Estrogenic activity and endocrine disrupting chemical (EDC) status in water obtained from selected distribution points in Pretoria and Cape Town

Report to the
Water Research Commission

by

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The publication of this report emanates from a project titled *Estrogenic activity and endocrine disrupting chemical (EDC) status in water obtained from selected distribution points in Pretoria and Cape Town* (WRC Project No. K8/993).

EXECUTIVE SUMMARY

BACKGROUND

As stated in the South African Constitution everyone has the right to have access to an environment that is not harmful to their health or well-being. This includes a constant supply of clean, safe water. There is public perception that drinking water supplied by some municipalities is of poor quality, with the result that more people are resorting to bottled water for health and safety reasons. However, the potential migration of plasticisers and non-intentionally added substances from the plastic bottles into the water is of concern.

Endocrine disrupting chemicals (EDCs) comprise one group of pollutants that can enter aquatic systems. They can therefore occur in the water that is treated to produce drinking water. Ineffective treatment can result in them occurring in drinking water.

EDCs which exhibit estrogenic activity have diverse chemical structures varying in estrogenic potency. Exposure to mixtures of chemicals, each present at or even below their individual no observed adverse effect levels (NOAELs) can still lead to adverse effects. The more chemicals that are present in the mixture the more concern is warranted. A number of diseases are associated with long-term chronic exposure to low doses of EDCs which include, cancer, cardiovascular diseases, diabetes and reproductive disorders. Other notable effects are evident in fetal life through to early childhood development and include secondary sexual developmental changes, neurobehavioral alterations and immune disorders. Concern increases when humans are exposed to mixtures of similar-acting EDCs particularly during sensitive windows of development.

This project is Phase 1 of a larger project consisting of three phases. Phase 1 includes the identification of sampling points for drinking water at various distribution points/reservoirs in Pretoria (Tshwane) and Cape Town, the assessment of estrogenic activity in the selected sample points as well as target chemical analysis. Phase 2 is funded by CANSA and includes the identification of different brands of bottled water, bioassays for estrogenic activity as well as target chemical analysis. Phase 3 will comprise a scenario-based health risk assessment, based on the data collected from the estrogenic bioassays and chemical analysis.

AIMS

The following were the aims of this project (Phase 1):

1. To identify the main water supply reservoirs, distribution points and specific sampling points for the Pretoria/Tshwane metropolitan and Cape Town metropolitan areas.
2. To screen water samples from selected distribution points/reservoirs for estrogenic activity using the recombinant yeast estrogen screen (YES) and the T47D-KBluc reporter gene bioassay.
3. To identify possible target chemicals that are present in the drinking water samples.
4. To determine target chemical concentrations present in drinking water samples collected from the identified collection points.
5. To evaluate the applicability of the YES and T47D-KBluc assays as recommended by the WRC Toolbox project (de Jager et al., 2011) to screen for estrogenic activity in drinking water.

METHODOLOGY

Water samples collected from 20 distribution points in Pretoria and Cape Town (10 each) were extracted using solid phase extraction. The bioassays were done according to the WRC Toolbox project for estrogenic activity using the YES and T47D-KBluc bioassays. The target chemical analysis (Bisphenol-A, 4 Nonylphenol, selected phthalates, estradiol, ethinyl-estradiol and estrone) was done using GC-MS.

RESULTS AND DISCUSSION

Estrogenic activity up to 0.167 ng/ℓ EEq was found in the T47D-KBluc bioassays in 22 of the 40 samples. These are well below the 0.7 ng/ℓ EEq value for drinking water which has been proposed as a trigger value necessitating further investigation and testing. Bisphenol-A was detected in all but five samples at concentrations ranging between0.004 ng/ℓ and 0.067 ng/ℓ. 17ß-Estradiol was detected in all samples at concentrations ranging between 0.035 ng/ℓ and 0.426 ng/ℓ. It should be borne in mind that results of the bioassays and chemical analysis cannot be compared directly.

CONCLUSIONS

The study achieved each of its five aims. The bioassays indicated some estrogenic activity in the samples from the water distribution points in Pretoria and Cape Town. The presence of Bisphenol-A and 17ß-Estradiol, which are known estrogens, measured in the chemical analysis is consistent with this finding. This study also showed that the YES and T47D-KBluc assays are suitable to be used as screening assays for estrogenic activity in drinking water as recommended by the WRC Toolbox project.

RECOMMENDATIONS

Although the EEqs of the bioassays were below the recommended trigger value, the fact that BPA and E2 were present is still of concern due to the possible effects of chronic low dose exposure. The development of a monitoring strategy is therefore recommended. There is concern regarding the detection levels for certain target chemicals, research into developing methods that can address this should be considered.

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Collaborators	Affiliation
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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS & ABBREVIATIONS

abs	Absorbance
BPA	Bisphenol-A
DBP	Dibutyl phthalate
DHEA	Bis(2-ethylhexyl) phthalate
d-16 BPA	Deuteratedbisphenol-A
EDC	Endocrine disrupting chemical
Eeq	Estradiol equivalents
EE2	Ethinylestradiol
E1	Estrone
E2	17ß-Estradiol
GC-MS	Gas chromatography-mass spectrophotometry
hERα	Human estrogen receptor alpha
LOD	Limit of detection
LOQ	Limit of quantification
MSTFA	N-Methyl-N-(trimethylsilyl)triflouroacetamide
MtBE	Methyl tertiarybutyl ether
Nd	Not detected
NOAEL	No observed adverse effect level
NP	Nonylphenol
PAH	Poly aromatic hydrocarbon
PNEC	Predicted No Effect Concentration
rpm	Revolutions per minute
RT	Retention time
SIM	Single ion monitoring
SPE	Solid phase extraction
YES	Recombinant yeast estrogen screen

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

People are exposed to multiple potentially hazardous environmental contaminants as part of daily life. Contaminants are introduced into the environment from multiple sources, can be naturally occurring or man-made and are found in all types of environmental media such as ambient air, water, food and personal care products (Fox and Aoki, 2010). Reports of environmental contaminants with endocrine disrupting potential are common in the scientific literature. The World Health Organisation defined an endocrine disruptor as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (WHO/IPCS, 2002). Endocrine disrupting chemicals (EDCs) are of diverse chemical structures and vary in potency (Metzler et al., 1998). They can disturb the hormonal system by mimicking the occurrence of natural hormones, blocking their production or by inhibiting or stimulating the endocrine system (Soares et al., 2008). EDCs include natural chemicals (e.g. phytoestrogens) and synthetic chemicals used as industrial solvents/lubricants and their byproducts (e.g. polychlorinated biphenyls, polybrominated biphenyls, dioxins), plastics (e.g. Bisphenol-A), plasticisers (e.g. phthalates), pesticides (e.g. methoxychlor, chlorpyrifos, DDT), fungicides (e.g. vinclozolin) and pharmaceutical agents (e.g. diethylstilbestrol) (Diamanti-Kandarakis et al., 2009).

EDCs interact with steroid hormone receptors as analogues or antagonists (Diamanti-Kandarakis et al., 2009). The effects of EDCs on the endocrine system generally take place at lower doses than those necessary for acute toxic effects (Pinto and Reali, 2009). Adverse effects resulting from exposure to a mixture of several chemicals present at or below their individual no observed adverse effect levels (NOAELs) can be explained by the dose addition effect (Kortenkamp et al., 2007; Muncke, 2009). Therefore the more chemicals that are present in a mixture the more concern is indicated (Muncke, 2009). This concern increases when humans are exposed to mixtures of similar-acting EDCs and/or during sensitive windows of development. Health effects associated with long term chronic exposure to low doses of EDCs include reproductive disorders, cardiovascular diseases, diabetes, obesity, liver dysfunction and preneoplastic lesions (Diamanti-Kandarakis et al., 2009; Muncke, 2009; Murray et al., 2007; Erler and Novak, 2010). Toxicologically one of the more serious aspects of the estrogens is their association with developing cancer (Metzler et al., 1998; Diamanti-Kandarakis et al., 2009).

The South African Constitution states that everyone has the right to have safe access to an environment that is not harmful to their health or well-being. This includes a constant supply of clean, safe water (DWAF, 2005). However, population growth, urbanisation, industrial development, and associated changes in agricultural and other land-use practices contribute significantly to reducing water quality through naturally occurring and anthropogenic contamination (Falconer et al., 2006). EDCs can enter the aquatic environment via direct discharge into water, leaching, and storm water runoff. Natural hormones, including estrogens can be released into the environment via sewage effluent and from such sources such as agricultural and pharmaceutical activities (Falconer et al., 2006; Slabbert et al., 2008). Estrogenic activity has been found at varying concentrations in raw and treated water in South Africa. The results showed a reduction in estrogenic activity in the treated water compared to the source water, indicating that properly functioning drinking water treatment works were able to remove most EDCs from source water ((Slabbert et al., 2008; Genthe et al., 2010). Various steps in the water treatment process can remove estrogenic activity to some degree. The most effective removal results from activated carbon, ultraviolet irradiation, reverse osmosis and bio- and

photo-degradation and activated sludge treatment, with granular activated carbon being the most efficient method for the removal of EDCs from drinking water (Genthe et al., 2010). However, although water treatment plants treat water to ensure that water quality is improved and safe, chemicals can migrate from the water lines/pipes that transport water to the reservoir and to the home, thereby resulting in the contamination of the drinking water (Renner, 2010).

A common question posed by scientists and the public is whether or not bottled water is safer to drink than tap water. This stems from the public perception that drinking water from the tap is of poor quality and that mineral water is more hygienic and better from a nutritional point of view than tap water (Pinto and Reali, 2009). However, several studies have reported the presence of EDCs in mineral water (Pinto and Reali, 2009; Wagner and Oehlmann, 2009; Toyo'oka and Oshige, 2000; Li et al., 2010; Sax, 2010). Estrogenic contamination of mineral water can occur in various ways: contamination of the water source; contamination through the production process (e.g. estrogenic disinfectants used to clean the filling system); or the migration of EDCs from the packaging material (Wagner and Oehlmann, 2009).

Several international studies reported the presence of Bisphenol-A (BPA) and Nonylphenol (NP) in environmental and drinking water samples (Soares et al., 2008; Li et al., 2010; Berryman et al., 2004). BPA and NP were also detected in several brands of bottled water (Toyo'oka and Oshige, 2000; Li et al., 2010). Both chemicals are known endocrine disruptors (Erler and Novak, 2010; Soto et al., 1991; Soares et al., 2008). Phthalates also display endocrine disrupting properties and were reported to leach from plastic bottles into water contents (Sax, 2010).

Based on the scientific publications reporting estrogenic contamination of various water sources, there is an increasing need to monitor estrogenic activity in drinking water supplied by municipalities as well as in bottled water as an alternative source of drinking water. It is essential to determine the concentrations of EDCs in water samples in order to assess the associated risks. However, suitable analytical methods are not available for some EDCs and some substances may exert an effect on the environment below the detection limit of available analytical methods. Furthermore, the chemical content of water samples is often unknown and many potential endocrine disruptors exist as mixtures in the environment, possibly acting synergistically. It is therefore recommended that estrogenic activity in environmental samples also be assessed with bioassays, rather than relying on chemical analysis alone (Falconer et al., 2006; Leusch et al., 2010).

This project is Phase 1 of a larger project consisting of three phases:

- Phase 1 includes the identification of sampling points for drinking water at various distribution points/reservoirs in Pretoria (Tshwane) and Cape Town, the assessment of estrogenic activity in samples from these points as well as target chemical analysis. This document presents the results of this phase.

- Phase 2 is funded by the Cancer Association of South Africa(CANSA) and includes the identification of different brands of bottled water, bioassays for estrogenic activity as well as target chemical analysis.

