

# Oestrogenic activity in drinking waters from a rural area in the Waterberg District, Limpopo Province, South Africa

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## Abstract

In South Africa, limited data are available regarding possible oestrogenic activity in the aquatic systems and especially drinking water. Water in the rural areas is often contaminated with a complex mixture of toxic compounds originating from nearby industries, agriculture and households. In these rural areas the only access to drinking water is boreholes, natural springs and rivers. Thus human exposure to environmental contaminants in drinking water is potentially high. Two rural communities near Mokopane in the Waterberg district of the Limpopo Province were selected in order to screen for oestrogenic activity in drinking water sources in a rural area. Eleven 1 l water samples (Molekane n= 4; Sekuruwe n= 7), were collected in prepared glass bottles and extracted on a SPE C18 cartridge and reconstituted into ethanol. The recombinant yeast oestrogen screen was used to determine the oestrogenic activity in the extracts. 17 $\beta$ -estradiol (E2) was used as a positive control and the results were expressed as estradiol equivalents (EEq). The EEq of the water from both the communities ranged between 0.63 - 2.48 x10<sup>-9</sup> g/l. These concentrations are similar to other studies conducted in Korean river waters in rural and city areas and Flemish surface waters. The recombinant yeast screen confirmed oestrogenic activity in the drinking water samples; further investigation is necessary to determine the source of the contamination and association with impaired growth.

**Keywords:** recombinant yeast screen, oestrogenic activity, water, rural, Limpopo, South Africa

## Introduction

Worldwide, there are a number of reports on the presence of a variety of environmental chemicals with oestrogenic activity in aquatic systems. A number of industrial, agricultural and household chemicals released into the environment are believed to disrupt normal endocrine function in humans and animals (Colborn et al., 1993; Toppari et al., 1996, Jobling and Tyler, 2006). These endocrine disrupting chemicals (EDCs) include a wide range of molecules such as organochlorine pesticides, phthalates, alkylphenols, natural hormones and pharmaceuticals (Safe and Gaido, 1998). Endocrine disruption has been associated with wildlife with observations documenting oestrogenic, androgenic and anti-thyroid actions in fish found in sewage and paper mill effluents; exposure of alligators to agricultural chemicals; and exposure of birds, fish and mammals to complex mixtures of chemicals in North America and Europe (Guillette, 2006). In South Africa the first histological evidence of intersex in feral sharp-tooth catfish from oestrogen-polluted water was reported by Barnhoorn et al. (2004). These effects in response to ecologically relevant exposure levels have been observed in a number of vertebrate species (Guillette, 2006), for example infertility and behavioural changes in polar bears, beluga whales and laboratory animals (Colborn et al., 1993; Kamrin, 1996; Kilian et al., 2007).

Human health impacts that have been attributed to EDC exposure include abnormalities in male reproductive health (Aneck-Hahn et al., 2007; De Jager et al., 1999; De Jager et al.,

2001; Kamrin, 1996; Kilian et al., 2007; Phillips and Foster, 2008; Toppari et al., 1996); female reproductive health (Kirkhorn and Schenker, 2002; Sharpe and Skakkebaek, 1993); increased incidence of reproductive cancers (Sharpe and Skakkebaek, 1993); immunological effects (WHO, 2002); neurodevelopmental effects (Eskenazi et al., 2006) and thyroid disease (Phillips and Foster, 2008; WHO, 2002). More recently diabetes, obesity and anogenital distance have been identified as new biological endpoints (Phillips and Foster, 2008).

