Table of Contents

Declaration ii
Summary iii
Acknowledgements v
Table of Contents vi
List of Figures xi
List of Tables xv
List of Abbreviations xviii
Chapter 1: Literature Review 1
1.1. The endocrine system 1
1.2. Endocrine disrupting chemicals (EDCs) 3
1.2.1. Definition of EDCs 4
1.2.2. Classes of EDCs 4
1.2.3. Routes of exposure 5
1.2.4. Health effects associated with EDC exposure 5
1.2.5. EDC activity 6
1.2.5.1. How do EDCs disturb the hormonal system? 6
1.2.5.2. The EDC dose response curve 7
1.2.5.3. The mixture effect 8
1.2.5.4. Exposure to EDCs during critical windows 8
1.2.5.5. Vulnerable populations 9
1.2.6.6. Multigenerational and transgenerational effects 10
1.3. EDCs in the aquatic environment 10
1.3.1. Natural and synthetic steroids in sewage effluent 11
1.3.2. Pharmaceuticals and hospital effluents 13
1.3.3. Personal care products 14
1.3.4. Household products and industrial effluents 14
1.3.5. Agricultural effluents 15
1.4. Removal of EDCs through water treatment processes 16
1.4.1. Removal of EDCs from wastewater 17

© University of Pretoria

1.4.2. EDCs in drinking/tap water 18
1.5. Bottled water as an alternative to tap water 19
1.5.1. Sources of contamination of bottled water 20
1.5.1.1. Endocrine activity 21
1.5.1.2. Bisphenol A (BPA) 21
1.5.1.3. Nonylphenol (NP) 24
1.5.1.4. Phthalates 26
1.5.1.5. Di(2-ethylhexyl) adipate (DEHA) 29
1.5.2. Factors influencing the migration of chemicals from bottles into water content 29
1.6. Testing methodologies for EDCs 30
1.6.1. Sample preparation 31
1.6.2. Bioassays and non-cellular *in vitro* assays 33
1.6.3. Whole organism assays 35
1.6.4. Chemical analysis 35
1.7. Health risk assessment of EDCs 36
1.7.1. Hazard identification 37
1.7.2. Dose response assessment 38
1.7.3. Exposure assessment 38
1.7.4. Risk characterization 39
Chapter 2: Aims and objectives of the study 40
2.1. Hypothesis 40
2.2. Aims and objectives 40
2.2.1. Phase 1 – Distribution point water 40
2.2.2. Phase 2 – Bottled water 41
2.2.3. Phase 3 – Health risk assessment 41
Chapter 3: Materials and Methods 42
3.1. General laboratory procedures 43
3.1.1. Materials 43
3.1.2. Methods 43

© University of Pretoria

3.2. Phase 1 – Distribution point water 43
3.2.1. Site selection 43
3.2.2. Sample collection........ 45
3.2.2.1. Materials 45
3.2.2.2. Methods 45
3.2.3. Water extraction procedure........ 47
3.2.3.1. Materials 47
3.2.3.2. Methods 47
3.2.4. Bioassays for estrogenic activity 51
3.2.4.1. The recombinant yeast estrogen screen (YES) bioassay 51
3.2.4.1.1. Materials 51
3.2.4.1.2. Methods: Preparation of medium and stock solutions and maintenance of stock cultures........ 52
3.2.4.1.3. Methods: Assay procedure 53
3.2.4.1.4. Methods: Calculation of results 56
3.2.4.2. T47D-KBluc reporter gene bioassay 56
3.2.4.2.1. Materials 57
3.2.4.2.2. Methods: Preparation of medium and stock solutions and general cell culture procedures 57
3.2.4.2.3. Methods: Assay procedure 59
3.2.4.2.4. Methods: Calculation of results 61
3.2.5. Target chemical analyses 61
3.2.5.1. Materials 61
3.2.5.2. Methods: Preparation of stock solutions 62
3.2.5.3. Methods: Sample preparation and derivatization........ 62
3.2.5.4. Methods: UPLC-MS analysis 63
3.3. Phase 2 - Bottled water........ 63
3.3.1. Sample collection........ 63
3.3.2. Sample preparation 65
3.3.3. Extraction procedure........ 67

