

Determination of selected steroid estrogens in treated sewage effluent in the Umsunduzi (*Duzi*) River water catchment area

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Abstract

Steroid hormones, naturally synthesized by human and animals, as well as synthetic/plant-derived ones usually in contraception, may be eventually released into the environment, especially in excreta. Levels of these hormones have been detected in significant concentration in sewage effluent around the world. These compounds have the ability, at very low concentrations, to alter normal functioning of the endocrine system, which is responsible for growth and development in vertebrate systems. Their eventual discharge into water bodies can affect reproduction and development in wildlife. Recycling of waste water for human domestic consumption necessitates the need to monitor the water quality of the effluent, as well as a check for these estrogens. Treated sewage effluent from the Darvill Waste Water Works (DWWW) is discharged into the Umsunduzi River; re-use options are being investigated. Samples were collected and assayed for estrone and 17- β -estradiol (estradiol) by enzyme-linked immunosorbent assay (ELISA). The steroid hormone concentrations detected were similar to those reported for sewage effluent in Britain, Italy, Germany, Canada and The Netherlands. Preliminary removal efficiencies were noted to be comparable to those reported.

Keywords: Endocrine disrupting compound (EDC); Steroid estrogens; sewage effluent; ELISA; estradiol (E2); estrone (E1)

Introduction

Steroid hormones are biologically active compounds synthesized from cholesterol, with the common cyclopentan-o-perhydrophenanthrene ring in common [1]. Steroids include progestogens, glucocorticoids, mineralocorticoids, androgens, estrogens [2]. Natural steroids are secreted by the adrenal cortex, testis, and ovary, placenta in human and other animals. The estrogens estrone, estradiol (E2) and estrone (E1) (Figure 1), predominantly female hormones, are responsible for maintenance of reproductive organs and tissue, breast, skin and brain.

All humans and animals excrete hormones through their bodies; these hormones can end up in the environment through sewage discharge or animal waste disposal [2-5]. The steroid hormones,

chemically very stable, are excreted in the free form or as conjugates; the latter readily biotransform to the free conjugates [4,6].

Steroids have been detected in sewage treatment plant effluents and in surface water [7-11]. Their eventual presence in the environment poses a significant potential problem of interference with normal function of the endocrine systems, and can thus affect reproduction and development in wildlife. The steroids of major concern in the aquatic environment, due to their endocrine disrupting potential, are mainly the estrogens.

Several studies, in the United Kingdom [7,12-15], continental Europe, Japan and North America [16-18], have shown the reproductive abnormalities exhibited by fish consistent with exposure to concentrations of estrogen, estrogen chemicals and estrogen mimics present in treated sewage water; their presence may be found to be impacting a wide range of fish species [19-21].

These estrogen hormones have also been reported to be present at varying levels (Table 1) [22] in effluents from sewage treatment works in Britain [7], Germany [9], Canada [9], The Netherlands [23], Italy [24] and the US [25].

Against a global rainfall average of 870 mm per year, South Africa receives a pitiful 450 mm, making it the world's 30th driest country [26]. It has been reported that Durban could face water restrictions as early as next year [27]. There is thus a definite need to recycle water.

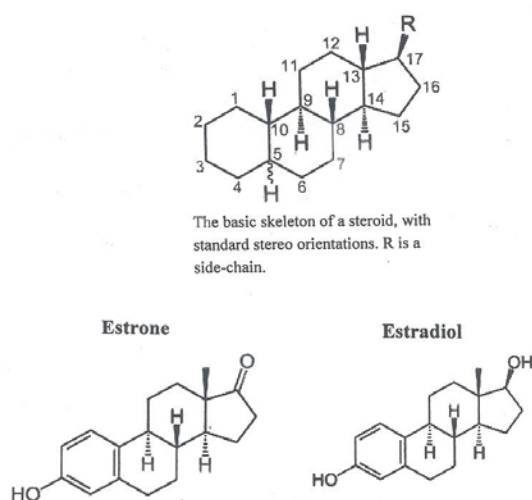


Figure 1: Structure of the steroid estrogens.