Currently only limited data are available on the levels of oestrogenic contamination in aquatic systems in South Africa. A pilot study to determine the presence of specific chemicals (p-nonylphenol (p-NP), polychlorinated biphenyls (PCBs) and organochlorine pesticides) known to have oestrogenic activity was performed on water samples from particular South African rivers and dams during the summer of 1998/99. The results indicated that there was oestrogenic contamination in a number of areas. A study done in November 2001 in the Rietvlei Nature Reserve reported oestrogenic activity between 0.31 and 2.1 ng/l in various surface and groundwater sites (Aneck-Hahn et al., 2008). A more comprehensive study done in the Reserve over a 2-year period indicated that the oestrogenic activity had increased with cytotoxicity (69%) being reported in the YES assay and oestrogenic activity between 0.16 and 1.92 ng/l. The more sensitive ER-Calux assay confirmed the oestrogenic activity between 0.32 and 16.0 ng/l (Bornman et al., 2007). This is a disturbing finding, in the light of the limited water resources in South Africa, as many humans and animals are dependent on these sources for their drinking water (De Jager et al., 2000; Bornman et al., 2007).

In South Africa the environments in rural and urbanised areas are often contaminated with a complex mixture of toxic compounds originating from industries, agriculture and private households (Aneck-Hahn, 2003). Many of these pollutants end

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up in surface waters, such as dams, rivers and eventually the ocean. Toxic contaminants may disturb the biological condition of aquatic ecosystems and be harmful for humans exposed through food or drinking water. In the rural areas the main sources of the communities' water for drinking and household purposes are local boreholes, natural springs and rivers.

Most chemical analytical methods are expensive and are not sufficient to identify all the environmental chemicals and to predict a combination of toxicity and bioavailability. A more appropriate strategy is to screen samples, using short-term, relatively inexpensive bioassays for indications of toxic effects and then to prioritise them for chemical analyses or more intensive studies (Aneck-Hahn, 2003). Many studies have focused on compounds that are oestrogen-receptor agonists including organochlorine pesticides, phthalates, alkylphenols, some natural hormones and some pharmaceuticals (Safe and Gaido, 1998). A number of assays have been developed to detect oestrogenic activity of chemicals, the Estrogen Screen (E-screen) (Soto et al., 1995), the Yeast Oestrogen Screen (YES-screen) (Routledge and Sumpter, 1996), the Estrogen Receptor mediated chemical activated luciferase gene expression (ER-Calux) (Legler et al., 1999) and the MCF-7-p-Vit-tk-Luc-Neo (MVLN) transgenic human cell line assay (Pons et al., 1990). These assays are also useful for detecting oestrogenic activity of complex environmental matrices such as effluents of wastewater treatment plants, or other industries or of surface waters (Desbrow et al., 1998; Witters et al., 2001; Van Den Belt et al., 2004). *In vitro* assays have advantages in terms of rapidity and low cost, saving on the use of animals (Phillips and Harrison, 1999).

The present study was an extension of a project initiated by the Department of Food and Hospitality Management of the Tshwane University of Technology (formerly Technikon Pretoria) on the nutritional status and dietary intake of children to determine whether poor diets increased the prevalence of stunting. The 2 rural villages Molekane and Sekuruwe in the Limpopo Province (formerly Northern Province) were selected because the people in these communities are poor, the unemployment rate is high and the lack of services and infrastructure could have an impact on chronic malnutrition among young children. The stunting rates among children in the Limpopo Province rural areas have been calculated to be 34% (Theron, 2000). The results of the study indicated that the nutritional diet, however, was not the cause of the stunted growth. This finding led to the hypothesis that the health status of the children was related to rapid urbanisation and informal settlements on the outskirts of

cities where there is a risk of exposure to chemical contaminants that may impair growth. This study was conducted to determine whether the drinking-water sources in these areas contain oestrogenic contaminants.