- Phase 3 will comprise a scenario based health risk assessment, based on the data collected from the estrogenic bioassays and chemical analysis.

1.2 PROJECT AIMS

The following were the aims of the project (Phase 1):

1. To identify the main water supply reservoirs, distribution points and specific sampling points for the Pretoria/Tshwane metropolitan and Cape Town metropolitan areas

2. To screen water samples from selected distribution points/reservoirs for estrogenic activity using the recombinant yeast estrogen screen (YES) and the T47D-KBluc reporter gene bioassay

3. To identify possible target chemicals that are present in the drinking water samples

4. To determine target chemical concentrations present in drinking water samples collected from the identified collection points

5. To evaluate the applicability of the YES and T47D-KBluc assays as recommended by the WRC Toolbox project (de Jager et al., 2011) to screen for estrogenic activity in drinking water

CHAPTER 2: METHODOLOGY

2.1 SITE SELECTION

Two study areas were identified, namely Pretoria (City of Tshwane) and Cape Town. These two cities were selected because both are large, well-functioning municipalities, but in different geographical areas, and with different possible sources of estrogenic contamination of their water sources. The main water supply reservoirs and distribution points in Pretoria and Cape Town were identified and ten representative sampling sites per city were selected. Samples were taken from selected distribution points/reservoirs and not from individual homes (i.e. not point of use). This was done to prevent the possibility of confounding factors in the form of the different types of piping used in private homes. For the purpose of confidentiality and sensitivity the sampling points were assigned a number for reporting purposes.

2.2 SAMPLE COLLECTION

Water samples were collected in January and April 2012 in triplicate in 1 litre glass Schott bottles, prepared by rinsing the bottles with HPLC grade methanol. The pH of the water was adjusted to 3 using concentrated HCl. Samples were stored at 4°C in the dark until extraction.

Figure 1: An example of a sample distribution point and sampling

2.3 WATER EXTRACTION PROCEDURE

Prior to extraction, samples were filtered through a glass wool and 0.45 micron filter to remove particulates. One litre of each sample was then concentrated using a solid phase extraction (SPE) procedure recommended by Oasis for the extraction of EDCs in water samples. The samples and extraction control were extracted onto pre-conditioned Oasis HLB reversed-phase SPE cartridges and eluted with 6 mℓ 10% methanols in methyl tertiarybutyl ether (MtBE). The solvent was evaporated to dryness under a gentle nitrogen stream and the sample residue reconstituted in 1 mℓ ethanol for analysis in the bioassays. Reconstituted samples were stored at -20°C.The cartridges for the chemical analysis of the target compounds were dried and sent to Stellenbosch University's CAF Laboratory for further elution and testing according to the protocol set out in Section 2.5.

2.4 BIOASSAYS FOR ESTROGENIC ACTIVITY

The bioassays to determine estrogenic activity in water samples were carried out according to the protocols described in the WRC EDC Toolbox project (de Jager et al., 2011).

2.4.1 The recombinant yeast screen assay

The recombinant yeastestrogen screen (YES) assay was developed to determine estrogenic activity in a variety of matrices. A yeast strain (Sacchromycescerevisiae) was genetically modified to contain the human estrogen receptor alpha (hERα). It also contains expression plasmids carrying the reporter gene lac-Z, encoding the enzyme β-galactosidase(Routledge and Sumpter, 1996).

Yeast stock cultures and growth medium were prepared using medium components described in the WRC EDC Toolbox project (de Jager et al., 2011).The growth medium was inoculated with 125 µℓ of the 10x concentrated yeast stock and incubated at 28˚C in a rotating water bath at 150-155 revolutions per minute (rpm) until turbid. Serial dilutions of the sample extracts and controls were made in ethanol (solvent), in 96 well microtiter plates. 10 µℓ Aliquots were then transferred to a second plate and allowed to evaporate to dryness. Aliquots (200 µℓ) of the assay medium containing the yeast and chromogenic substrate (CPRG) were then dispensed into each sample well. Each plate contained at least one row of blanks (assay medium and solvent ethanol) and a standard curve for 17ß-estradiol (E2), ranging from $1x10^{-8}$ M (2.724 µg/ℓ) to $1.19x10^{-15}$ M (3.24x10-13 g/ℓ). The plates were sealed with parafilm and placed in a naturally ventilated incubator at 32˚C for 3 to 5 days. Active ligands that bind to and activate the estrogen receptor result in the expression of the reporter gene Lac-Z which produces the enzyme β-galactosidase in a dose-dependent manner. This enzyme is secreted into the medium and metabolises the chromogenic substrate CPRG (normally yellow) into a red product, which can be measured by absorbance (Routledge and Sumpter, 1996).

After 3 days incubation the colour development of the medium was checked for a further 3 days (day 3 to 5) at an absorbance (abs) of 540 nm for colour change and 620 nm for turbidity of the yeast culture. The absorbance was measured on a Multiskan Spectrum v1.2 spectrophotometer to obtain data with the best contrast. After incubation the control wells appeared light orange in colour, due to background expression of ß-galactosidase and turbid due to the growth of the yeast. Positive wells were indicated by a deep red colour accompanied by yeast growth. Clear wells, containing no growth indicated lysis of the cells and colour varied. All experiments were performed in triplicate.

The following equation was applied to correct for turbidity:

Corrected value = test abs (540 nm) – [test abs (620nm) – median blank abs (620nm)]

The E2 standard curve was fitted (sigmoidal function, variable slope) using Graphpad Prism (version 4), which calculated the minimum, maximum, slope, EC50 value and 95% confidence limits. The detection limit of the yeast assay was calculated as absorbance elicited by the solvent control (blank) plus three times the standard deviation. Sample concentrations with absorbance values below the solvent control minus three times the standard deviation were considered as cytotoxic concentrations. The estradiol equivalents (EEq) of the samples were interpolated from the estradiol standard curve and corrected with the appropriate dilution factor for each sample.

The assay procedure is summarised in Figure 2.

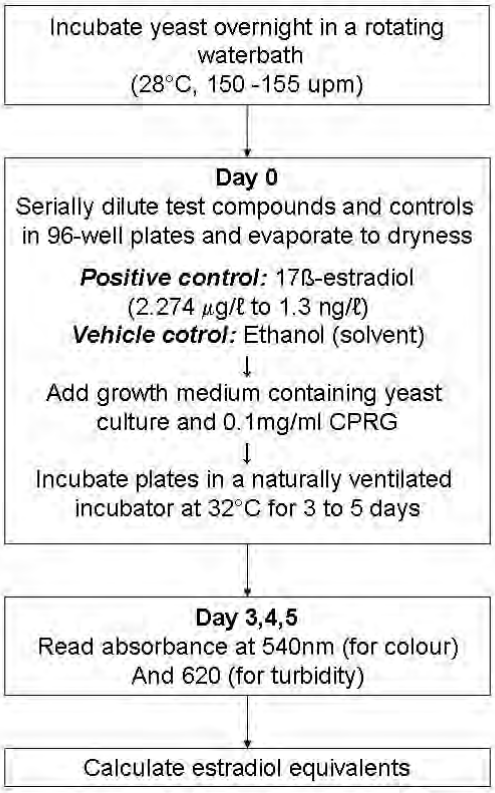

Figure 2: Recombinant Yeast Screen (YES) assay protocol (adapted from de Jager et al., 2011)

2.4.2 T47D-KBluc reporter gene assay

The T47D-KBluc reporter gene assay was developed to be a specific and sensitive assay for screening samples for estrogenic and anti-estrogenic activities. T47D human breast cancer cells naturally express estrogen receptor alpha and beta and were transfected with an estrogen-responsive element luciferase reporter gene construct. Active ligands that bind to the estrogen receptor result in the activation of the luciferase reporter gene and dose dependent production of the luciferase enzyme. When testing chemicals using the T47D-KBluc cells, an estrogen is defined as a chemical that induced a dose-dependent luciferase activity, which could be specifically inhibited by the anti-estrogen ICI 182,780 (Wilson et al., 2004).

T47D-KBluc cells were maintained in RPMI growth media supplemented with 2.5 g/ℓ glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/ℓ $NaHCO_3$, 10% fetal bovine serum (FBS), 100µg/mℓ penicillin, 100 U/mℓ streptomycin and 0.25 µg/mℓ amphotericin B. One week prior to the assay, cells were placed in growth media modified by replacement of 10% FBS with 10% dextran-charcoal treated FBS excluding antibiotic supplements (Wilson et al. 2004).

Cells were seeded at 5 x 10^4 cells per well in 96-well luminometer plates and allowed to attach overnight. Dosing dilutions were prepared in growth media containing 5% dextran-charcoal treated FBS and vehicle (ethanol) did not exceed 0.2%. Each plate contained agonist positive control (E2), negative control (solvent only), antagonist control (E2 plus ICI) and background control (solvent plus ICI). Each sample was tested alone as well as in the presence of 0.1 nME2 or ICI. Cells were incubated 24 h with 100 µℓ/well dosing solution at 37°C, with 5% CO_2.

After the incubation period, cells were washed with phosphate buffered saline at room temperature and lysed with 25 µℓ lysis buffer. Luciferase activity was determined using a microtiter plate luminometer and quantified as relative light units. Each well received 25 µℓ reaction buffer (25 m

Mglycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/mℓ BSA, pH 7.8), followed by 25 µℓ 1 mM D-luciferin 5 s later. Relative light units were converted to a fold induction above the vehicle control value.

The E2 standard curve was fitted (sigmoidal function, variable slope) using Graphpad Prism (version 4), which calculated the minimum, maximum, slope, EC50 value and 95% confidence limits. The EEq values of extracts with greater than a two-fold induction above the vehicle control were interpolated from the estradiol standard curve and corrected with the appropriate dilution factor for each sample.

The assay procedure is summarised in Figure 3.

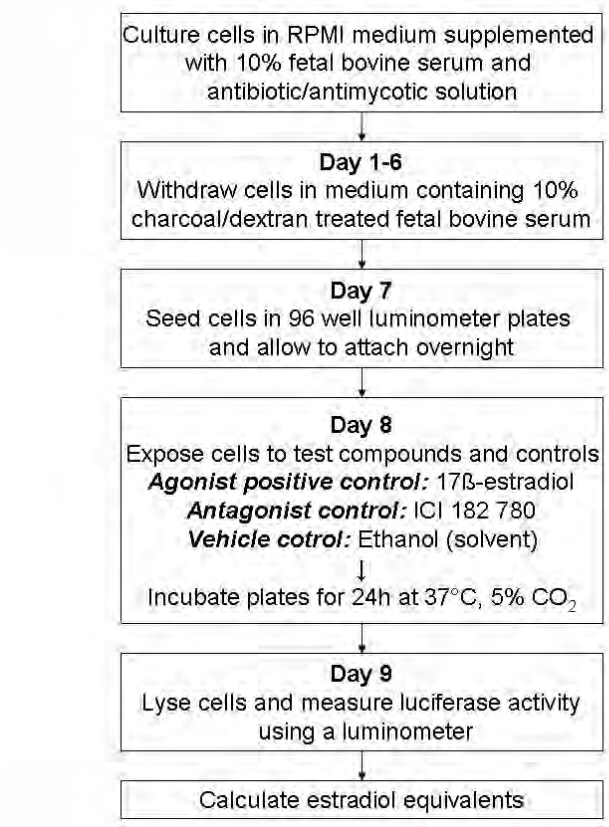

Figure 3: T47D-KBluc reporter gene assay protocol (adapted from de Jager et al., 2011)

2.5 GAS CHROMATOGRAPHY-MASS SPECTROPHOTOMETRY(GC-MS) DETERMINATION OF EDCS IN WATER SAMPLES

Drinking water samples were analysed using gas chromatography-mass spectrophotometry(GC-MS) for the compounds listed in Table 1. As per the method followed the samples were previously eluted from the SPE with 6 mℓ 10% (v/v) methanol/MtBE. The samples were then separated into two 3 mℓ fractions. One fraction was to be derivatised as per the protocol below. The other fraction was evaporated to dryness and reconstituted in 1 mℓ dicholoromethane and analysed by GC-MS. The purpose for separating the sample volume in two was to avoid the use of derivatisation reagents which were previously shown to interfere with the phthalate analysis of the sample (see analytical report in Appendix A). As the sample which is being derivatised is not being monitored for any target compounds which may be present in the derivatisation reagent there is little concern for contamination.