© University of Pretoria

3.3.4. Bioassays for estrogenic activity 67
3.3.5. Target chemical analyses 67
3.4. Phase 3 - Health Risk Assessment 67
3.5. Ethical considerations 69
Chapter 4: Pilot study 70
4.1. Assessment of the target chemicals in the YES and T47D-KBluc bioassays. 70
4.2. Validation of extraction method 72
4.3. Recoveries of target chemicals using UPLC-MS 75
Chapter 5: Results 76
5.2. Phase 1 – Distribution point water 76
5.2.1. Bioassays for estrogenic activity 76
5.2.2. Target chemical analyses 77
5.2.2.1. Bisphenol A (BPA) 79
5.2.2.2. Nonylphenol (NP) 81
5.2.2.3. Di(2-ethylhexyl) adipate (DEHA) 82
5.2.2.4. Dibutyl phthalate (DBP) 84
5.2.2.5. Di(2-ethylhexyl) phthalate (DEHP) 86
5.2.2.6. Diisononyl phthalate (DINP) 89
5.2.2.7. 17β-Estradiol (E_2) 92
5.2.2.8. Estrone (E_1) 94
5.2.2.9. Ethynylestradiol (EE_2) 96
5.3. Phase 2 - Bottled water 98
5.3.1. Mineral composition 98
5.3.2. Bioassays for estrogenic activity 99
5.3.3. Target chemical analyses 100
5.3.3.1. Bisphenol A (BPA) 100
5.3.3.2. Nonylphenol (NP) 100
5.3.3.3. Di(2-ethylhexyl) adipate (DEHA) 101
5.3.3.4. Dibutyl phthalate (DBP) 102
5.3.3.5. Di(2-ethylhexyl) phthalate (DEHP) 103
5.3.3.6. Diisononyl phthalate (DINP) 104
5.3.3.7. 17β-Estradiol (E_2) 105

© University of Pretoria

5.3.3.8. Estrone (E_1) ... 105
5.3.3.9. Ethynylestradiol (EE_2) ... 106
5.4. Comparison between distribution point and bottled water ... 107
5.5. Phase 3 - Health Risk Assessment ... 116
Chapter 6: Discussion and Conclusions ... 139
6.1. General discussion on methods ... 139
6.2. Distribution point water ... 140
6.3. Bottled water ... 147
6.4. Comparison between distribution point and bottled water ... 155
6.5. Health risk assessment ... 156
6.6. Environmental and Public Health considerations ... 160
6.7. Conclusions ... 165
6.8. Recommendations ... 166
References ... 170
Appendix A: Bottled water sample codes ... 198
Appendix B: Ethics approval ... 199

© University of Pretoria

List of Figures

Figure 1.1: An overview of the endocrine system and some of the hormones produced by endocrine glands and organs with secondary endocrine function 2
Figure 3.1: Flow diagram of the three phases of the project 42
Figure 3.2: Examples of distribution points and sampling 46
Figure 3.3: Solid phase extraction apparatus 48
Figure 3.4: Procedural steps for solid phase extraction... 50
Figure 3.5: An example of dosing and reading yeast screen bioassay plates 55
Figure 3.6: Procedural steps for the YES bioassay 55
Figure 3.7: Procedural steps for the T47D-KBluc reporter gene bioassay 60
Figure 3.8: Incubator setup for the exposure of bottled water samples to simulated sunlight 66
Figure 4.1: Estrogenic response of target chemicals in the YES bioassay............. 71
Figure 4.2: Estrogenic response of target chemicals in the T47D-KBluc bioassay . 71
Figure 5.1: Chromatogram of BPA standard (10 µg/L).. 79
Figure 5.2: BPA calibration curve 79
Figure 5.3: Chromatogram of NP standard (500 ng/L) 81
Figure 5.4: NP calibration curve 81
Figure 5.5: Chromatogram of DEHA standard (300 µg/L) 82
Figure 5.6: DEHA calibration curve 82
Figure 5.7: Chromatogram of DBP standard (300 µg/L).. 84
Figure 5.8: DBP calibration curve... 84
Figure 5.9: Chromatogram of DEHP standard (300 µg/L) 86
Figure 5.10: DEHP calibration curve 86
Figure 5.11: Chromatogram of DINP standard (100 µg/L)..................................... 89
Figure 5.12: DINP calibration curve.. 89
Figure 5.13: Chromatogram of E_2 standard (500 ng/L) 92
Figure 5.14: E_2 calibration curve 92
Figure 5.15: Chromatogram of E_1 standard (500 ng/L) 94
Figure 5.16: E_1 calibration curve 94
Figure 5.17: Chromatogram of EE_2 standard (500 ng/L) 96
Figure 5.18: EE_2 calibration curve 96

© University of Pretoria

Figure 5.19: Comparison of distribution point and bottled water using the T47D-KBluc bioassay 107