## Materials and methods

### Study area

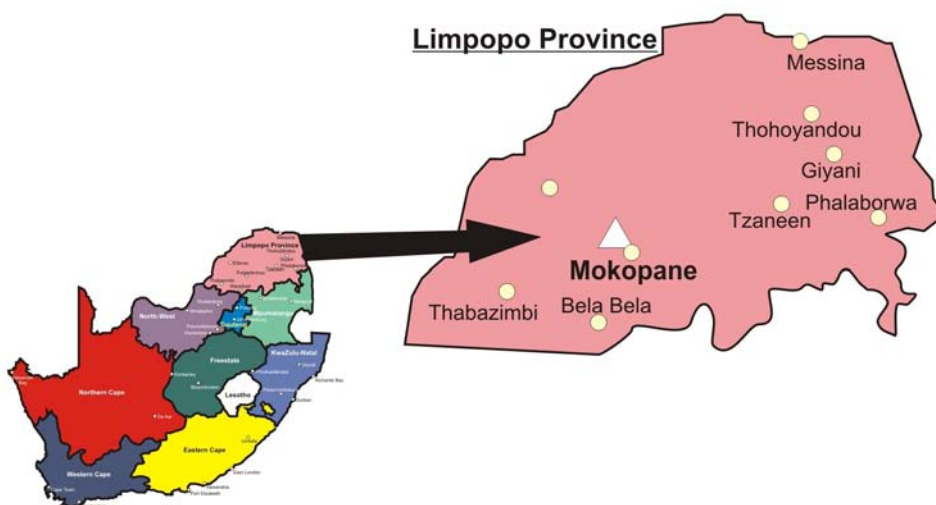
The Limpopo Province covers approximately one tenth of the total surface area of South Africa (Fig. 1). It comprises an area of 116 824 km<sup>2</sup>, only 14% of which is arable land (Meintjes et al., 1995). Molekane is a rural community in the Waterberg district of the Limpopo Province. It is located 27 km north of Mokopane (Potgietersrus) and has approximately 800 households. Sekuruwe also situated in the Waterberg district, is 32 km north of Mokopane, 10 km from Molekane and has approximately 500 households. These two rural villages were selected because of the increasing prevalence of stunted growth in the children (Theron, 2000; Theron et al., 2007).

These 2 communities depend mostly on groundwater. Piped water is only available to 17% of the population, whereas 40% of the population rely on public taps and at least 11% of the population rely on dams, rivers, streams and springs for their water supply (Theron, 2000). There is no running water in the houses but those living nearby fetch it from the main line at communal taps or borehole pumps, using any available container, from plastic buckets to metal oil drums, and carry it back home. A few people own their own boreholes, from which they sell water to the community when the public water sources dry up. The most common toilet available is a pit latrine (Theron, 2000). There are no municipal waste-removal containers for refuse collection and household refuse is dumped alongside the road, burned or buried.

A peri-urban region near Pretoria had been selected as a control site for this study. Unfortunately this area could not be used as a control site as the levels of oestrogenic activity in the area were similar to those obtained in this study (Aneck-Hahn et al., 2008).

### Sample collection

In November 2001 the University of Pretoria project team collected the water samples from the 2 communities, Molekane (n=4) and Sekuruwe (n=7), on the same day in a once-off



**Figure 1**  
The two rural communities are situated in the Waterberg, Limpopo Province, north of Mokopane (Potgietersrus).

△: indicates the study area, where Molekane and Sekuruwe are situated

Sample site	Description of water source	EEq (g/ℓ)*
<b>Molekane</b>		
Mol 1	Mine water from tap (soccer field/shop)	0.68 x 10 <sup>-9</sup>
Mol 2	Borehole into plastic drum (personal borehole)	1.82 x 10 <sup>-9</sup>
Mol 3	Borehole water (personal borehole)	1.30 x 10 <sup>-9</sup>
Mol 4	Spring (and runoff from rain water)	2.29 x 10 <sup>-9</sup>
<b>Sekuruwe</b>		
Sek 1	Tank (large plastic tank)	<dl
Sek 2	Borehole (personal borehole)	2.15 x 10 <sup>-9</sup>
Sek 3	Spring I (South side of town, main)	2.48 x 10 <sup>-9</sup>
Sek 4	Spring II (North side of town)	0.63 x 10 <sup>-9</sup>
Sek 5	Plastic drum	1.90 x 10 <sup>-9</sup>
Sek 6	Disei shop (water pump)	0.83 x 10 <sup>-9</sup>
Sek 8	Spring (West)	< dl