Table 1: Target compounds monitored for in GC-MS analysis and whether analytes were derivatised

Analyte	Abbreviation	Derivatised
Estrone	E1	Yes
β-Estradiol	E2	Yes
Ethinylestradiol	EE2	Yes
Dibutyl phthalate	DBP	No
Bis(ethyl hexyl) phthalate	DHEP	No
Bis(ethyl hexyl) adipate	DHEA	No
Bisphenol-A	BPA	Yes
4-Nonylphenol	4-NP	Yes

2.5.1 Derivatisation

The 3 mℓ fraction was evaporated to dryness and reconstituted in 100 µℓ 10% (v/v) methanol/MtBE containing α-cholestane as the internal standard at concentration of 1 µg/ml. The reconstituted samples were then evaporated to dryness with nitrogen at 40°C. 100 µℓ of 2% methoxyamine in pyridine was added and the samples were then derivatised for 30 minutes in an oven maintained at a temperature of 50°C. The vials were allowed to cool to room temperature and then evaporate to dryness. The final derivatisation was obtained by adding 100 µℓ of 0.05 M triethylamine in pyridine and 50 µℓ of N-Methyl-N-(trimethylsilyl)triflouroacetamide(MSTFA) followed by heating for 1 hour at 80°C. The derivatives were allowed to cool to room temperature before being transferred onto a GC vial with an insert.

2.5.2 Standard handling and derivatisation

The stock solution of the compounds of interest from the pure compounds was dissolved in methanol. All the dilutions for the calibration were done in 10% (v/v) methanol/MtBE since the samples were eluted from the SPE cartridges with that solvent. The standards were not extracted with the SPE. The standards were evaporated in the same manner as the samples. The same derivation procedure was as above.

2.5.3 GC-MS analysis

GC-MS experiments were conducted on an Agilent 6890N GC Coupled with an Agilent 5975 MS. Parameters for GC-MS analyses are as in Table 2.

Table 2: GC-MS parameters for water sample analysis

Instrument: Agilent 6890N GC Coupled with an Agilent 5975 MS			
Column: Rtx®-5MS (30 m, 0.25 mm ID, 0.25 µm film thickness)			
Instrument settings			
Injector temperature	250°C		
Injection volume	5 µℓ		
Injection mode	Splitless		
Purge flow	50 mℓ/min		
Purge time	5.00 min		
Total flow	33.9 mℓ/min		
Flow rate	1.2 mℓ/min		
Gas saver	On		
Saver flow	50.0 mℓ/min		
Saver time	10.00 min		
Carrier gas	Helium		
MS mode	EI+ (SIM)		
Scanning mass range	40 to 800 m/z		
Oven temperature program			
Oven Ramp	**°C/min**	**Temp (°C)**	**Hold (min)**
Initial		40	0
Ramp 1	10	260	3
Ramp 2	25	280	7
Ramp 3	50	330	6.2
Total Run time: 40.00 min			

CHAPTER 3: RESULTS AND DISCUSSION

3.1 BIOASSAYS FOR ESTROGENIC ACTIVITY

No estrogenic activity or cytotoxicity was observed in any of the samples in the YES assay. The results obtained in the T47D-KBluc assay are summarised in Table 3.

Table 3: Estrogenic activity of water extracts collected from selected distribution points in Pretoria and Cape Town

Sample reference		Estradiol equivalents (EEq) in ng/ℓ	
		January 2012	April 2012
Pretoria	PTA01	0.167 ± 0.054 *	0.044 ± 0.010 *
	PTA02	0.155 ± 0.059 *	0.052 ± 0.003
	PTA03	<dl	0.015 ± 0.001
	PTA04	<dl *	<dl
	PTA05	<dl	<dl
	PTA06	<dl	0.015 ± 0.004
	PTA07	<dl *	<dl
	PTA08	<dl	<dl
	PTA09	0.028 ± 0.002 *	<dl
	PTA10	0.011 ± 0.003 *	<dl
Cape Town	CT01	<dl	<dl
	CT02	0.005 ± 0.001	<dl
	CT03	0.002 ± 0.001	<dl
	CT04	0.013 ± 0.001	0.005 ± 0.0004
	CT05	0.011 ± 0.004	0.003 ± 0.0002
	CT06	0.004 ± 0.001	<dl
	CT07	0.002 ± 0.001	0.005 ± 0.001
	CT08	0.013 ± 0.004	0.012 ± 0.003
	CT09	0.042 ± 0.005	0.049 ± 0.026
	CT10	0.005 ± 0.0004	<dl

* Cytotoxicity observed in 100x concentrated sample

<dl Below detection limit of the assay

Estrogenic activity was measured in 22 of the water samples using the T47D-KBluc assay. However, none were above the trigger value of 0.7 ng/ℓ for estrogenic activity in drinking water Genthe et al., (2010) proposed a South African framework for guideline development for EDCs in drinking water. The proposed framework recommended a precautionary risk-based approach based on the World Health Organisation risk assessment approach. A trigger value of 0.7 ng/ℓw as derived for estrogenic activity in drinking water. If the trigger value is exceeded, further investigation and testing of the water is necessitated. The framework also suggested a tiered approach to screening and testing of water samples rather than testing for specific target chemicals.

Estrogenic activity has previously been found at varying concentrations in raw and treated water in South Africa. The results indicated a reduction in estrogenic activity in the treated water compared to the source water, showing that properly functioning drinking water treatment works were able to

remove most EDCs from source water (Slabbert et al., 2008; Genthe et al., 2010). In this study the highest EEq value (0.167 ± 0.054 ng/ℓ) was measured in the PTA01 sample in January 2012. The EEqs found in this study were lower than that found by Stanford et al., (2010) in the United States (EEq: 0.19-0.77 ng/ℓ). However, similar values were obtained by Slabbert et al., (2008) (EEq: <dl - 0.18 ng/ℓ) and Genthe et al., (2010) (EEq: <dl -0.3 ng/ℓ) in South African water.

Seven of the water samples collected in Pretoria had cytotoxicity when concentrated 100 times. It should be noted that cytotoxicity could mask estrogenic activity or result in it being underestimated. Therefore estrogenic activity at sites PTA01 (Jan & Apr); PTA02 (Jan); PTA09 (Jan) and PTA10 (Jan) could be underestimated due to the presence of cytotoxicity.

The reason why the T47D-KBluc assay revealed estrogenic activity while the YES assay did not could be because the yeast cells contain only the ERα, whereas the T47D-KBluc cells contain both the endogenous ERα and ERß. This makes the T47D-KBluc assay more sensitive for estrogenic activity than the YES assay.

Bioassays are useful screening tools for water samples as the specific chemical nature is not always known. Drinking water samples may contain a mixture of chemicals with diverse activities (including (anti)-estrogenic and (anti)-androgenic). The effects of chemical mixtures cannot always be elucidated from their concentrations therefore bioassays are an important tool when examining the presence of, and integrating the effects of complex mixtures of EDCs. The bioassays in this study measured the resultant estrogenic activity of the sample as a whole.

It should therefore be noted that EEqs are indicators of activity in a complex mixture. The composition of drinking water is constantly changing due to external factors (like seasonal changes, rain, chemical spills, etc.) and should therefore be monitored regularly for estrogenic activity.

3.2 GC-MSTARGETED ANALYSIS

3.2.1 Limits of Detection and Quantification

The limit of detection (LOD) is defined as the concentration that corresponds to the sum of the mean of blanks and three times the standard deviation. LOD was determined by calculating the area under the peak at for each analyte using 5 blanks (using MilliQ water). The limit of quantification (LOQ) is defined as the sum of the mean of 5 replicate blank values at each analyte retention time and 10 times the standard deviation. The LOD and LOQ for the respective analytes are given in Table 4.

Table 4: LOD and LOQ for analytes

	4NP	DBP	BPA	DHEA	DHEP	E1	E2	EE2
LOD (ng/ℓ)	3.2	5.7	13	8.7	4.4	8.8	6.2	3.0
LOQ (ng/ℓ)	11	19	42	29	15	29	21	10

3.2.2 Measured concentrations

The results of the chemical analyses for BPA, E1, E2, EE2, DBP, DHEP, DHEA and 4NP are summarised in Tables 5-8. The single ion monitoring (SIM) chromatogram of the standard and an example of a test sample for each chemical measured are shown in figures 4-11.

3.2.2.1 Bisphenol-A

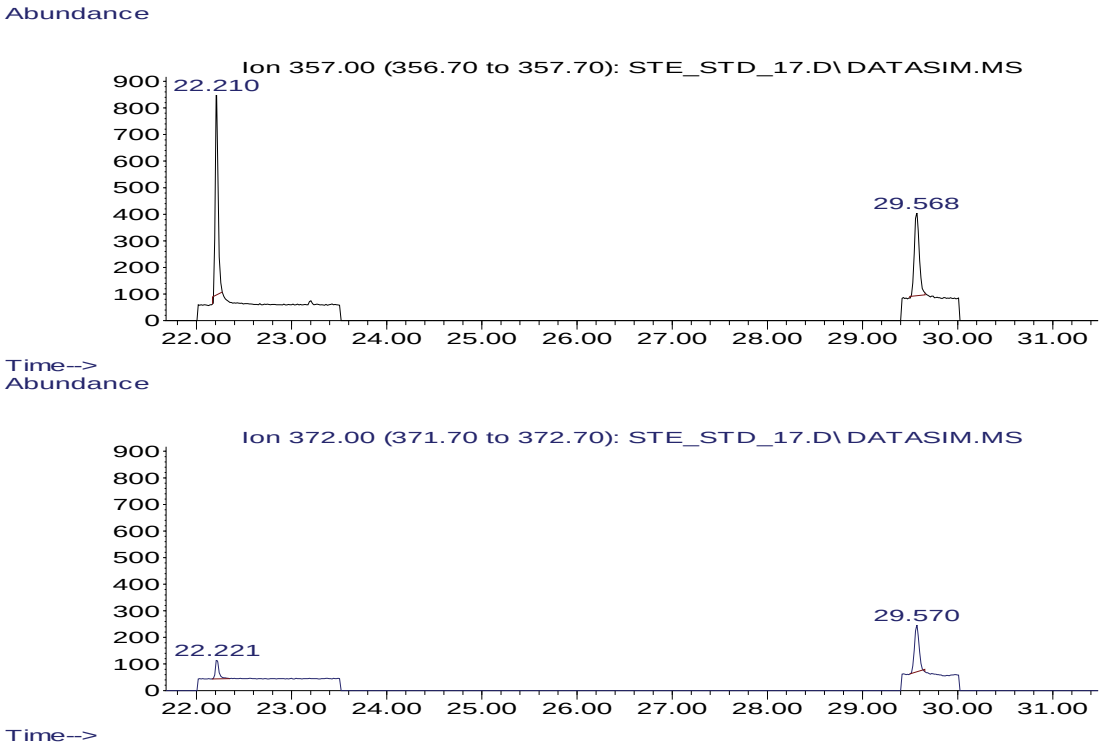

Figure 4: The SIM chromatogram of the BPA standard (top) at a concentration of 1.56 ng/ℓ (ions 372 and 357) at retention time of 22.21 min and that of α-cholestane (mz 372 and 357) as internal standard (bottom) at retention time of 29.56 min