Figure 5.20: Comparison of BPA concentrations in distribution point and bottled water 108

Figure 5.21: Comparison of DEHA concentrations in distribution point and bottled water 109

Figure 5.22: Comparison of DBP concentrations in distribution point and bottled water 110

Figure 5.23: Comparison of DEHP concentrations in distribution point and bottled water 111

Figure 5.24: Comparison of DINP concentrations in distribution point and bottled water 112

Figure 5.25: Comparison of E_2 concentrations in distribution point and bottled water 113

Figure 5.26: Comparison of E1 concentrations in distribution point and bottled water 114

Figure 5.27: Comparison of EE2 concentrations in distribution point and bottled water. 115

Figure 5.28: Monte Carlo simulation of Pretoria distribution point water indicating the probability distributions of reasonable maximum EEq values in ng/L 118

Figure 5.29: Monte Carlo simulation of Cape Town distribution point water indicating the probability distributions of reasonable maximum EEq values in ng/L 119

Figure 5.30: Monte Carlo simulation of bottled water indicating the probability distributions of reasonable maximum EEq values in ng/L 119

Figure 5.31: Monte Carlo simulation of HQ risks posed by BPA to people consuming distribution point water from Pretoria 120

Figure 5.32: Monte Carlo simulation of HQ risks posed by BPA to people consuming distribution point water from Cape Town 121

Figure 5.33: Monte Carlo simulation of HQ risks posed by BPA to people consuming bottled water 121

Figure 5.34: Monte Carlo simulation of HQ risks posed by DEHA to people consuming distribution point water from Pretoria 122

Figure 5.35: Monte Carlo simulation of HQ risks posed by DEHA to people consuming distribution point water from Cape Town 123

© University of Pretoria

Bestpfe.com

Figure 5.36: Monte Carlo simulation of HQ risks posed by DEHA to people consuming bottled water 123

Figure 5.37: Monte Carlo simulation of cancer risks posed by DEHA to people consuming distribution point water from Pretoria 124

Figure 5.38: Monte Carlo simulation of cancer risks posed by DEHA to people consuming distribution point water from Cape Town.. 125

Figure 5.39: Monte Carlo simulation of cancer risks posed by DEHA to people consuming bottled water 125

Figure 5.40: Monte Carlo simulation of HQ risks posed by DBP to people consuming distribution point water from Pretoria.. 126

Figure 5.41: Monte Carlo simulation of HQ risks posed by DBP to people consuming distribution point water from Cape Town 127

Figure 5.42: Monte Carlo simulation of HQ risks posed by DBP to people consuming bottled water.. 127

Figure 5.43: Monte Carlo simulation of HQ risks posed by DEHP to people consuming distribution point water from Pretoria 128

Figure 5.44: Monte Carlo simulation of HQ risks posed by DEHP to people consuming distribution point water from Cape Town.. 129

Figure 5.45: Monte Carlo simulation of HQ risks posed by DEHP to people consuming bottled water 129

Figure 5.46: Monte Carlo simulation of cancer risks posed by DEHP to people consuming distribution point water from Pretoria 130

Figure 5.47: Monte Carlo simulation of cancer risks posed by DEHP to people consuming distribution point water from Cape Town.. 131

Figure 5.48: Monte Carlo simulation of cancer risks posed by DEHP to people consuming bottled water 131

Figure 5.49: Monte Carlo simulation of HQ risks posed by DINP to people consuming distribution point water from Pretoria 132

Figure 5.50: Monte Carlo simulation of HQ risks posed by DINP to people consuming distribution point water from Cape Town.. 133

Figure 5.51: Monte Carlo simulation of HQ risks posed by DINP to people consuming bottled water 133

Figure 5.52: Monte Carlo simulation of HQ risks posed by E_2 to people consuming distribution point water from Cape Town 134

© University of Pretoria

Figure 5.53: Monte Carlo simulation of HQ risks posed by E_1 to people consuming distribution point water from Pretoria (95th percentile) 135
Figure 5.54: Monte Carlo simulation of HQ risks posed by E_1 to people consuming distribution point water from Pretoria (average) 136
Figure 5.55: Monte Carlo simulation of HQ risks posed by E_1 to people consuming distribution point water from Cape Town 137
Figure 5.56: Monte Carlo simulation of HQ risks posed by E_1 to people consuming bottled water.. 137
Figure 5.57: Monte Carlo simulation of HQ risks posed by EE_2 to people consuming distribution point water from Pretoria 138
Figure 5.58: Monte Carlo simulation of HQ risks posed by EE_2 to people consuming bottled water.. 138