\* Based on the  $EC_{50}$  value of dose response curves obtained for 17 $\beta$ -estradiol (E2) and the test samples.

dl: detection limit of the assay

sampling exercise. Samples of surface water and groundwater pumped from boreholes were collected with a stainless steel cup and placed into 1 ℓ glass Schott Suprax bottles (Cat. No. 21 802 54 56) that had been pre-washed with ethanol (Cat. No. 27,0741, Sigma-Aldrich) and covered with tin foil. The samples were placed on ice and taken back to the laboratory on the same day and kept at 4°C until extracted. There was no contamination or contact with the plastic lid of the bottles.

These samples were representative of the water sources available for consumption by the residents in the communities, their livestock and for other domestic use (Table I). Molekane 1 (Mol 1) was tap water supplied to the local soccer club from the nearby platinum mine. This water was also collected in and stored in plastic drums by the local community residents. Molekane 2 (Mol 2) was water collected from a personal household borehole, which is subsequently stored in a plastic drum. Molekane 3 (Mol 3) was also collected from a personal household borehole, but in a different area of the community. Molekane 4 (Mol 4) was a spring in the dry riverbed at the entrance to the community. This spring is used by both humans and animals as a water source for drinking and other domestic needs.

Sekuruwe 1 (Sek 1) was water from the nearby town, Moko-pane, which was delivered by truck and pumped into and stored in a large plastic tank. Sekuruwe 2 (Sek 2) was collected from a household that had a personal borehole. There are 3 natural springs (Sek 3, 4 and 8) situated on the outskirts of the village. These springs are used by the residents of Sekuruwe and animals as a water source for drinking and other domestic needs. Specifically, Sek 8 is a spring that is situated to the west of the community across the tarred road with no houses or animal pens in close proximity. Water is collected from this spring in cans or drums and transported on a donkey cart to the community. Sekuruwe 5 (Sek 5) was water from a plastic drum that was standing outside a house; this water had been collected from the public borehole supplying the village. There was a public water pump situated in front of the local general store (Sek 6; Disei Shop) at the entrance to the informal settlement. This site was selected as it served as one of the major community water-collection points.

## Sample preparation

### Water extraction procedure

All glassware for sample storage, filtration and extraction of water samples was washed in chromic acid and rinsed once in methanol (Cat. No. 34860, Riedel-de Haën) and twice in ethanol (Cat. No. 27,0741, Sigma-Aldrich). For extraction and enrichment of potential oestrogen-like compounds a solid phase extraction (SPE) was performed. A volume of 1 ℓ was passed in 2 subsequent steps, through a glass wool filter (Cat. No. 000904, Macherey-Nagel), through a 0.45  $\mu$ m, 47 mm sterile filter (Cat. No. E04WG047S1, Osmonics, MicronSep) to remove particulates. The samples were extracted onto a preconditioned Chromabond C18 ec, SPE cartridge (Cat. No. 730 014, Macherey-Nagel), at a flow rate of 10 ml/min (Routledge et al., 1998). Control water (Renalcare® water prior to autoclaving (Aneck-Hahn et al., 2005)) was included to assess possible oestrogenic contamination from the SPE cartridge. The samples were then eluted from the cartridge with 3.5 ml methanol into sterile glass tubes (Cat. No. 13099, Pierce, amber reacti-vials™, 5 ml). The methanol was then evaporated under a gentle nitrogen stream (Beresford, 1999) using a Pierce, Reacti-Vap and a Reacti-Therm heating and stirring module. The sample residue was reconstituted with 1ml ethanol and placed into sterile amber glass bottles (Cat. No. 154515, Chromatography research supplies, 4ml), and stored at -20°C prior to analysis.