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The Darvill Waste Water Works, Pietermaritzburg, is the main sewage treatment plant for the Pietermaritzburg area, serving over 300 000 people. Treated sewage is then discharged directly into the adjacent Umsunduzi (*Duzi*) River. The process currently treats 75 ML/d through an activated sludge process, the modified Johannesburg biological nutrient removal process [28].

This river joins the Umgeni River, which is the main water supply to the Inanda dam. The latter supplies raw water to Durban Heights and Wiggins potable water treatment works, to supply potable water to the greater Durban area. Currently the dam supplies about 300 ML/d.

As the Inanda impoundment ($\sim 240 \times 10^6 \text{ m}^3$) has a retention time in the order of 2 years [29], estrogenic contaminants are not currently a concern [30]. However, direct recycling options are being considered.

A pilot project is currently investigating the use of membrane bioreactors (MBR), followed by advanced treatment, to treat waste water to a standard where it is drinkable [31]. The necessity of monitoring of the water quality is therefore a critical requirement to assess performance of the MBR.

Internationally, and also in South Africa, treated waste water effluent has to comply with certain legal requirements before discharge into natural water courses. However, there is currently no legislation in South Africa as regards maximum allowable levels of these estrogen hormones in water matrices.

To date, the only report on the estrogen levels in treated sewage effluent in South Africa was by Swart and Pool [32], in the Kuils River Water Catchment Area. Their results are listed in Table 1, under "South Africa". They reported 10.6 ng/L, for estrone, and 4.7 ng/L, for 17- β -estradiol Table 2, using the Enzyme-linked immunosorbent assay (ELISA) [33,34] technique.

Our main aim was therefore to determine, the presence, if any, of these steroid estrogens in the current (conventional) Darvill treated sewage effluent, and Umsunduzi River up- and down-stream and their corresponding concentrations. Some preliminary findings regarding the analytical techniques available, and the treatment plant efficiency of removal is also briefly reported on.

Methodology

Sample collection

As per the recommended procedure [32], glass bottles (250 mL)

Estrogen hormone	Country	Minimum (ng/L)	Maximum (ng/L)
17- β -Estradiol	UK	2.7	48
	Germany	ND [#]	3
	Canada	ND [#]	64
	Japan	3.2	55
	South Africa	ND	4.7
Estrone	UK	1.4	76
	Germany	ND [#]	70
	Canada	ND [#]	48
	South Africa	< 0.2	10.6
	Netherlands	< 0.4	47
	Italy	2.5	82.1

[#]ND = not detected

Table 1: Minimum and maximum levels of Estrogen hormones in selected treated sewage effluents collected from several countries.

Sample Date	Sample Site description	Estrone (ng/L)	% Removal	17- β -Estradiol (ng/L)	% Removal
11/03/2010	Darvill influent	13		20	
	Darvill effluent	3	77	5	75
	Duzi River u/s of Darvill	2		1	
	Duzi River d/s of Darvill	2		2	
01/11/2010	Darvill influent	30		33	
	Darvill effluent	11	63	9	73
	Duzi River u/s of Darvill	7		12	
	Duzi d/s of Darvill	2		4	
01/02/2011	Darvill influent	42		57	
	Darvill effluent	16	62	18	68
	Duzi River u/s of Darvill	3		3	
	Duzi River d/s of Darvill	5		5	

u/s = control sample taken upstream of the Darvill WWW

d/s = control sample taken downstream of the Darvill WWW

Table 2: Estrogen levels in sewage influent and treated sewage effluent at DWWW.

were initially washed sequentially with: detergent, rinsed with running tap water, rinsed 4 times with distilled water and finally rinsed with 25 mL HPLC grade ethanol (99.5% purity, from Merck). Inverted bottles and caps were allowed to dry on a drying rack. The head of the bottle was covered with foil before the cap was screwed on. In the absence of amber bottles, the clear glass was covered with foil. Collected water can be stored for 3 days at 4°C. No additions to the sample were required. Samples were immediately couriered "same day", packed with ice packs, to the testing laboratory, after collection.

"Control" or reference samples were collected from 2 points of the Duzi River: one sample just upstream (u/s) of the Darvill sewage treatment works, and another, downstream (d/s) of the Darvill effluent mixing zone.