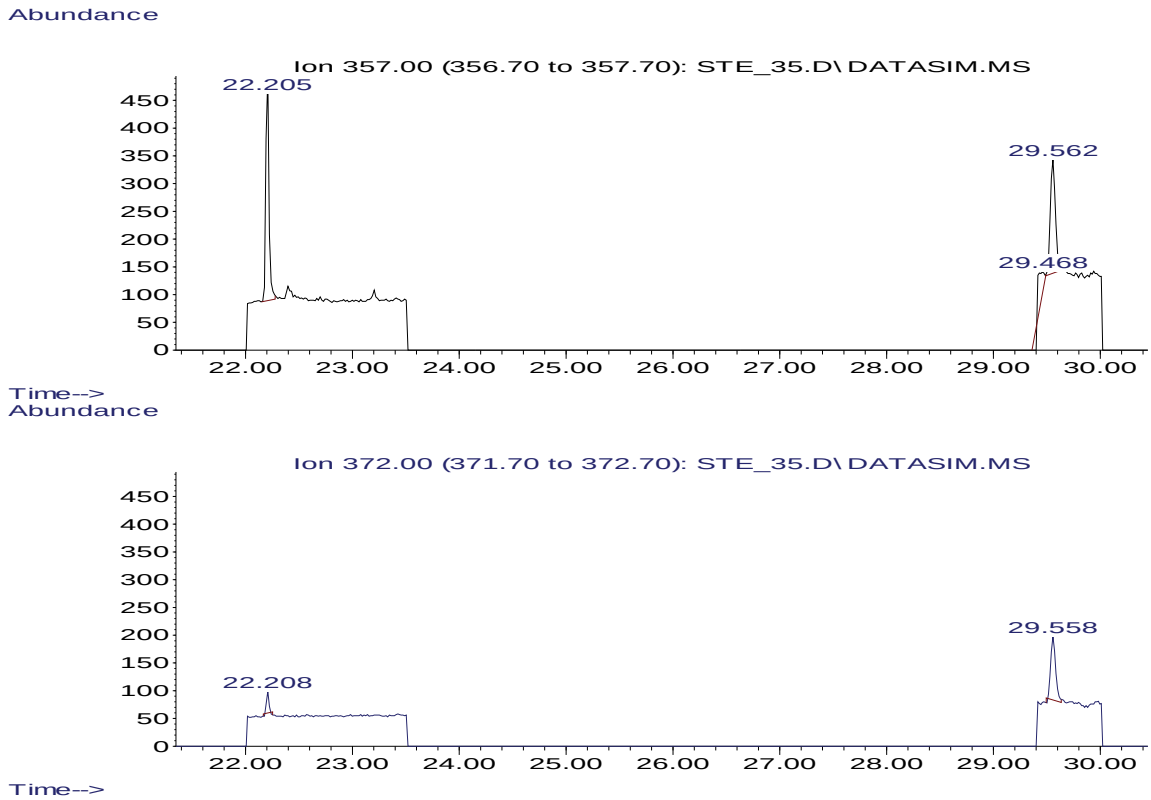

Figure 5: The SIM chromatogram of sample 35 monitoring for BPA (ions 372 and 357) (top) at retention time of 22.21 min and that of α-cholestane (mz 372 and 357) as internal standard (bottom) at retention time of 29.56 min

Table 5: Concentration of Bisphenol-A in drinking water samples

		Bisphenol-A	
Sample #	**Sample Identification**	**Conc (ng/ℓ) of injected sample** [a]	**Conc (ng/ℓ)** [b]
1	Jan 2012 PTA 01	23.64	0.04728
2	Jan 2012 PTA 02	16.37	0.03274
3	Jan 2012 PTA 03	18.68	0.03736
4	Jan 2012 PTA 04	<LOD	<LOD
5	Jan 2012 PTA 05	16.55	0.0331
6	Jan 2012 PTA 06	<LOD	<LOD
7	Jan 2012 PTA 07	15.05	0.0301
8	Jan 2012 PTA 08	Nd	Nd
9	Jan 2012 PTA 09	13.35	0.0267
10	Jan 2012 PTA 10	<LOD	<LOD
11	Apr 2012 PTA 01	23.28	0.04656
12	Apr 2012 PTA 02	17.71	0.03542
13	Apr 2012 PTA 03	Nd	Nd
14	Apr 2012 PTA 04	<LOD	<LOD
15	Apr 2012 PTA 05	<LOD	<LOD
16	Apr 2012 PTA 06	<LOD	<LOD
17	Apr 2012 PTA 07	<LOD	<LOD
18	Apr 2012 PTA 08	<LOD	<LOD
19	Apr 2012 PTA 09	<LOD	<LOD
20	Apr 2012 PTA 10	<LOD	<LOD
21	Apr 2012 CT 01	<LOD	<LOD
22	Apr 2012 CT 02	<LOD	<LOD
23	Apr 2012 CT 03	Nd	Nd
24	Apr 2012 CT 04	<LOD	<LOD
25	Apr 2012 CT 05	<LOD	<LOD
26	Apr 2012 CT 06	33.99	0.06798
27	Apr 2012 CT 07	Nd	Nd
28	Apr 2012 CT 08	22.34	0.04468
29	Apr 2012 CT 09	<LOD	<LOD
30	Apr 2012 CT 10	<LOD	<LOD
32	Jan 2012 CT 01	<LOD	<LOD
33	Jan 2012 CT 02	<LOD	<LOD
34	Jan 2012 CT 03	<LOD	<LOD
35	Jan 2012 CT 04	<LOD	<LOD
36	Jan 2012 CT 05	<LOD	<LOD
37	Jan 2012 CT 06	<LOD	<LOD
38	Jan 2012 CT 07	<LOD	<LOD
39	Jan 2012 CT 08	14.43	0.02886
40	Jan 2012 CT 09	<LOD	<LOD
41	Jan 2012 CT 10	Nd	Nd
31	JAN + APR Control	<LOD	<LOD

[a] The values in this column are indicative of the concentration of the sample injected into the GC-MS apparatus. Due to the SPE protocol the sample has, however, been concentrated (1000 times concentration factor) and split and hence the concentration factor and separation of the sample needs to be taken into account

[b] The concentration of the target compound as present in the original samples

Nd: Not detected

3.2.2.2 Estradiol, ethinylestradiol and estrone

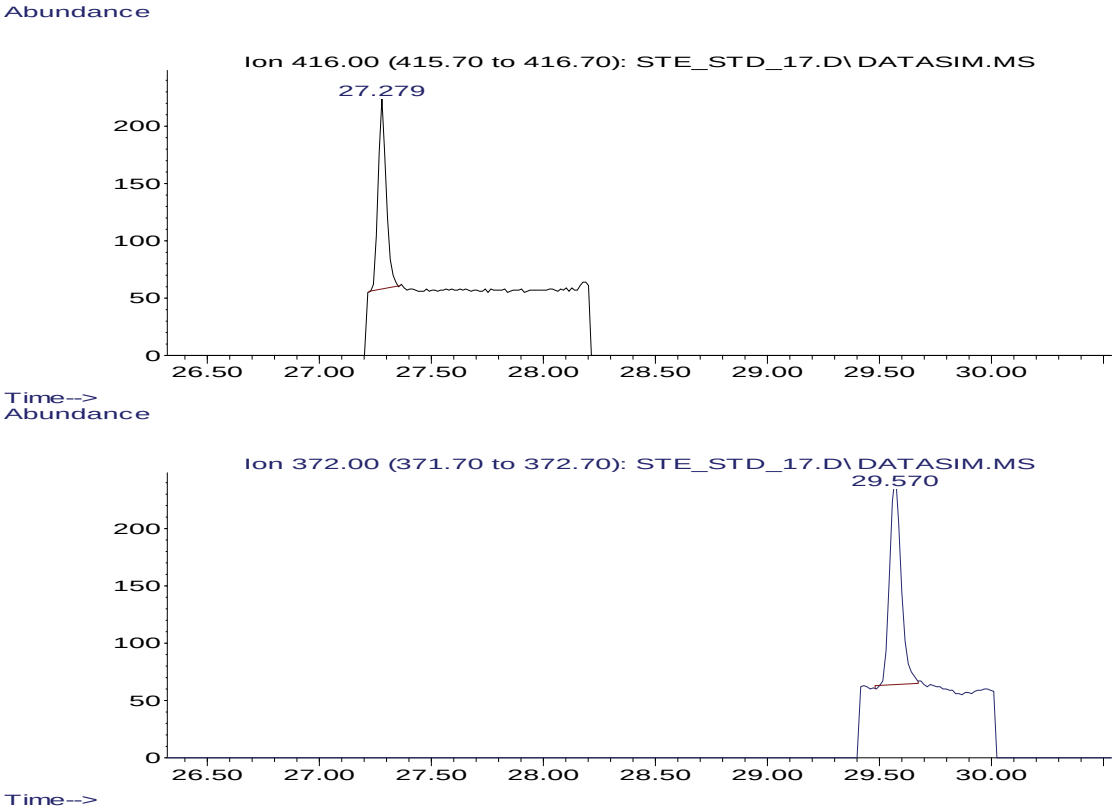

Figure 6: The SIM chromatogram of the β-Estradiol standard (top) at a concentration of 0.912 ng/ℓ (mz 416) at retention time of 27.279 min and that of α-cholestane (mz 372 and 357) as internal standard (bottom) at retention time of 29.56 min

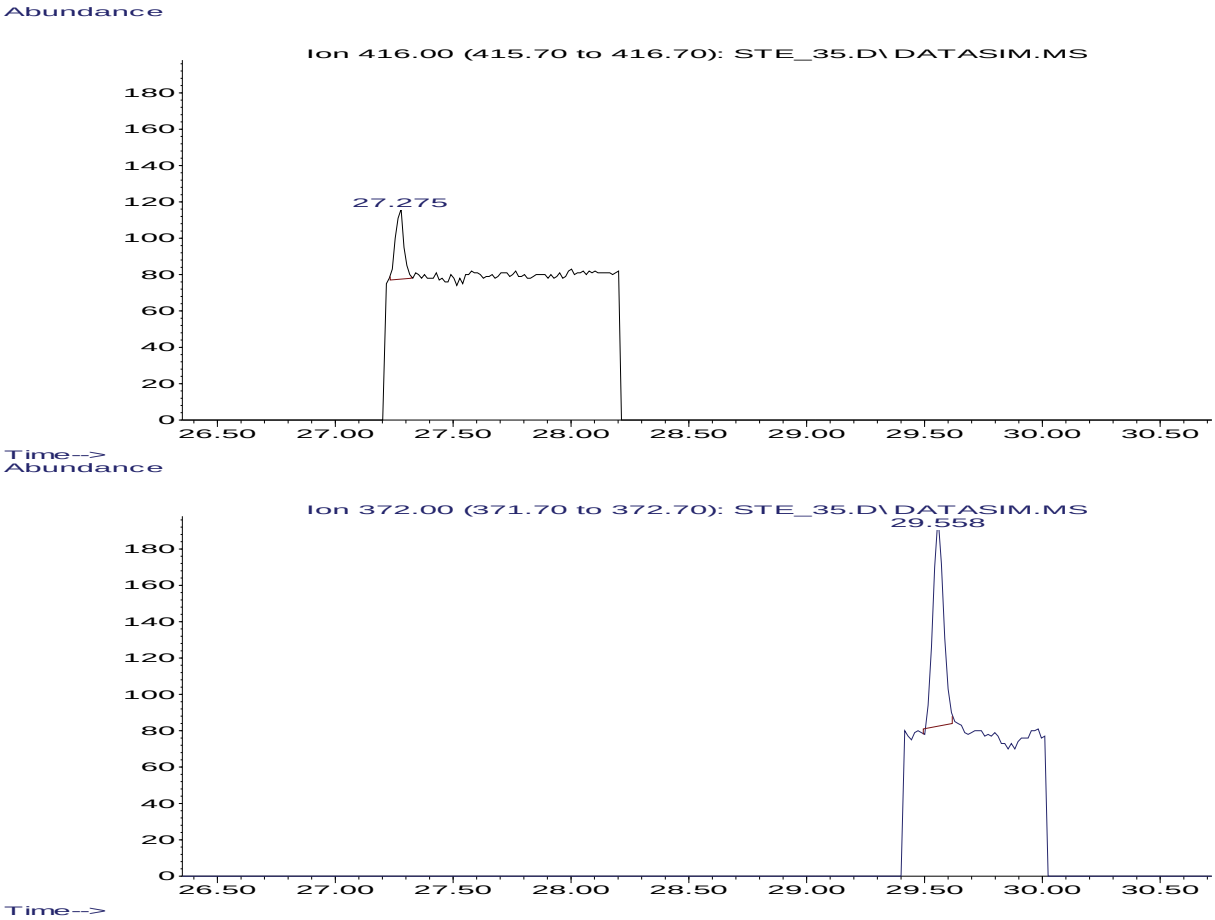

Figure 7: The SIM chromatogram of sample 35 monitoring for β-Estradiol (mz 416) (top) at retention time of 27.279 min and that of α-cholestane (mz 372 and 357) as internal standard (bottom) at retention time of 29.56 min