### Recombinant yeast screen bioassay (RCBA)

The Recombinant Yeast Screen Bioassay (RCBA) (including details of medium components) previously described by Routledge and Sumpter (1996), was used to test water for oestrogenic activity. In this system, yeast cells transfected with the human oestrogen receptor- $\alpha$  (ER- $\alpha$ ) gene, together with expression plasmids, containing oestrogen responsive elements and the *lac-Z* reporter gene encoding the enzyme  $\beta$ -galactosidase, were incubated in a medium containing 17 $\beta$ -estradiol and the chromogenic substrate, chlorophenol red- $\beta$ -d-galactopyranoside (CPRG). Active ligands, which bind to the receptor, induce  $\beta$ -galactosidase ( $\beta$ -gal) expression and these cause the CPRG

(yellow) to change into a red product that can be measured by absorbance.

The assay was carried out by the Andrology Laboratory at the University of Pretoria according to the standard assay procedure found in Routledge and Sumpter (1996) with modifications to the method described in Aneck-Hahn et al. (2005). In this assay 17  $\beta$ -estradiol (E2) was used as the calibration standard. The E2 standard curve was fitted (sigmoidal function, variable slope) using Graphpad Prism (version 2.01), which calculated the minimum, maximum, slope, EC<sub>50</sub> value and 95% confidence limits. The oestrogenic activity was reported as the 50% (EC<sub>50</sub>) effect concentration using non-linear regression with a sigmoid curve fitting. The detection limit of each test plate (ranging between 0.6 and 2.7 ng/l) was calculated as absorbance elicited by the solvent control (blank) plus three times the standard deviation.

The dose-response curves for the extracted samples and the E2 (standard) curves were used to calculate the estradiol equivalent (EEq) values of the samples. Based on the dose-response curves for E2 and the test sample, the EE of each sample was calculated using the EC<sub>50</sub> value (absorbance) of the sample. The concentration was then adjusted using the appropriate dilution factor.

## Results

No oestrogenic activity was measured in the extraction control samples, thus contamination of the cartridges during the extraction process can be excluded. Yeast growth was checked at an absorbance of 620nm. Compared to the reference, none of the samples showed a decrease in cell density, therefore no cytotoxicity was present. The sample was considered positive for oestrogenic activity when three or more consecutive observations were above the detection limit of the assay. The oestrogenic activity (EEqs) of the samples is based on the EC<sub>50</sub> value of the dose response curves obtained for 17 $\beta$ -estradiol (E2) and the test sample.

### Molekane

All the samples from Molekane had oestrogenic activity. Surface water from the mine tap, Mol 1 had the lowest oestrogenic activity (0.68 x 10<sup>-9</sup> g/l EEq) while the samples from the groundwater sources were all above 1ng/ l EEq. The spring in the dry river bed, Mol 4 (2.29 x 10<sup>-9</sup> g/l EEq), had the highest oestrogenic activity (Table I).

### Sekuruwe

Two of the samples (Sek 1; Sek 8) showed no oestrogenic activity as the EC<sub>50</sub> absorbance for both samples was below the calculated detection limit of the assay, even before the dilution factor was considered. The rest of the samples had EEqs ranging between 0.63 x 10<sup>-9</sup> and 2.48 x 10<sup>-9</sup>g/ l (Table I). The main spring to the south of the community (Sek 3) had the highest EEq (2.48 x 10<sup>-9</sup> g/l) and the borehole (Sek 2) had the lowest EEqs (0.83 x 10<sup>-9</sup> g/l). Water that had been collected from a public borehole pump in a plastic drum (Sek 5) also had an EEq of 1.90 x 10<sup>-9</sup> g/ l, while a sample from a personal borehole (Sek 2) situated in the garden and close to the house had an EEq of 2.15 x 10<sup>-9</sup>g/l.