© University of Pretoria

List of Tables

Table 3.1: Selected sampling points for Pretoria (City of Tshwane) 44

Table 3.2: Selected sampling points for Cape Town 44

Table 3.3: Instrument and method for UPLC-MS analysis...................................... 63

Table 3.4: Source, treatment and additional information of the selected brands of bottled water for Phase 2 64

Table 4.1: Relative potencies of target chemicals 72

Table 4.2: Comparison of extraction methods 73

Table 4.3: Estrogenic activity and E_2 and BPA concentrations of spiked water samples using different extraction methods 74

Table 4.4: Recoveries of target chemicals.. 75

Table 5.1: Estrogenic activity of water extracts collected from selected distribution points in Pretoria using the T47D-KBluc bioassay 76

Table 5.2: Estrogenic activity of water extracts collected from selected distribution points in Cape Town using the T47D-KBluc bioassay... 76

Table 5.3: Detection limits (dl) and quantification limits (ql) for the target chemicals using UPLC-MS 78

Table 5.4: BPA concentrations in water extracts collected from selected distribution points in Pretoria 80

Table 5.5: BPA concentrations in water extracts collected from selected distribution points in Cape Town... 80

Table 5.6: DEHA concentrations in water extracts collected from selected distribution points in Pretoria 83

Table 5.7: DEHA concentrations in water extracts collected from selected distribution points in Cape Town 83

Table 5.8: DBP concentrations in water extracts collected from selected distribution points in Pretoria 85

Table 5.9: DBP concentrations in water extracts collected from selected distribution points in Cape Town... 85

Table 5.10: DEHP concentrations in water extracts collected from selected distribution points in Pretoria 87

© University of Pretoria

Table 5.11: DEHP concentrations in water extracts collected from selected distribution points in Cape Town 88

Table 5.12: DINP concentrations in water extracts collected from selected distribution points in Pretoria 90

Table 5.13: DINP concentrations in water extracts collected from selected distribution points in Cape Town 91

Table 5.14: E_2 concentrations in water extracts collected from selected distribution points in Pretoria 93

Table 5.15: E_2 concentrations in water extracts collected from selected distribution points in Cape Town.. 93

Table 5.16: E_1 concentrations in water extracts collected from selected distribution points in Pretoria 95

Table 5.17: E_1 concentrations in water extracts collected from selected distribution points in Cape Town.. 95

Table 5.18: EE_2 concentrations in water extracts collected from selected distribution points in Pretoria 97

Table 5.19: Mineral composition of the selected brands of bottled water analysed in this study 98

Table 5.20: Estrogenic activity of selected bottled water extracts stored at different temperatures and light conditions using the T47D-KBluc bioassay.......................... 99

Table 5.21: BPA concentrations in selected bottled water extracts stored at different temperatures and light conditions 100

Table 5.22: DEHA concentrations in selected bottled water extracts stored at different temperatures and light conditions.. 101

Table 5.23: DBP concentrations in selected bottled water extracts stored at different temperatures and light conditions 102

Table 5.24: DEHP concentrations in selected bottled water extracts stored at different temperatures and light conditions.. 103

Table 5.25: DINP concentrations in selected bottled water extracts stored at different temperatures and light conditions 104

Table 5.26: E_1 concentrations in selected bottled water extracts stored at different temperatures and light conditions 105

Table 5.27: EE_2 concentrations in selected bottled water extracts stored at different temperatures and light conditions 106

© University of Pretoria

Table 5.28: Exposure parameters used in the human health risk calculations 116

Table 5.29: Health risk assessment of distribution point and bottled water 117

Table 6.1: Comparison of estrogenic activity in drinking water from various countries 141

Table 6.2: Comparison of target chemicals in drinking water from various countries 143

Table 6.3: Comparison of the estrogenic activity in bottled water from various countries... 149

Table 6.4: Comparison of target chemicals in bottled water from various countries 150

© University of Pretoria

List of Abbreviations

AA-EQS	Annual-average environmental quality standards
ADD	Average daily dose
ADI	Acceptable daily intake
APEO	Alkylphenol polyethoxylate
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
β	Oral potency factor
BBP	Benzyl butyl phthalate
BPA	Bisphenol A
BPF	Bisphenol F
BPS	Bisphenol S
BSA	Bovine serum albumin
BW	Body weight
CAF	Central Analytical Facility
c/d FBS	Charcoal/dextran treated FBS
C_{medium}	Concentration of substance in water
CPRG	Chlorophenol red-β-D-galactopyranoside
CSIR	Council for Scientific and Industrial Research
$CuSO_4$	Copper (II) sulphate
DBP	Dibutyl phthalate
dBPA	Deuterated BPA
DCM	Dichloromethane
ddH_2O	Double distilled water
DDT	Dichlorodiphenyltrichloroethane
DEHA	Di(2-ethylhexyl) adipate
DEHP	Di(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DIBP	Diisobutyl phthalate
DIDP	Diisodecyl phthalate