Table 6: Concentration of β-Estradiol in drinking water samples

		β-Estradiol	
Sample #	**Sample Identification**	**Conc (ng/ℓ) of injected sample** [a]	**Conc (ng/ℓ)** [b]
1	Jan 2012 PTA 01	178.81	0.35762
2	Jan 2012 PTA 02	34.89	0.06978
3	Jan 2012 PTA 03	81.48	0.16296
4	Jan 2012 PTA 04	76.16	0.15232
5	Jan 2012 PTA 05	162.03	0.32406
6	Jan 2012 PTA 06	107.66	0.21532
7	Jan 2012 PTA 07	108.03	0.21606
8	Jan 2012 PTA 08	21.09	0.04218
9	Jan 2012 PTA 09	184.46	0.36892
10	Jan 2012 PTA 10	84.19	0.16838
11	Apr 2012 PTA 01	105.78	0.21156
12	Apr 2012 PTA 02	46.63	0.09326
13	Apr 2012 PTA 03	18.49	0.03698
14	Apr 2012 PTA 04	77.53	0.15506
15	Apr 2012 PTA 05	58.63	0.11726
16	Apr 2012 PTA 06	141.35	0.2827
17	Apr 2012 PTA 07	52.95	0.1059
18	Apr 2012 PTA 08	137.53	0.27506
19	Apr 2012 PTA 09	174.05	0.3481
20	Apr 2012 PTA 10	17.69	0.03538
21	Apr 2012 CT 01	101.97	0.20394
22	Apr 2012 CT 02	107.38	0.21476
23	Apr 2012 CT 03	133.12	0.26624
24	Apr 2012 CT 04	105.25	0.2105
25	Apr 2012 CT 05	116.29	0.23258
26	Apr 2012 CT 06	129.16	0.25832
27	Apr 2012 CT 07	49.49	0.09898
28	Apr 2012 CT 08	172.63	0.34526
29	Apr 2012 CT 09	213.29	0.42658
30	Apr 2012 CT 10	154.44	0.30888
32	Jan 2012 CT 01	76.78	0.15356
33	Jan 2012 CT 02	153.9	0.3078
34	Jan 2012 CT 03	86.49	0.17298
35	Jan 2012 CT 04	97.8	0.1956
36	Jan 2012 CT 05	118.08	0.23616
37	Jan 2012 CT 06	111.3	0.2226
38	Jan 2012 CT 07	107.7	0.2154
39	Jan 2012 CT 08	186.62	0.37324
40	Jan 2012 CT 09	67.2	0.1344
41	Jan 2012 CT 10	25.34	0.05068
31	JAN + APR Control	83.93	0.16786

[a] The values in this column are indicative of the concentration of the sample injected into the GC-MS apparatus. Due to the SPE protocol the sample has, however, been concentrated (1000 times concentration

factor) and split and hence the concentration factor and separation of the sample needs to be taken into account

[b] The concentration of the target compound as present in the original samples

Nd: Not detected

Table 7: Concentration of ethinylestradiol and estrone in drinking water samples

	EthinylEstradiol (EE2)			Estrone (E1)	
Sample #	**Sample Identification**	**Conc (ng/ℓ)**	**Sample #**	**Sample Identification**	**Conc (ng/ℓ)**
1	Jan 2012 PTA 01	Nd	1	Jan 2012 PTA 01	Nd
2	Jan 2012 PTA 02	Nd	2	Jan 2012 PTA 02	Nd
3	Jan 2012 PTA 03	Nd	3	Jan 2012 PTA 03	Nd
4	Jan 2012 PTA 04	Nd	4	Jan 2012 PTA 04	Nd
5	Jan 2012 PTA 05	Nd	5	Jan 2012 PTA 05	Nd
6	Jan 2012 PTA 06	Nd	6	Jan 2012 PTA 06	Nd
7	Jan 2012 PTA 07	Nd	7	Jan 2012 PTA 07	Nd
8	Jan 2012 PTA 08	Nd	8	Jan 2012 PTA 08	Nd
9	Jan 2012 PTA 09	Nd	9	Jan 2012 PTA 09	Nd
10	Jan 2012 PTA 10	Nd	10	Jan 2012 PTA 10	Nd
11	Apr 2012 PTA 01	Nd	11	Apr 2012 PTA 01	Nd
12	Apr 2012 PTA 02	Nd	12	Apr 2012 PTA 02	Nd
13	Apr 2012 PTA 03	Nd	13	Apr 2012 PTA 03	Nd
14	Apr 2012 PTA 04	Nd	14	Apr 2012 PTA 04	Nd
15	Apr 2012 PTA 05	Nd	15	Apr 2012 PTA 05	Nd
16	Apr 2012 PTA 06	Nd	16	Apr 2012 PTA 06	Nd
17	Apr 2012 PTA 07	Nd	17	Apr 2012 PTA 07	Nd
18	Apr 2012 PTA 08	Nd	18	Apr 2012 PTA 08	Nd
19	Apr 2012 PTA 09	Nd	19	Apr 2012 PTA 09	Nd
20	Apr 2012 PTA 10	Nd	20	Apr 2012 PTA 10	Nd
21	Apr 2012 CT 01	Nd	21	Apr 2012 CT 01	Nd
22	Apr 2012 CT 02	Nd	22	Apr 2012 CT 02	Nd
23	Apr 2012 CT 03	Nd	23	Apr 2012 CT 03	Nd
24	Apr 2012 CT 04	Nd	24	Apr 2012 CT 04	Nd
25	Apr 2012 CT 05	Nd	25	Apr 2012 CT 05	Nd
26	Apr 2012 CT 06	Nd	26	Apr 2012 CT 06	Nd
27	Apr 2012 CT 07	Nd	27	Apr 2012 CT 07	Nd
28	Apr 2012 CT 08	Nd	28	Apr 2012 CT 08	Nd
29	Apr 2012 CT 09	Nd	29	Apr 2012 CT 09	Nd
30	Apr 2012 CT 10	Nd	30	Apr 2012 CT 10	Nd
31	JAN + APR Control	Nd	31	JAN + APR Control	Nd
32	Jan 2012 CT 01	Nd	32	Jan 2012 CT 01	Nd
33	Jan 2012 CT 02	Nd	33	Jan 2012 CT 02	Nd
34	Jan 2012 CT 03	Nd	34	Jan 2012 CT 03	Nd
35	Jan 2012 CT 04	Nd	35	Jan 2012 CT 04	Nd

36	Jan 2012 CT 05	Nd	36	Jan 2012 CT 05	Nd
37	Jan 2012 CT 06	Nd	37	Jan 2012 CT 06	Nd
38	Jan 2012 CT 07	Nd	38	Jan 2012 CT 07	Nd
39	Jan 2012 CT 08	Nd	39	Jan 2012 CT 08	Nd
40	Jan 2012 CT 09	Nd	40	Jan 2012 CT 09	Nd
41	Jan 2012 CT 10	Nd	41	Jan 2012 CT 10	Nd

Nd: Not detected

3.2.2.3 Dibutyl phthalate, bis (ethyl hexyl) phthalate and bis (ethyl hexyl) adipate

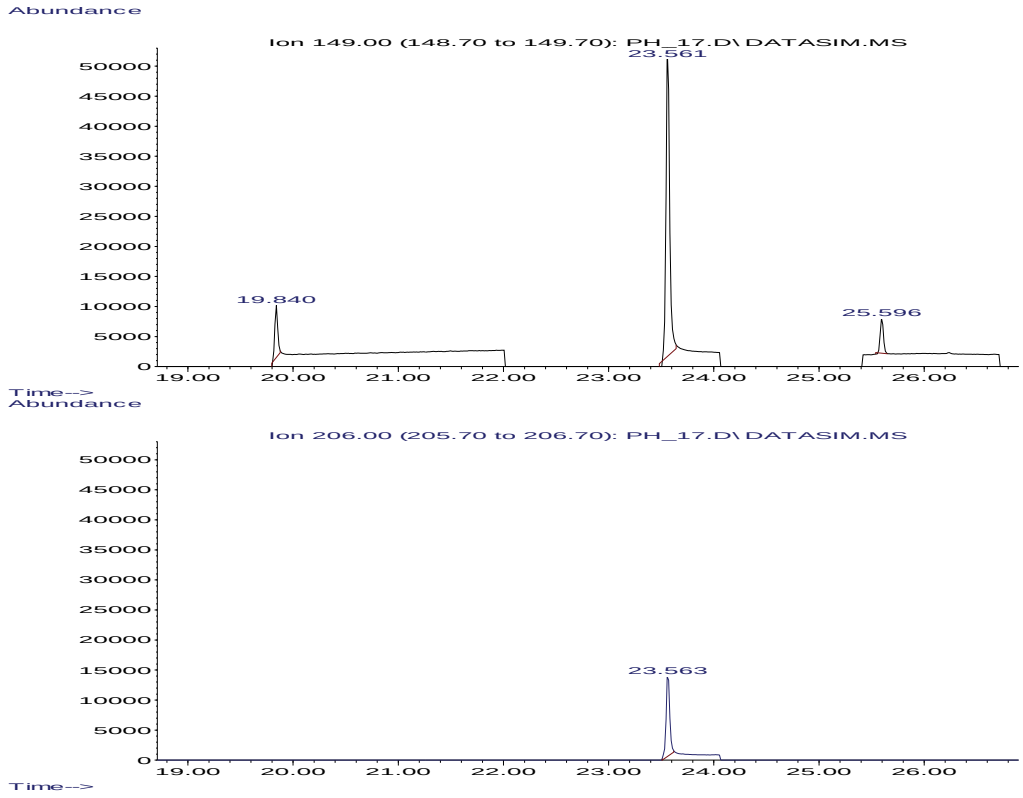

Figure 8: The SIM chromatogram for the DBP and DEHP standards (top) at a concentration of 2.14 ng/ℓ and 1.25 ng/ℓ at retention times of 20.74 and 25.59 min respectively and that of BBP as internal standard (bottom) at retention time of 23.57 min