## Discussion

This once-off sampling study indicated that there was oestrogenic activity in all except 2 samples (Sek 1 and Sek 8). The

reason for this could be that Sek 1 was treated water suitable for drinking from the nearby town, Mokopane. While Sek 8 was groundwater from a spring situated a distance away from the community, where water is collected in containers (metal and plastic) and taken back to the community on a donkey cart. No animals graze in the area and there is no other agricultural activity in the vicinity of the spring, limiting possible oestrogenic contamination.

The Molekane community lies adjacent to the platinum mine which has a slimes dam and the water is used to irrigate a nearby soccer field, but is also used by the community for drinking purposes. The pollution from the mine may be the source of the oestrogenic activity (EEq: 0.68 x 10<sup>-9</sup> g/l) found in this sample (Mol 1), the low activity could be due to an anti-oestrogenic response. Metals are capable of binding to the ligand-binding domain (LBD) of the oestrogen receptor alpha (ER $\alpha$ ) and then block the binding of 17 $\beta$ -estradiol to this domain (Darbre, 2006). When attached to estradiol, metal ions (palladium and platinum) have been found to increase the relative binding affinity of ligand to receptors of MCF-7 cells (Darbre, 2006; Jackson et al., 2001). Metallo-oestrogens may also give rise to oestrogen-agonistic responses in cells (Darbre, 2006).

Of concern are the EEq-values of the remainder of the Molekane and Sekuruwe samples which ranged between 0.68 x 10<sup>-9</sup> and 2.29 x 10<sup>-9</sup> g/l and 0.63 x 10<sup>-9</sup> to 2.48 x 10<sup>-9</sup> g/l respectively. These are not surface water samples but groundwater samples indicating a high level of oestrogenic pollution of the groundwater system in the area in and bordering the communities. These equivalents were slightly lower than those found in a study determining oestrogenic activity (EEq: 10<sup>-9</sup> -10<sup>-8</sup> M) in river water samples from different provinces in South Africa, using a reporter gene assay (ER-Calux) (De Jager et al., 2002). It is to be expected as the YES assay is not as sensitive as the ER Calux which contains both ER $\alpha$  and ER $\beta$  (Bornman et al., 2007; Leusch, 2008). However, the oestrogenic activity was in the same range (0.31 to 2.1 ng/l) as those in the Rietvlei Nature Reserve sampled at the same time of the year using the YES assay (Aneck-Hahn et al., 2008) and other South African studies (Table 2). The other 3 springs (Mol 4, Sek 3 and 4) are all close to the informal settlements, suggesting that the source of oestrogenic contamination could be from the settlements themselves. The oestrogenic activity in the study also corresponds with a number of international studies summarised in Table 2.

EDCs are used in certain polycarbonate and the epoxy resin linings of food and beverage containers (Palanza et al., 2008), Krishnan et al. (1993) found that Bisphenol-A had leached from plastic flasks during autoclaving and Aneck-Hahn et al. (2005) found that sterile bottled water after autoclaving also exhibited oestrogenic activity. In rural communities water is often stored in any available container. Plastic containers such as the one used in Mol 2 and old oil drums could be possible sources of oestrogenic contamination. There is no municipal waste- removal service available, refuse is dumped, burned or buried. Basic services are lacking and the most common toilet available is a pit latrine. It is known that the presence of oestrogenic compounds in sewage represents one of the best examples of linkage between effects and exposure to EDCs (Damstra et al., 2002; Sarmah et al., 2006). Leachates from municipal solid waste landfills can also severely contaminate groundwater and surface waters with EDCs; this includes compounds from plastics like Bisphenol A, nonylphenol and other products (Zhang et al., 2009). Therefore there is a likelihood that the source of the oestrogenic activity in the ground-