© University of Pretoria

DINP	Diisononyl phthalate
dl	Detection limit
DMP	Dimethyl phthalate
DNA	Deoxyribonucleic acid
E_1	Estrone
E_2	17β-Estradiol
E_3	Estriol
ED	Exposure duration
EDCs	Endocrine disrupting chemicals
EE_2	Ethynylestradiol
EEC	European Economic Commission
EEq	Estradiol equivalents
ELISA	Enzyme-linked immunosorbent assay
ELRA	Enzyme-linked receptor assay
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen-responsive element
EtOH	Ethanol
EU	European Union
FBS	Fetal bovine serum
FDA	Food and Drug Administration
$Fe_2(SO_4)_3$	Ferric sulphate
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography-tandem mass spectrometry
hAR	Human androgen receptor
HBSS	Hank's buffered salt solution
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
hERα	Human estrogen receptor alpha
hERβ	Human estrogen receptor beta
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
HQ	Hazard quotient

© University of Pretoria

HRGC-(NCI)-MS	High-resolution gas chromatography with negative chemical ionization mass spectrometry
IL-GC-FID(MS)	Ionic liquid gas chromatography associated with flame ionization detection or mass spectrometry
IQ	Intelligence quotient
IR	Daily intake rate
IT	Ion-trap
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
LADD	Lifetime average daily dose
LC-ESI/MS/MS	Liquid chromatography-electrospray ionization tandem mass spectrometry
LC-FTMS	Liquid chromatography-Fourier transform mass spectrometry
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Lft	Lifetime
LLE	Liquid-liquid extraction
LPME	Liquid phase microextraction
LVI-GC-MS	Large volume injection and capillary gas chromatography coupled to mass spectrometry
MeOH	Methanol
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MtBE	Methyl tertiarybutyl ether
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
$(NH_4)_2SO_4$	Ammonium sulphate
NIEHS	National Institute of Environmental Health Sciences
NOAELs	No observed adverse effect levels
NP	Nonylphenol
NPLC-ESI-MS	Normal-phase liquid chromatography electrospray ionization mass spectrometry
OP	Octylphenol
PBS	Phosphate buffered saline

© University of Pretoria

PCBs	Polychlorinated biphenyls
PES	Polyethersulfone
PET	Polyethylene terephthalate
POCIS	Polar organic compounds integrative sampler
PVC	Polyvinyl chloride
ql	Quantification limit
QqQ	Triple quadrupole
Q-TOF	Quadupole time of flight
RBA	Relative binding assay
RfD	Reference dose
RIANA	RIver ANAlyser
RLU	Relative light units
RNA	Ribonucleic acid
SANBWA	South African National Bottled Water Association
SBSE-LD	Stir bar sorptive extraction with liquid desorption
SCCoR	Single cell coactivator recruitment
SD	Standard deviation
SPE	Solid phase extraction
SPME	Solid phase microextraction
TiPED	Tiered Protocol for Endocrine Disruption
TQMS	Triple-quadrupole mass spectrometry
UK	United Kingdom
UNEP	United Nations Environment Programme
UPLC-MS	Ultra-performance liquid chromatography-mass spectrophotometry
US	United States
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization
WRC	Water Research Commission
YES	Yeast estrogen screen

© University of Pretoria

Chapter 1: Literature Review

Humans are exposed to multiple potentially hazardous chemicals as part of daily life through air, water, food and dermal absorption. Some of these chemicals are endocrine disruptors that can disturb the endocrine system and may result in adverse health effects. This literature review will give a brief background on the endocrine system and how endocrine disrupting chemicals (EDCs) can disturb the endocrine system to cause adverse effects. It will focus on EDCs in the aquatic environment, especially in municipal drinking water and bottled water as an alternative source of drinking water. Bioassays for EDC activity, target chemical analysis and human health risk assessment for EDCs will also be discussed.