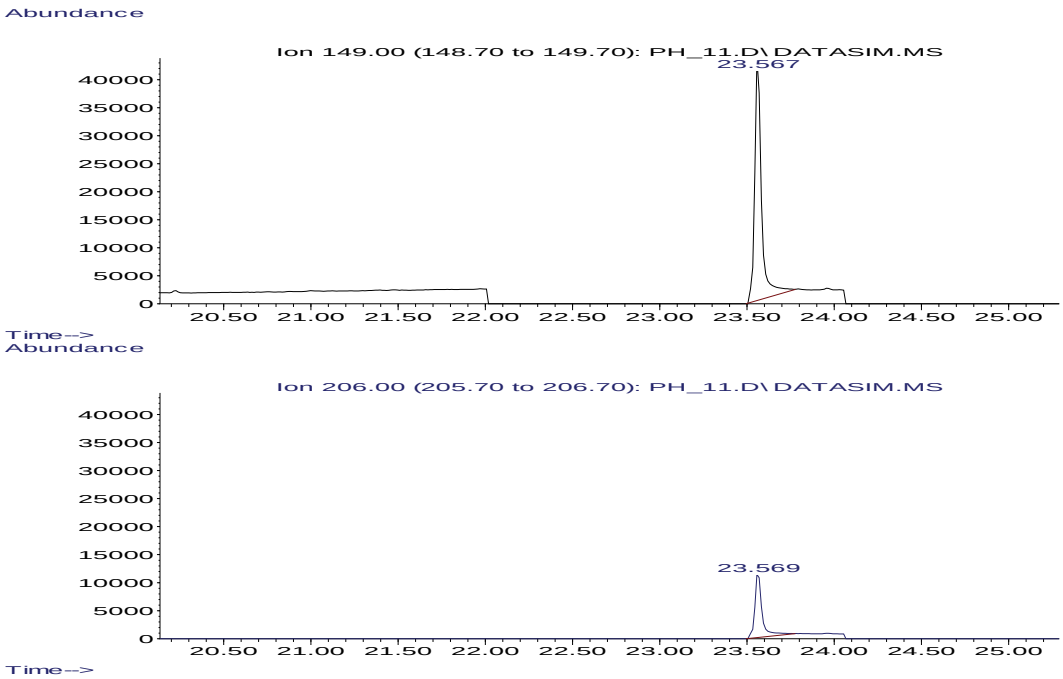

Figure 9: The SIM chromatogram for sample 11 showing the absence of both DBP and DEHP at retention times of 20.74 min and 25.59 min respectively and that of BBP as internal standard at retention time of 23.57 min

DHEP, DBP and DHEA were not detected in the samples. The same trend as seen in the chromatograms of sample 11 was representative of all 40 samples analyzed.

Table 8: Concentration of DBP, DHEP and DHEA in drinking water samples

Sample #	Sample Identification	Dibutyl phthalate (DBP) Conc (ng/ℓ)	Bis (ethyl hexyl) phthalate (DHEP) Conc (ng/ℓ)	Bis (ethyl hexyl) adipate (DHEA) Conc (ng/ℓ)
1	Jan 2012 PTA 01	Nd	Nd	Nd
2	Jan 2012 PTA 02	Nd	Nd	Nd
3	Jan 2012 PTA 03	Nd	Nd	Nd
4	Jan 2012 PTA 04	Nd	Nd	Nd
5	Jan 2012 PTA 05	Nd	Nd	Nd
6	Jan 2012 PTA 06	Nd	Nd	Nd
7	Jan 2012 PTA 07	Nd	Nd	Nd
8	Jan 2012 PTA 08	Nd	Nd	Nd
9	Jan 2012 PTA 09	Nd	Nd	Nd
10	Jan 2012 PTA 10	Nd	Nd	Nd
11	Apr 2012 PTA 01	Nd	Nd	Nd
12	Apr 2012 PTA 02	Nd	Nd	Nd
13	Apr 2012 PTA 03	Nd	Nd	Nd
14	Apr 2012 PTA 04	Nd	Nd	Nd
15	Apr 2012 PTA 05	Nd	Nd	Nd
16	Apr 2012 PTA 06	Nd	Nd	Nd
17	Apr 2012 PTA 07	Nd	Nd	Nd
18	Apr 2012 PTA 08	Nd	Nd	Nd
19	Apr 2012 PTA 09	Nd	Nd	Nd
20	Apr 2012 PTA 10	Nd	Nd	Nd
21	Apr 2012 CT 01	Nd	Nd	Nd
22	Apr 2012 CT 02	Nd	Nd	Nd
23	Apr 2012 CT 03	Nd	Nd	Nd
24	Apr 2012 CT 04	Nd	Nd	Nd
25	Apr 2012 CT 05	Nd	Nd	Nd
26	Apr 2012 CT 06	Nd	Nd	Nd
27	Apr 2012 CT 07	Nd	Nd	Nd
28	Apr 2012 CT 08	Nd	Nd	Nd
29	Apr 2012 CT 09	Nd	Nd	Nd
30	Apr 2012 CT 10	Nd	Nd	Nd
31	JAN + APR Control	Nd	Nd	Nd
32	Jan 2012 CT 01	Nd	Nd	Nd
33	Jan 2012 CT 02	Nd	Nd	Nd
34	Jan 2012 CT 03	Nd	Nd	Nd
35	Jan 2012 CT 04	Nd	Nd	Nd
36	Jan 2012 CT 05	Nd	Nd	Nd
37	Jan 2012 CT 06	Nd	Nd	Nd
38	Jan 2012 CT 07	Nd	Nd	Nd
39	Jan 2012 CT 08	Nd	Nd	Nd
40	Jan 2012 CT 09	Nd	Nd	Nd
41	Jan 2012 CT 10	Nd	Nd	Nd

Nd: Not detected

3.2.2.4 4-Nonyl phenol

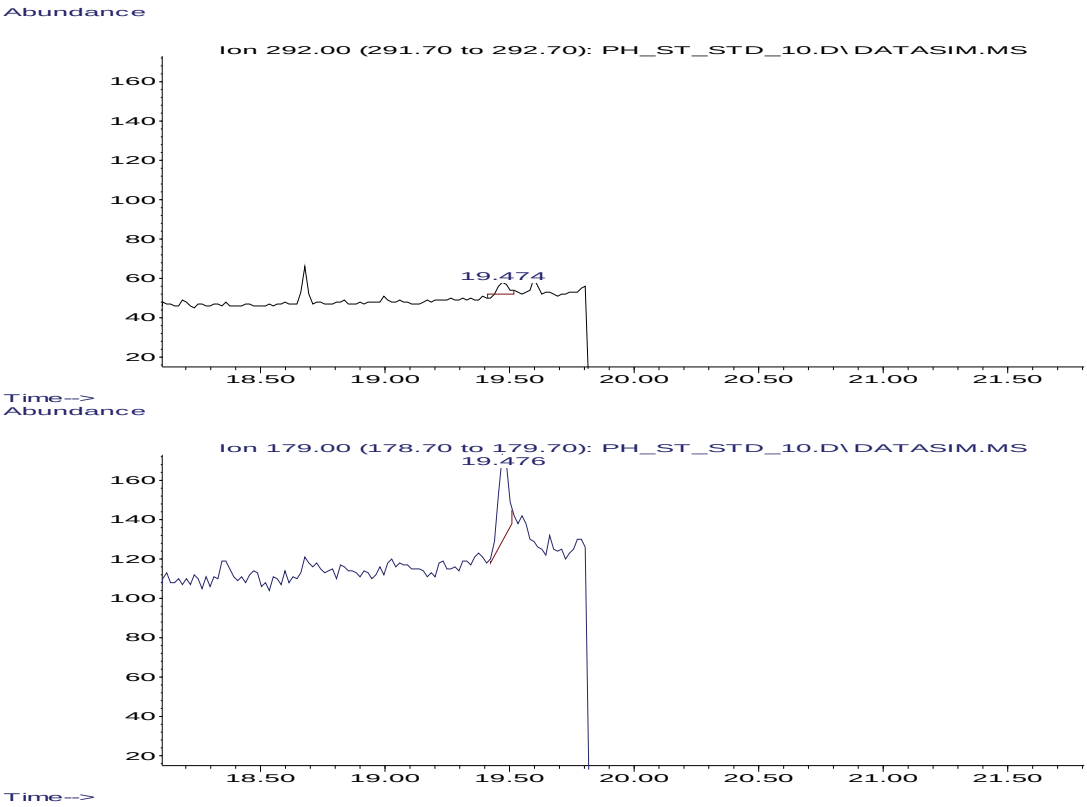

Figure 10: The SIM chromatogram for the 4-NP standard at a concentration of 1.11 ng/ℓ (mz 272 and 179) at retention time of 19.47 min

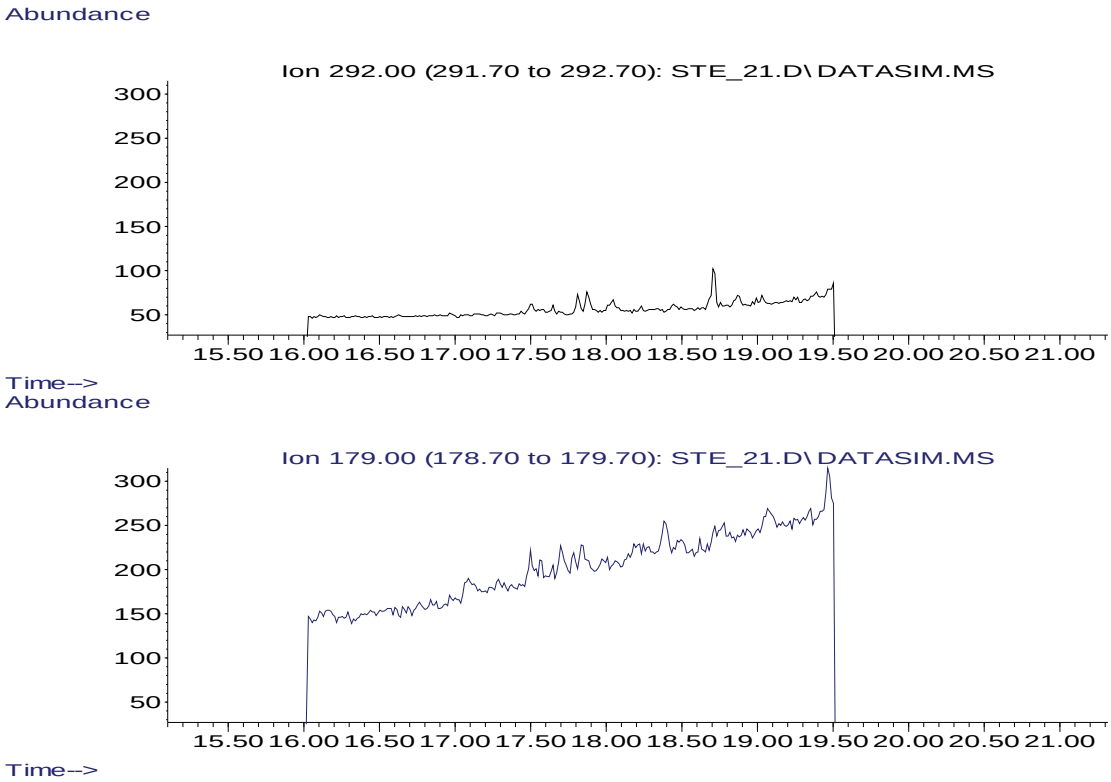

Figure 11: The SIM chromatogram of sample 21 monitoring for 4-NP (mz 179 and 292). A signal for both ions was absent between 16.00 mins and 19.5 mins. No 4-NP could be detected.

3.2.3 Discussion

4-NP was not detected in the samples as can be seen in figure 11. The same trend as seen in the chromatograms of sample 21 was representative of all 40 samples analyzed.