As the city of Pietermaritzburg lies in the Duzi catchment upstream of the Darvill WWW, and as sewer problems are quite frequent, some detectable estrogens were considered likely.

Analytical method for assay: ELISA

The methods used for extracting and assaying environmental water samples for estrogenic compounds are described in detail in the paper by Swart and Pool [32]. These tests were extensively validated [32] using spiked, real water samples and the standards provided in the commercial kits. For estrone, the recovery averaged, over the range 15-2000 ng/L, $102.5 \pm 5.7\%$. Intra-assay and inter-assay variation was $5.5 \pm 0.3\%$ and $8.2 \pm 0.7\%$ respectively. For estradiol, the recovery averaged, over the range 25-2000 ng/L, $95.8 \pm 9.0\%$. Intra-assay and inter-assay variation was $8.9 \pm 1.0\%$ and $3.9 \pm 0.1\%$, respectively [32].

Samples were assayed for estradiol and estrone by ELISA, using the estradiol ELISA kit (cat. No. RE52041 IBL, Germany) and the estrone ELISA kit (Cat. No. DB 52051 IBL, Germany). The detection limits for both these estrogens are 1 ng/L [35].

Accuracy study

β -Estradiol and estrone (minimum 98% purity) were purchased from Sigma-Aldrich (via local agents, Capital Lab Supplies). Methanol (gradient grade for liquid chromatography) was purchased from Merck.

Statistical data analysis

Student t-tests [36] were used to examine significant differences of

the mean assay values between the two control sites and the influent/effluent samples. A significance level of $P < 0.050$ was used.

Results

The observed tests results for the Darvill WWW to date are summarized in Table 2. The statistical data analysis for the comparison of the means of the Darvill samples with both control samples are summarized in Table 3.

Estrone level in sewage effluent

The mean influent level is 28 ng/L (Standard Deviation (SD) = 15) for estrone. The corresponding mean effluent level is 10 ng/L (SD = 7).

For the Duzi River control samples, the mean level is 4 ng/L (SD = 3) and 3 ng/L (SD = 2) for the samples upstream and downstream of Darvill, respectively.

Estradiol level in sewage effluent

The mean influent level is 37 ng/L (SD = 19) for 17- β - estradiol. The corresponding mean effluent level is 11 ng/L (SD = 7).

For the Duzi River control samples, the mean level is 5 ng/L (SD = 6) and 4 ng/L (SD = 2) for the samples upstream and downstream of Darvill, respectively.

Removal efficiencies

The calculated mean sewage works removal efficiencies are: 67% (SD = 8) for estrone and 72% (SD = 4) for 17- β -estradiol.

Discussion

Occurrence and removal efficiency of E1 and E2

The steroidal sex hormones, like estradiol and estrone, are naturally excreted into the environment from human and animal sources. Recent research has shown that such EDC's are present in municipal wastewater effluent. Untreated domestic sewage contains large concentrations of estrogen (40-300 ng/L), which is highly variable, depending on the source and dilution [43].

In primary treated wastewater, estradiol has been found to range from "not detected" (ND) to 150 ng/L. For estrone, the range is 7.3-132 ng/L. In secondary treated effluent, the reported ranges are ND-43, and ND-108 ng/L, for estradiol and estrone [43]. The four main removal pathways for EDC's are: adsorption onto suspended solids,

aerobic and anaerobic biodegradation, chemical (abiotic) degradation via processes such as hydrolysis, and volatilization. In general, research has supported the strongest role for adsorption and biodegradation for the removal of such compounds.

The octanol-water partitioning coefficients (K_{ow}) of hormones suggest that they should sorb to mixed liquor suspended solids (MLSS) before significant degradation occurs. Sorption to solids has been found to be an important removal mechanism. The highest estrogenic activity was found in the digested biosolids. Hormones were adsorbed onto biosolids as the primary removal mechanism with the estradiol concentration of the biosolids 1,000 times greater than the secondary effluent concentration.

Biodegradation of estradiol and estrone has also been demonstrated. Effluent concentrations of estrone were reported at higher levels than influent concentrations for four of six sampling events, likely due to oxidation of estradiol to estrone during the treatment process. Similar activated sludge batch experiments found that estradiol was removed with concomitant increase in estrone. Estrone was biodegraded in both studies with longer incubation.