1.1. The endocrine system

A healthy endocrine system is needed for humans and wildlife to develop and function normally and to reproduce.[1] The endocrine system consists of various ductless glands that secrete hormones directly into the blood. The hormones are circulated to all parts of the body and act on target organs to regulate body functions.[1] The endocrine glands include the pituitary gland, thyroid gland, parathyroid glands, adrenal glands, islets of Langerhans of the pancreas, and gonads (testes and ovaries). Other organs, such as the heart, body fat, muscle, liver, intestines and kidneys also secrete hormones and have secondary endocrine functions.[1] An overview of the endocrine system and some of the hormones produced are given in Figure 1.1.

Bestpfe.com

© University of Pretoria

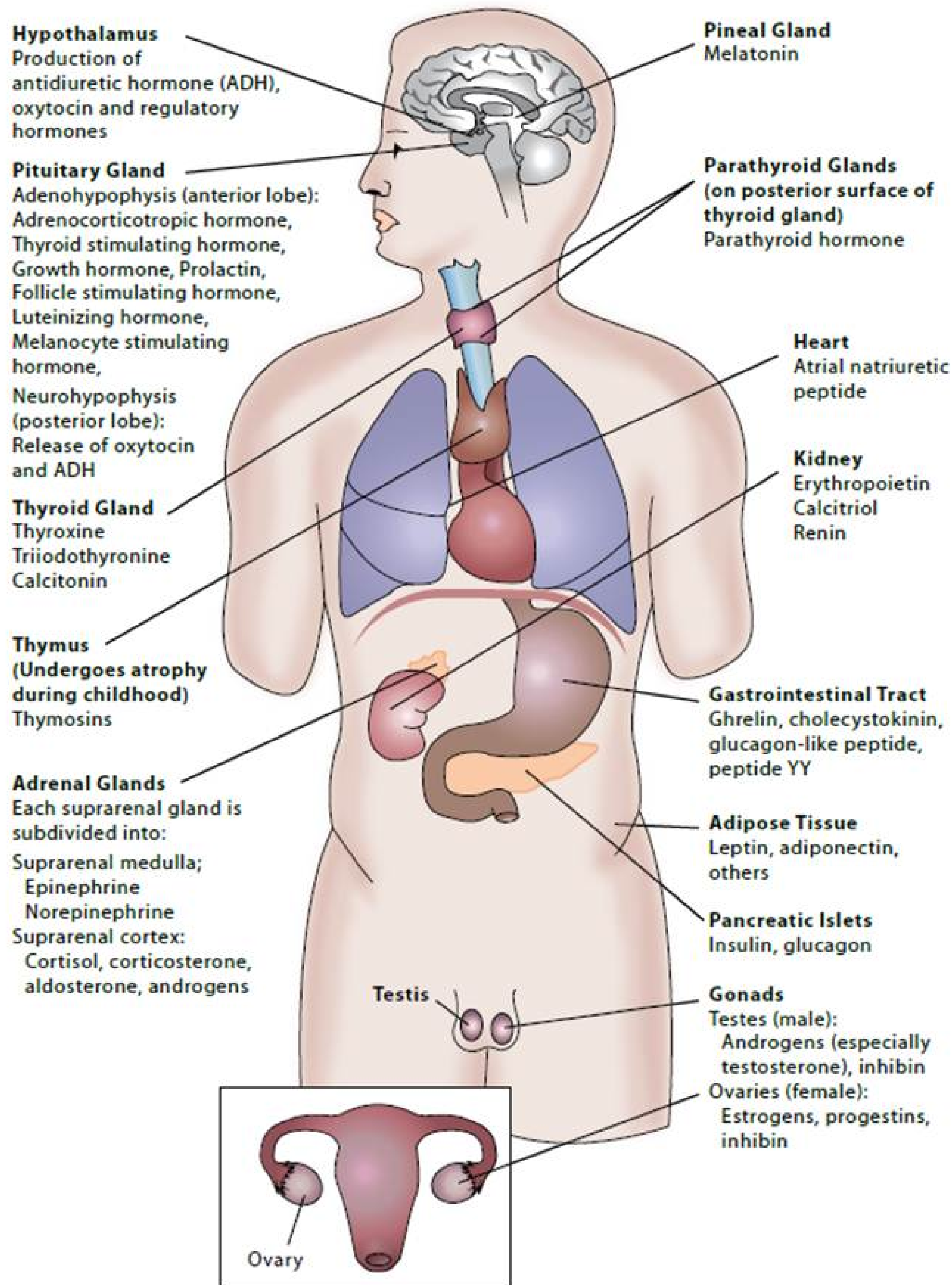


Figure 1.1: An overview of the endocrine system and some of the hormones produced by endocrine glands and organs with secondary endocrine function. Available from: http://www.unep.org/chemicalsandwaste/UNEPsWork/StateoftheScience/tabid/105913/Default.aspx