E1, EE2 DHEP, DBP, DHEA and 4-NP were not detected in any of the samples analysed in spite of advances made with respect to analytical methods to determine EDCs in water samples. These chemicals are usually present at extremely low levels in drinking water which could account for the phthalates and nonylphenol not being detected. Method development needs to take place to get to the concentration range of ng/ℓ to pg/ℓ in order to detect these compounds. As suggested by Kortenkamp et al. (2007) although chemicals are considered “safe” below their NOAELs when present in a mixture of similar-acting chemicals they may act additively or synergistically to cause adverse health effects.

BPA is used to manufacture plastics for poly-carbonate bottles and epoxy resin liners for food and beverage cans. Polycarbonates are useful in industry due to their strong durability. However researchers have reported deleterious effects on animals and humans when released from the polycarbonates due to its estrogenic properties (Erler and Novak, 2010). EDCs like BPA, alkylphenols (e.g. NP), phthalates and poly aromatic hydrocarbons (PAHs) can leach from water supply lines into drinking water (Rahman et al., 2009).

Several international studies reported the presence of BPA in drinking water (Table 9). This study detected BPA in 11 of the drinking water samples at a concentration ranging between 0.027ng/ℓ (ppt) and 0.067 ng/ℓ. These levels are below the newly derive Predicted No Effect Concentration (PNEC) of 0.06 µg/ℓ BPA in fresh water (Wright-Waters et al., 2011; Santhi et al., 2012).

Table 9: Levels of BPA found in drinking water from other countries

BPA concentration (ng/ℓ)	Country	Reference
0.3-2	Germany	Kuch and Ballschmiter, 2001
Nd *	Northern Italy	Loos et al., 2007
15-36	China	Shao et al., 2008; Huang et al., 2012
38.9-55.8	China	Zhou et al., 2009; Huang et al., 2012
Nd *	US	Benotti et al., 2009
Nd *	Iran	Jafari et al., 2009
160	Brazil	Sodre et al., 2010
2.3-317	South China	Li et al., 2010
3.5-59.8	Malaysia	Santhi et al., 2012

Nd Not detected

* BPA only detected in source water, not in drinking/tap water

Despite the advances in conventional water treatment technology many synthetic and natural organic compounds have been detected in drinking water (Benotti et al., 2009). Various steps in the water treatment process can remove estrogenic activity to some degree. The most effective removal results from activated carbon, ultraviolet irradiation, reverse osmosis and bio- and photo-degradation and activated sludge

treatment, with granular activated carbon being the most efficient method for the removal of EDCs from drinking water (Genthe et al., 2010). However, it is unlikely that a single water treatment process will be able to remove all traces of organic contaminants (Stanford et al., 2010). There may well be a risk associated with the presence of EDC steroids in drinking water, especially as many drinking water treatment plants use source water impacted by wastewater (Benotti et al., 2009). In this study E2 was detected in all samples at a concentration range of between 0.035 ng/ℓ and 0.426 ng/ℓ. These levels are lower than those found by Kuch and Ballschmitter, (2001), but higher than Jafari et al., (2009) (Table 10).

Table 10: Levels of E2 found in drinking water from other countries

E2 concentration (ng/ℓ)	Country	Reference
0.2-2.1	Germany	Kuch and Ballschmiter, 2001
Nd *	Northern Italy	Loos et al., 2007
Nd *	US	Benotti et al., 2009
0.002	Iran	Jafari et al., 2009

Nd Not detected

* E2 only detected in source water, not in drinking/tap water

The extraction control was below the detection limit in the bioassays, however low levels of E2 were measured with GC-MS. The reasons for this need to be investigated as there are a number of possible contributing factors including; the EDS filter may not have been effective in removing the E2 from the source water; or contamination of the solvents used in elution and reconstitution.

CHAPTER 4: CONCLUSIONS & RECOMMENDATIONS

4.1 CONCLUSIONS

The bioassay results of this study indicate that estrogenic activity was present in 22 of the 40 samples over the two sampling periods. BPA was detected by GC-MS in 11 of the drinking water samples at concentrations ranging between 0.027 ng/ℓ (ppt) and 0.067 ng/ℓ. E2 was detected in all samples at concentrations ranging between 0.035 ng/ℓ and 0.426 ng/ℓ. BPA and E2 are known to have estrogenic activity, which supports the finding of the T47D-KBluc bioassay. It should be borne in mind that results of the bioassays and chemical analysis cannot be directly compared. While the bioassays indicate the total activity of the complex mixture, the chemical analysis is aimed at testing for the presence of specifically selected target chemicals and cannot account for the presence and the possible interactions between all the chemicals in the sample. Therefore bioassays should always be done in conjunction with chemical analyses.

The distribution point water samples ranged from below detection to a maximum of 0.167 ng/ℓEEq. These are well below the recommended trigger value of 0.7 ng/ℓ EEqs in drinking water as proposed by Genthe et al., (2010).

This study showed that the YES and T47D-KBluc assays are suitable as screening assays for estrogenic activity in drinking water as recommended by the WRC Toolbox project (de Jager et al., 2011).

4.2 RECOMMENDATIONS

Based on the findings and conclusions of this study the following recommendations are suggested

- Although the EEqs of the bioassays were below the recommended trigger value, the fact that BPA and E2 were present is still of some concern due to the possible effects of chronic low dose exposure. The fact that EDCs were detected in the drinking water indicates that these chemicals are entering the system at some point. Therefore a monitoring strategy using both bioassays and chemical analysis is recommended for not only the two chemicals detected but should include other EDCs as well. This will give a measure of contamination of the distribution points over a period of time but will also indicate acute contamination episodes.

- The local drinking water treatment facilities should be responsible for this and the monitoring could take place quarterly to ensure that the drinking water processes are functioning optimally.

- E1, EE2 DHEP, DBP, DHEA and 4-NP were not detected in any of the samples analysed but may well be present at levels below the current detection limits. Further development of the chemical analysis methodology is recommended in order to obtain lower detection limits for EDCs in water sources and specifically drinking water.

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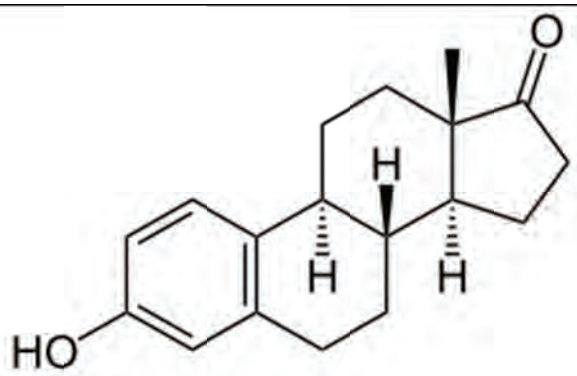
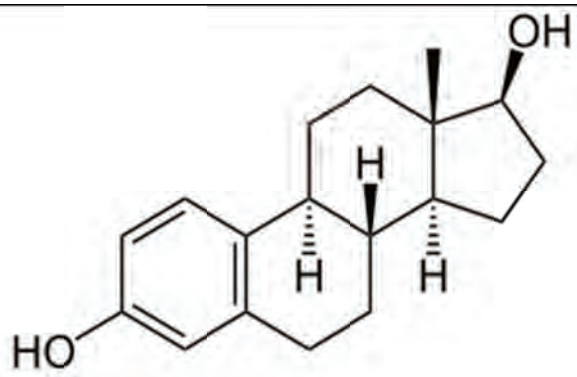
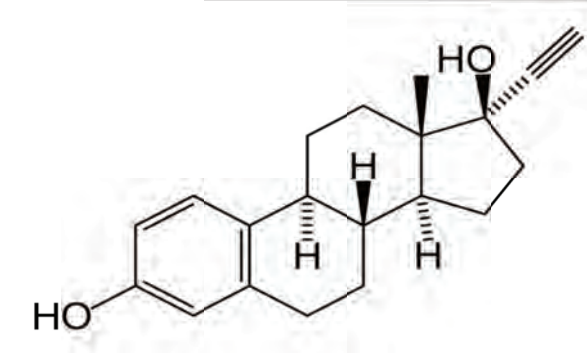
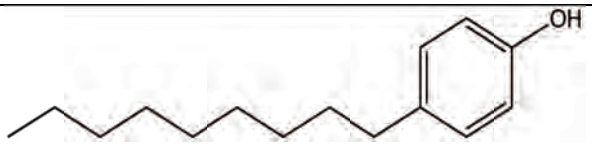
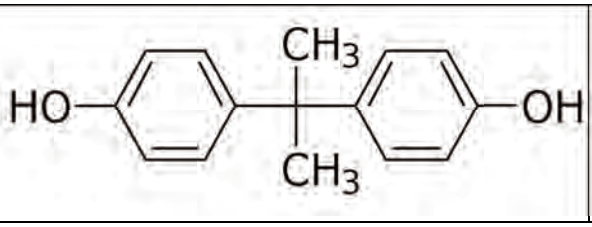
APPENDIX A: Report on analyses of water samples

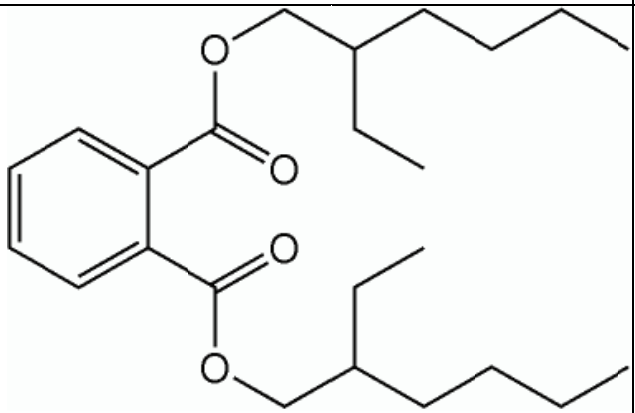
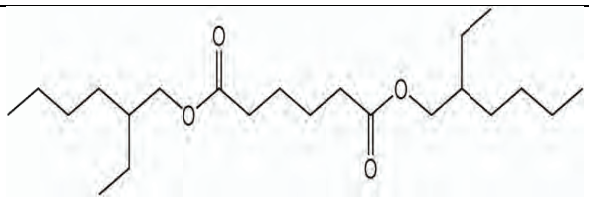
By: Prof P Swart and Mr B Truebody
03 October 12

Specific water extracted samples were presented on solid phase extraction cartridges for the analyses for 8 targeted chemical species: Bisphenol-A (BPA), 4-*n*-nonylphenol (4NP), dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate (DHEP), bis(2-ethylhexyl) adipate (DHEA), estradiol (E2), estrone (E1) and ethinylestradiol (EE2) (Table 1). Targeted chemical analysis of these species was conducted using gas chromatography-mass spectrophotometry (GC-MS).

Table 1: Structures and mass spectrometric properties of EDC analysed in water extracts.

Compound	Structure	Quantitative ion (after MSTFA derivatisation)
Estrone (E1) MW: 270		$[(CH_3)_3\text{-Si-O-}C_{18}H_{21}O]^+$ MW: 342
Estradiol (E2) MW: 272		$[(CH_3)_3\text{-Si-O-}C_{15}H_{19}]^+$ MW: 285
Ethinylestradiol (EE2) MW: 296		$[(CH_3)_3\text{-Si-O-}C_{18}H_{23}\text{-O-Si-}(CH_3)_3]^+$ MW: 425
4-nonylphenol (4NP) MW: 270		$[(CH_3)_3\text{-Si-O-}C_6H_4\text{-}C(CH_3)_2]^+$ MW: 179

Bisphenol-A (BPA) MW: 270		$[(CH_3)_3Si\text{-}O\text{-}C_6H_4\text{-}C(CH_3)\text{-}C_6H_4\text{-}O\text{-}Si(CH_3)_3]^+$ MW: 357

Compound	Structure	Quantitative ion
Dibutyl phthalate (DBP) MW: 278		$[C_8H_5O_3]^+$ MW: 149
Bis(2-ethylhexyl) phthalate (DHEP) MW: 270		$[C_8H_5O_3]^+$ MW: 149
Bis(2-ethylhexyl) adipate (DHEA) MW: 370		$[C_6H_9O_3]^+$ MW: 129

Materials and methods

Stock solutions of DBP, DHEP, BPA, 4NP, E1, E2, EE2 and DHEA were prepared in analyte grade acetone at a concentration of 1 mg/mℓ. A stock solution of deuterated Bisphenol-A (*d*-16 BPA)(internal standard) was prepared in analyte grade methanol at a concentration of 1 mg/mℓ. All stock solutions were light protected and stored at -18°C.Due to the use of DBP, DHEP and DHEA in commercial plastics and their propensity to leach out of the plastic matrix, all glassware was soaked in acetone for 18 hours and placed in an oven at 200°C overnight in order to prevent contamination.