Unfortunately a fair amount of research reporting removal of estrogenic activity in wastewater treatment facilities fails to report operational parameters at the facilities studied. The latter omission has made it difficult to correlate the relationship between operational parameters and percent removal. Many studies have suggested that increased solids retention times (SRT's) result in an improved removal of hormones in wastewater treatment facilities. Hydraulic retention time (HRT) has also been found to have positive correlation with hormone removal. Higher SRT's resulted in greater percentage removal of the hydrophobic compounds being studied and suggested a minimum SRT is required for removal of such EDC's.

The ability of advanced treatment technologies, like reverse osmosis and nanofiltration membrane, membrane bioreactors, ultraviolet disinfection, and activated carbon adsorption, to remove such EDC compounds, is an area of active research.

Removal of EDC's during advanced treatment technology

MBR technology is often considered a promising development in wastewater treatment, integrating biological degradation of waste products with membrane filtration. These treatment systems are effective in removing organic and inorganic compounds as well as biological contaminants from wastewater. Steroid removal rates of 90% were achieved in membrane bioreactors with nitrification and denitrification [45]. Biological degradation has been cited as the most important factor in the removal of estrogens and other endocrine disruptors in membrane bioreactors [45]. It was found that two membrane bioreactors, Zenon and Mitsubishi, provided almost complete removal of the steroid estrogens [45].

Estrogen analysis

Estrogenic hormones in water can be determined by various techniques, such as gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), GC-MS/MS, high performance liquid chromatography (HPLC), HPLC-MS and HPLC-MS/MS [37,38]. Although these methods are reliable, some of their disadvantages are high initial capital cost of the equipment, complex derivatisation, extensive sample clean-up, purification and the requirement for staff with high technical expertise. One advantage of the HPLC-MSMS method is the ability to screen for various other compounds of different classes, like the steroid estrogens, and other endocrine disrupting

Mean Comparison	Z	t at 95% (P = 0.050)	Null hypothesis $H_0: \mu_1 = \mu_2$	Result/decision
Estrone				
u/s vs influent	2.717	2.132	$Z > t$	Reject H_0 : means are different
d/s vs influent	2.861	2.132	$Z > t$	Reject H_0 : means are different
u/s vs effluent	1.289	2.132	$Z < t$	Accept H_0 : means not different
d/s vs effluent	1.665	2.132	$Z < t$	Accept H_0 : means not different
17-β- Estradiol				
u/s vs influent	2.782	2.132	$Z > t$	Reject H_0 : means are different
d/s vs influent	2.992	2.132	$Z > t$	Reject H_0 : means are different
u/s vs effluent	1.127	2.132	$Z < t$	Accept H_0 : means not different
d/s vs effluent	1.666	2.132	$Z < t$	Accept H_0 : means not different

u/s = control sample taken upstream of the Darvill WWW

d/s = control sample taken downstream of the Darvill WWW

Table 3: Statistical data analysis for comparison of mean results for samples and controls.

compounds (EDC's) in the same run, not possible by the ELISA [33, 34], which is compound class-specific.

Due to the fact that Umgeni Water Laboratory Services do not currently offer these tests, a study was conducted to establish testing centers in the country for the purposes of routine monitoring of these steroid estrogens present in the influent and effluent at DWWW. Three centers were initially found to conduct these tests: Du Buisson (Gas chromatography-mass spectrometry (GC-MS) method) [39] (2009), FDA Laboratories [40] (Swemmer, 2010) (HPLC-tandem mass spectrometry (MS) method) and University of Western Cape (UWC) [32] (Pool, 2010) (ELISA method). It was more recently established that the University of the Free State (UFS)/LiquidTech [41] (Kemp, 2011) (HPLC-tandem mass spectrometry method) also offers estrogen tests.

The sensitivity of the HPLC-MSMS method for these hormones varies from 1000 ng/L [39,41] to as low as 10 ng/L [40] (Swemmer, 2010), at best.