© University of Pretoria

Hormones bind to specific hormone receptors to produce effects in the body. Steroid and thyroid hormones bind to nuclear receptors to regulate gene expression, and protein and amine hormones bind to membrane receptors to produce effects via a second messenger system. Different cell types express different combinations of hormone receptors and receptors for a specific hormone might be predominantly in one cell type (e.g. receptors for thyroid stimulating hormone are predominantly in the thyroid gland) or may be found in different cell types throughout the body (e.g. insulin receptors). Some hormones can bind to more than one receptor type and the same receptor may have different effects in different cells. Some hormones only act during specific times during development or in adulthood because their receptors are only expressed during specific times in the life cycle.[1,2]

Physiological processes controlled by hormones include growth and developmental processes, metabolism, homeostatic processes, development and functioning of the reproductive organs, sexual characteristics and libido, development of personality and higher nervous functions, body responses to stress and immune responses.[1,3]

1.2. Endocrine disrupting chemicals (EDCs)

Endocrine-related diseases and disorders in humans are increasing, as can be seen from the larger proportion of young men with low semen quality, the increased incidence of genital malformations (e.g. cryptorchidisms and hypospadias), a global increase in endocrine-related cancers (e.g. testicular, prostate, breast, ovarian, endometrial and thyroid) and a higher prevalence of obesity and type 2 diabetes over the past 40 years.[1] Endocrine-related effects are also evident in wildlife populations, like the developmental deformities, reproductive anomalies and decline in numbers of some wildlife populations.[1,4,5] Increases in reproductive diseases and decline in reproductive function have been observed in a relatively short time frame (since the mid-20th century) and cannot be explained by genetic changes.[6] A sharp increase in the production and use of natural and synthetic chemicals occurred over roughly the same period.[6] Laboratory studies identified numerous chemicals with endocrine disrupting properties and evidence suggests that these EDCs may contribute to the endocrine-related effects seen in humans and wildlife.[1]

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

1.2.1. Definition of EDCs

Although various definitions for EDCs exist, the definition provided by the World Health Organization (WHO) is widely accepted and also functional in the context of environmental and human risk assessment.[7] The WHO 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".[8]

1.2.2. Classes of EDCs

EDCs are structurally very diverse and comprise of a variety of chemical classes.[9] EDCs are natural or synthetic chemicals that can be divided into the following groups:

- Natural hormones and their metabolites
  (e.g. 17β-estradiol (E_2), estriol (E_3), estrone (E_1))
- Phytoestrogens and mycoestrogens
  (e.g. isoflavones, coumestans, zearalenone)
- Food additives
  (e.g. propyl gallate used as antioxidant/preservative, tartrazine used as yellow food colouring)
- Pharmaceutical agents
  (e.g. diethylstilbestrol (DES), 17α-ethynylestradiol (EE_2))
- Personal care products
  (e.g. oxybenzone in sun screen, triclosan used as antimicrobial in soap, cosmetics, toothpaste, etc.)
- Industrial solvents/lubricants and combustion byproducts
  (e.g. polychlorinated biphenyls, polybrominated biphenyls, dioxins)
- Plastics and plasticizers
  (e.g. bisphenol A (BPA), phthalates)
- Pesticides and fungicides
  (e.g. methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT), vinclozolin)
- Metals
  (e.g. cadmium, arsenic, lead, mercury)[9-13]

© University of Pretoria

1.2.3. Routes of exposure

According to the United Nations Environment Programme (UNEP) and the WHO "the well-being of future human and wildlife generations depends on safe environments".[1] However, people are exposed to multiple potentially hazardous environmental contaminants, including EDCs, as part of daily life.[14] Humans are exposed to EDCs in water, air, soil and food through ingestion, inhalation and dermal absorption.[15] Personal care products and medical devices are other sources of EDC exposure.[16] EDCs are sometimes deliberately applied for a specific purpose. For example, synthetically produced bovine growth hormone is legally administered to dairy cows in the United States (US) to increase milk yield.[17] Some people are occupationally exposed to EDCs, for example people who work with pesticides or industrial chemicals.[10] Exposure can also be inadvertently, for example from the migration of EDCs from food packaging into food.[17]