**TABLE 2**  
**Literature data on oestrogen levels in different water sources**

Country	Water source	Oestrogenic activity	Bioassay used	Reference
Japan	Sewage treatment effluents	5-15 ng/l	YES	Matsui et al., 2000
	Lake water	1 ng/l	YES	Matsui et al., 2000
Korea	River water	0.5 pg/l - 7.4 ng/l	E-screen	Oh et al., 2000
Korea	River water	0.005-1.92 ng/l	E-screen	Oh et al., 2006
China, Lake Taihu	Lake water	4.8-12.1 ng/l	MVLN	Shen et al., 2001
China, Lake Taihu	Lake water	2.2-8.3 ng/l	HGELN*	Shen et al., 2001
Belgium	Rivers	10 ng/l	YES	Witters et al., 2001
United Kingdom	Streams	0- 26.5 ng/l	YES	Matthiessen et al., 2006
South Africa	Surface, ground and river water	0.31-2.1 ng/l	YES	Aneck-Hahn et al., 2008
South Africa	Small industry effluents	0.001-5.8 ng/l	YES	Mahomed et al., 2008
South Africa	Surface, ground and river water	0.16- 1.92 ng/l	YES	Bornman et al., 2007
South Africa	Surface, ground and river water	0.32-16.0 ng/l	ER-Calux®	Bornman et al., 2007

\* HGELN: (*HeLa Gal-ER-Luc-Neo*) transgenic human cell line for oestrogenic activity

water could be due to contamination as a result of infiltration of domestic effluents and raw sewage. It is unlikely that agricultural runoff would contribute much, as there is very little farming activity in the region, other than a small amount of subsistence farming.

Published information on fish studies (Metcalf et al., 2001; Seki et al., 2005; Young et al., 2002) shows that exposure to average long-term EEq concentrations in excess of 1 ng/l, if bioavailable, are likely to cause ovotestis and other oestrogen-induced intersexual abnormalities (e.g. Vitellogenin induction) in some fish (Matthiessen et al., 2006). This has also been observed in South Africa in Rietvlei Nature Reserve (Barnhoorn et al., 2004; Bornman et al., 2007). Van Wyk et al. (2005) found that exposing male *Oreochromis mossambicus* to estrogen for 3 d caused plasma vitellogenin (Vtg) to increase. Using the *in vitro* *Xenopus laevis* liver culture Vtg bioassay estrogenic activity was detected in different types of water in the Western Cape and Windhoek, Namibia (Van Wyk et al., 2005).

Measuring human exposure to EDCs (e.g., alkylphenols, phthalate esters and bisphenol-A) is difficult because the data are still sparse. There is, however, considerable concern because these compounds are so ubiquitous in the modern environment. Not only were some nonylphenol ethoxylates found in water in New Jersey, USA (Toppari et al., 1996), but more importantly also in South African drinking water (<0.005 to 0.05 mg/l; EEqs: 10<sup>-9</sup>-10<sup>-8</sup> M) (De Jager et al., 2002).

Other rural communities in South Africa may already suffer from health effects caused by exposure to chemicals like DDT, used for malaria vector control (Aneck-Hahn et al., 2007). Water is a potential source for human exposure to EDCs, although it is probably not a major exposure pathway. In developing countries, where drinking water is not generally treated it may be contaminated with industrial and naturally occurring chemicals. It must therefore be taken into account that the community might be exposed to EDCs through diet (in these communities a relatively high soybean intake), the environment (air and water pollution), domestic products (household cleaning products) and occupation. The routes of exposure of the poverty-stricken rural communities will be similar, as even the drinking water was found to be oestrogenic. In fact these communities seem to be at higher risk due to the fact that they lack proper water services in the area. Together with other sources of exposure, the potential for health risks needs further investigation.

## Conclusion

To our knowledge, and despite the limitations of the study, this is the first report on oestrogenic potential in drinking water in the Limpopo Province, South Africa. The origin and identity of the oestrogenic compounds are as yet unknown. It may well be argued that EDC exposure may at least in part contribute to the high incidence of stunted growth in the study population. These preliminary findings indicate a need for further study of these water sources to identify the chemicals responsible for the contamination of and oestrogenic activity in drinking water.

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