The ELISA assay was adequately validated by Swart and Pool [32], with good limits of detection (1 ng/L for both estrone and estradiol), and the work was also published in an international journal. For these reasons, it was initially decided to send samples to the University of Western Cape.

Currently, there is no World Health Organization (WHO) levels or limits for 17- β -estradiol and estrone, or any of the other estrogen hormones, in their Guidelines for Drinking Water Quality (3rd Edition WHO, 2008) [42]. However, 17- β -estradiol, estrone and 17- α -ethinylestradiol are all included in the Environmental Protection Agency (EPA) Candidate List 3 (CCL 3) [43]. This is a list of contaminants, 104 in total, that are currently not subject to any proposed or promulgated national primary drinking water regulations, that are known or anticipated to occur in public water systems, and which may require regulation under the Safe Drinking Water Act (SDWA) in the United States [43] (EPA). These three hormones are also part of the list verified as potential EDC's in the recent study by Benotti et al. [44]. They also form part of the EDC package test performed by LiquidTech, University of the Free State.

Reported raw sewage influent levels in the literature range from: 0.5 to 670 ng/L for estrone, < 0.3 to 224 ng/L for 17- β -estradiol, 2-660 ng/L for estril and 0.4-13 ng/L for 17- α -ethinylestradiol [11,45].

The first study on estrogen hormones levels, conducted in South Africa, was by Poole and Swart [32], on the sewage effluents from the treatment plants in the Kuils River Water Catchment Area (Table 1) [32].

Our preliminary results (Table 2) are also similar to the lower range detected in sewage effluent from Britain, Italy, Germany, Canada and The Netherlands (Table 1) [22]. However, concentrations as low as 1 ng/L of 17- β -estradiol led to vitellogenin [46,47] in male trout [48]; it was observed that ova formed in the testis of Japanese medaka [49] at a low concentration of 4 ng/L of 17- β -estradiol [50]. This indicates that even these observed low concentrations may remain a concern.

Comparison of the mean control samples with the Darvill influent samples showed a significant difference for both estrone and estradiol (Table 3). However, the control samples were not significantly different when compared to the Darvill treated effluent samples. The relatively lower estrogen levels in the effluent are to be expected due to the removal efficiency of the activated sludge process at the sewage treatment works.

With the activated sludge treatment process, a study of the literature indicates that estrone will not be completely removed. The reported removal performance for estrone shows variation - 61% has been reported in the literature, whilst > 85 % removal efficiency has been reported for 17- β -estradiol [11,45]. Preliminary results obtained for the DWWW shows a similar removal efficiency of 67%, for estrone, and of 72% for 17- β -estradiol.

Over the sampling period, the Darvill area sampling data (Table 2) indicated significant variation in concentrations received (up to three times) in the raw sewage. Removal efficiencies however remained constant. The data confirm the presence of estrogens in the river upstream as well as downstream of the sewage treatment works, indicating sources in the city of Pietermaritzburg. Even when effluent concentrations were relatively high (01/02/2011), downstream concentrations remained almost unaffected, at least partly due to significant dilution during the summer rainfall period.

The ELISA test method for estrogen assay appears to be more sensitive than the HPLC tandem MS method: 1 ng/L can be detected for both estrone and 17- β -estradiol by ELISA [22,32], while the corresponding reported limits of quantitation are generally, at best, 10 ng/L for the HPLC-MSMS technique. This appears to be inadequate for environmental quantification requirements.

To check the accuracy of the ELISA assay, real samples from the Darvill WWT were taken and spiked at 20 ng/L with a commercial composite of E1 and E2, prepared in methanol. They were submitted to UWC for assay of E1 and E2 by ELISA. The results are summarized in Table 4.

Imprecision: The Darvill Final and Pilot Plant permeate are relatively cleaner matrices (much less suspended solids compared to Darvill raw and we should expect better precision. However, 25 and 33% relative standard deviation (RSD) was calculated for estrone and 17- β -estradiol. For Darvill raw, which has relatively more suspended solids, the corresponding imprecision was 10 and 0% RSD, for estrone and 17- β -estradiol, respectively. However, only 2 replicates were submitted ($n = 2$). The average imprecision (unspiked and spiked samples) observed is: 16% for estrone and 22% for 17- β -estradiol.