A working solution of the standards BPA, 4NP, DHEP, DBP, DHEA, E1, E2 and EE2 for GC-MS was prepared in analyte grade acetone. A calibration series of BPA, 4NP, DHEP, DBP, DHEA, E1, E2 and EE2 was prepared with concentrations of 10 ng/ℓ, 50 ng/ℓ, 100 ng/ℓ, 500 ng/ℓ, 1 000 ng/ℓ, 5 000 ng/ℓand 10 000 ng/ℓ. A working solution of the standards DHEP, DBP and DHEA for GC-MS was prepared in analyte grade acetone. The internal standard, *d*-16 BPA, was added to each working solution to a final concentration of 5 µg/mℓ. All working solutions were prepared fresh whenever a GC-MS experiment was conducted.

Standards and eluents from SPE were transferred to 5 mℓ reaction vials and 100 ng internal standard added. Derivatisation of standard working solutions for GC-MS was conducted according to Wilding et al. (1) in order to increase the volatility of 4NP, BPA and steroids and improve chromatographic separation. Solvent was then evaporated and to each standard and sample a solution of 100 µℓ*N*-methyl-trimethylsilylflouroacetamide (MSTFA), 100 µℓ pyridine and 10 µℓ trimethylchlorosilane (TMCS) was added. The standards and samples were then incubated for 20 mins at 80°C. The derivatives were stored at room temperature until GC-MS analysis. The derivitisation protocol calls for pyridine as solvent to increase the hydrogenaBisphenol-Abstraction from the derivitised samples and to enhance reaction with the MSTFA.

The samples analysed showed that specifically the pthalates, DBP, DHEA and DHEP were over-estimated by orders of magnitude when compared to the bio-assays performed. The cause of this overestimation was investigated and it was found that the pyridine, supplied to us as the highest analytical grade, was contaminated with DBP, DHEA and DHEP.

GC-MS, using single ion monitoring for ions corresponding to DBP, DHEA and DHEP of 100 µℓ of a 6 month batch of pyridine and a newly-opened batch of pyridine was conducted. Under SIM conditions peaks at retention times of 19.3, 23.4 and 25.0 mins can be observed for both the older pyridine (fig. 1) and more recently purchased pyridine (fig. 2) corresponding to DBP, DHEA and DHEP respectively. Retention times of targeted chemicals in the pyridine GC-MS analysis are comparable with that of the DBP, DHEA and DHEP standards (fig. 3).

These results clearly indicate that the pyridine is the cause of the pthalate overestimation and that the only way to prevent are-occurrence of this overestimation of phthalates and adipate a new method of derivatisation will be developed without using pyridine. The alternative method of derivatisation to be explored will be acylation (2), using heptafluorobutyric anhydride. We have already conducted trial runs and the results are extremely promising. By avoiding the use of pyridine, any contaminants which would result in an overestimation of the concentration of the targeted chemical species will be mitigated.

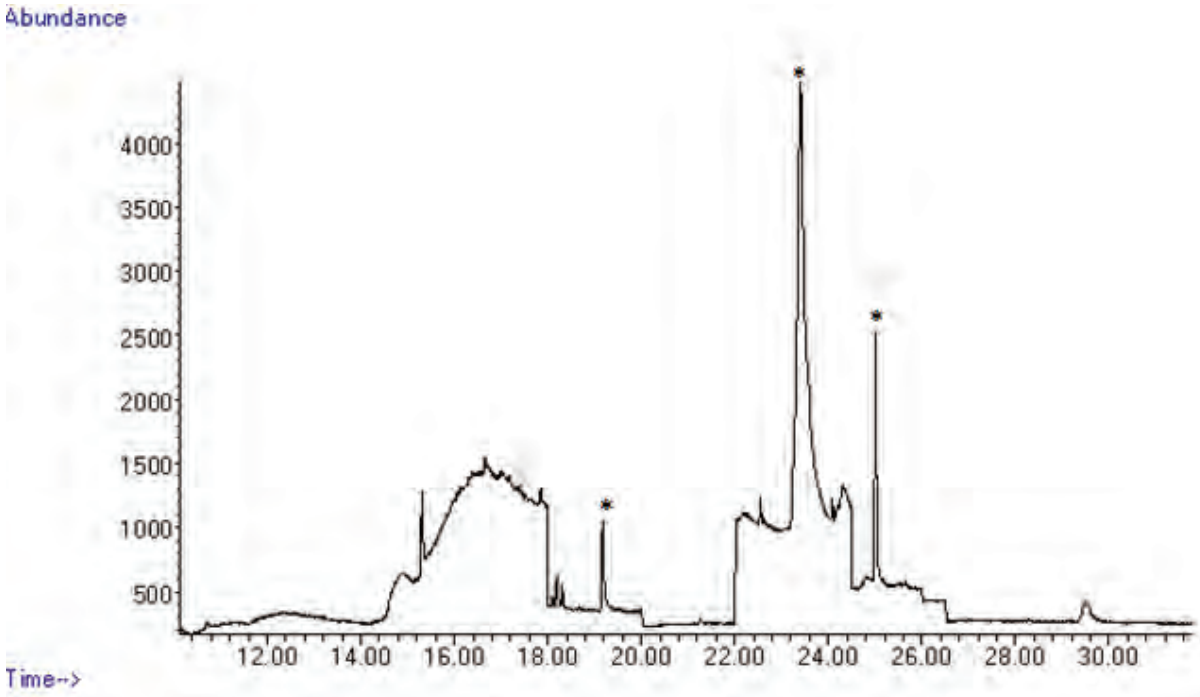


Figure 1: SIM chromatogram of 6 month old pyridine. Asterisks indicate peaks of DBP (19.3 mins), DHEA (23 mins) and DHEP (25 mins).

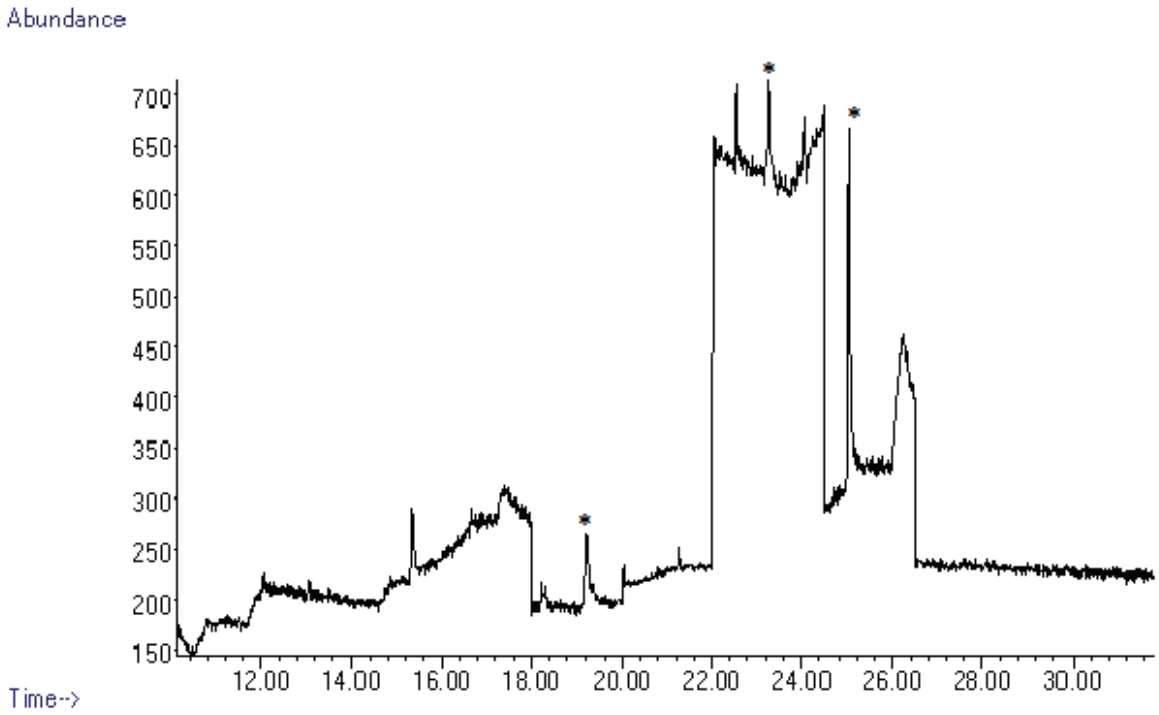


Figure 2: SIM chromatogram of recently opened pyridine. Asterisks indicate peaks of DBP (19.3 mins), DHEA (23 mins) and DHEP (25 mins).

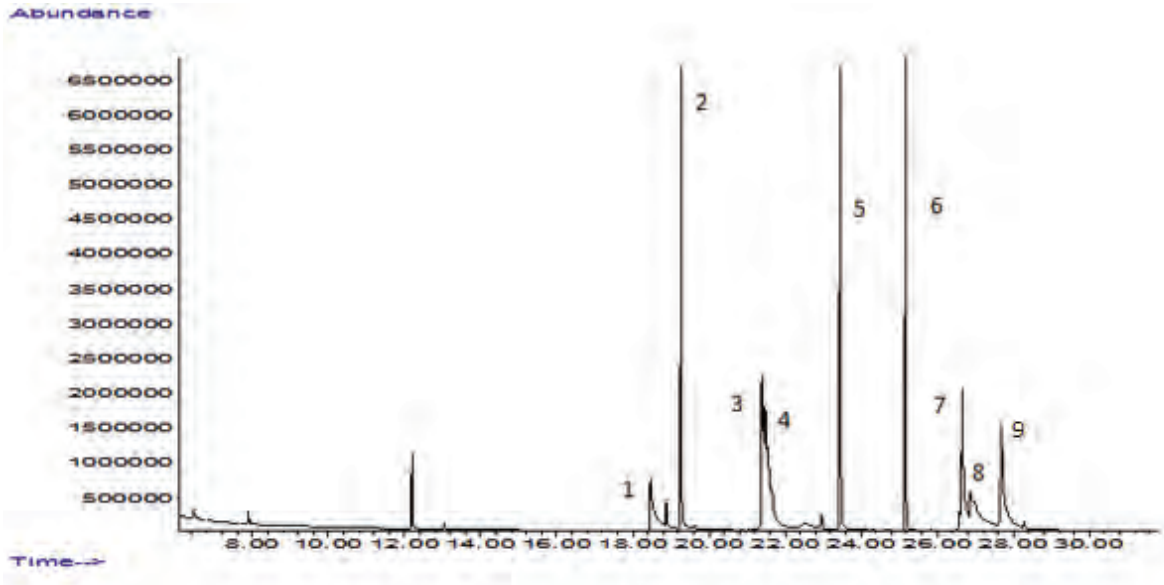


Figure 3: Total ion chromatogram of EDC standards. 1) 4NP, 2) DBP, 3) BPA, 4) dBPA, 5) DHEA, 6) DHEP, 7) E1, 8) E2, 9) EE2.

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