presence of significant amounts of steroid hormones released back into the environment from sewage treatment plants. Detailed surveys are necessary to understand the distribution of steroid hormones in the environment. Significant levels of these steroid hormones do have the potential to cause endocrine disruption, not only in animals but in humans as well. Future work must be directed to test whether these samples are biologically active through *in vivo* and *ex vivo* studies.

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