Accuracy (by recovery): Abnormally high recovery was obtained for estrone for Darvill raw spiked (180%) and for Duzi u/s Darvill spiked for estradiol (155%). Very low recovery was obtained for estrone for Duzi u/s Darvill and for estrone for Duzi d/s Darvill. The average recovery (accuracy) is: 86% for estrone and 105% for 17- β -estradiol.

One possible reason for these discrepancies is that spiking must be done with steroid in dimethyl sulfoxide solvent. Alcohols, like methanol, which was used in our "spiking" study, do not result in consistent recoveries – due to steroid being at local high and precipitate at point of entry into sample [35].

The overestimation caused by matrices in environmental samples is considered to be an inherent problem with some ELISA methods. More recent related research on ELISA test methods and kits for estrogen assay have been reported [50-53]. Hirobe et al. [54] developed ten kinds of ELISA systems for quantitative analysis of endocrine disruptors, surfactants and estrogens for the analysis of environmental and biological samples; these samples included influent and effluent samples from a sewage treatment plant as well. Good correlations were observed between the ELISA's and instrumental analytical methods, like high performance liquid chromatography, and tandem mass spectrometry in all cases. They [54] recommended that a proper

Sample number	Actual Description/site	Estrone (ng/L)	% Recovery/ Accuracy	17-β-Estradiol (ng/L)	% Recovery/ Accuracy
1	Darvill influent/raw	39		57	
2	Darvill influent/raw duplicate	45		57	
	mean	42		57	
	SD*	4		0	
	RSD%*	10		0	
3	Darvill influent/raw spiked	78	180	70	65
4	Darvill effluent/ final	13		22	
5	Darvill effluent/final duplicate	18		14	
	mean	16		18	
	SD	4		6	
	RSD%	25		33	
6	Darvill effluent/final spiked	34	90	41	115
7	Duzi upstream Darvill	3		3	
8	Duzi upstream Darvill spiked	13	50	34	155
9	Duzi downstream Darvill	5		5	
10	Duzi downstream Darvill spiked	10	25	22	85
11	Pilot plant permeate/final	34		7	
12	Pilot plant permeate/final duplicate	29		11	
	mean	32		9	
	SD	4		3	
	RSD%	13		33	
	<u>Overall means</u>	precision	accuracy	precision	accuracy
		10	180	0	65
		25	90	33	115
		13	50	33	155
			25		85
	mean	16	86	22	105
	SD	8	68	19	39

*RSD = relative standard deviation

*SD = standard deviation

Table 4: Accuracy check of the ELISA test.

cleanup method can eliminate such problems of over- or under-estimation because of cross reactivity or matrix effects: for example, use of dichloromethane as elution solvent of choice for solid phase extraction.

The obstacle faced by Swart and Pool [32] was the fact that the commercial ELISA kits were optimized for quantitation of estrogen in blood serum. However, their validation work of these kits for steroid hormone assay in sewage effluent showed that the kits are highly repetitive, with minimal inter- and intra-assay ELISA kit interference. This was further supported by good parallelism between dilution curves of the kit standards and sewage effluent samples [32].

There are plans to have the ELISA method set up “in-house” at Umgeni Water laboratory. In the light of a recent publication [55], the option of using GC-MS for the subsequent assay of these compounds at Laboratory Services, Umgeni Water is being seriously considered. Until this, or another suitable test method is developed and validated “in-house”, samples will be outsourced to UWC in the interim.

Conclusion

To date, this is the second report on detection of steroid estrogens

in water catchment areas in South Africa, and also the first report for the Umsunduzi (*Duzi*) River water catchment area, Kwa-Zulu-Natal region.

The steroid hormone concentrations detected were similar to those reported for treated sewage effluent in Britain, Italy, Germany, Canada and The Netherlands. Percentage removals were consistent but significant variation was noted in samples over the one year period.

Further sampling for estrogen monitoring at DWWW, is required on a long-term basis (\pm 1-2 years). The accuracy, and imprecision, of the test methods and results also need to be better evaluated. The latter will assist in making more definitive, meaningful conclusions.

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