Reports of EDCs in the environment are common in the scientific literature. Some EDCs are persistent in the environment, while others are degraded rapidly.[9] As many EDCs have an anthropogenic source, higher concentrations are expected near urbanized or industrial areas.[18] EDCs can travel in all environmental mediums (air, water, sediment and live tissue) and have been detected thousands of miles from their source and even in areas where they were never used.[7,10] They can also bioaccumulate and get incorporated into the food chain, leading to biomagnification, especially in species at the top level of the food chain.[10,12] Persistent pollutants can accumulate in human fatty tissues and women excrete some of the accumulated chemicals into the fat of breast milk and across the placenta to the fetus.[19] Every individual is exposed to EDCs to some degree and has measurable levels of multiple environmental chemicals in his/her body which may include phthalate plasticizers, dioxins, furans, polychlorinated biphenyls (PCBs), metals and pesticides.[6,19] EDCs are therefore globally ubiquitous, making human exposure to environmental EDCs inevitable.[7]

1.2.4. Health effects associated with EDC exposure

Model organisms are mostly used to predict the adverse effects of EDCs on human health, but epidemiologic and clinical studies provide complimentary sources of information.[6] Scientific literature suggests that EDCs may have an adverse effect on

© University of Pretoria

thyroid function, brain function, metabolism, insulin and glucose homeostasis, bone function, learning and behaviour, reproduction and immune function.[1,20,21]

Some of the adverse health effects associated with EDCs include the following:

- Reproductive disorders and malformations, including non-descended testes, cryptorchidism and poor semen quality in males, endometriosis and polycystic ovarian syndrome in females and infertility[1,6,10,22-26]
- Learning and behaviour problems, for example reduced intelligence quotient (IQ) scores and attention deficit/hyperactivity disorder[1,10,27,28]
- Cardiovascular diseases[1,10,29]
- Immune-related disorders, for example autoimmune diseases, asthma, allergies and greater susceptibility to infections[1,30-32]
- Diabetes[1,3,10,21,24,29]
- Obesity[1,10,21,24,27,33-35]
- Liver dysfunction[29]
- Preneoplastic lesions[10]
- Cancer, including prostate, testicular, breast, ovarian, endometrial, vaginal and thyroid cancer[1,10,35-39]

1.2.5. EDC activity

1.2.5.1. How do EDCs disturb the hormonal system?

EDCs are of diverse chemical structures and vary in potency.[13,37] They can disturb the hormonal system by mimicking the natural hormones, blocking their production or by inhibiting or stimulating the endocrine system.[40] Some EDCs can exhibit more than one mechanism of action. [41] The effects of EDCs can be receptor dependent or receptor independent.[9,42]

EDCs have been shown to interact with the androgen receptor, estrogen receptor (ER), thyroid hormone receptor, progesterone receptor, arylhydrocarbon receptor, peroxisome proliferator-activated receptor and the glucocorticoid receptor.[24] They interact with steroid hormone receptors as agonists or antagonists.[2,10] Biphasic activity has also been reported, for example some phytoestrogens can act as agonists at low concentrations and as antagonists at high concentrations.[13] Some

© University of Pretoria

EDCs can interact with multiple hormone receptors.[1] For example, BPA binds and activates the ER, but also binds and inhibits the androgen and thyroid hormone receptors at higher concentrations.[43] On the other hand, various EDCs can potentially bind to the same receptor. The gap in the ligand-binding domain of the human ER is almost twice the size required for E_2, allowing for various other molecules, for example BPA, to bind to the receptor.[44]

Receptor independent activities of EDCs include the modification of hormone receptor levels or interfering with the synthesis, metabolism, transport or degradation of hormones.[2,9] For example, by activating or blocking metabolizing enzymes, EDCs can alter the concentrations of hormones.[9,42] EDCs can also have a direct effect on genes and epigenetic programming.[24]

1.2.5.2. The EDC dose response curve

Dose response curves for EDCs are mostly sigmoidal, as the dose increases the response will increase in a logarithmic manner until saturated.[1,2] An equivalent change in concentration will have a proportionally greater effect at the low end of the curve than at the high end of the curve, so a small change at low concentrations will have a significant impact.[2] Once the receptors are saturated, no further increase in the response will be observed.[2] The dose response curve can also be non-monotonic (bi-phasic), resembling a U-shaped (with maximal responses at high and low doses) or inverted U-shaped curve (with maximal responses at intermediate doses).[1,24,45] One of the first non-monotonic dose-responses for endocrine disruptors was reported by Vom Saal et al.[46] who showed that fetal exposure to estradiol and DES resulted in increased prostate mass at low doses, but not at higher doses, creating an inverted-U dose-response relationship. In a review by Vandenberg et al.[47] that included more than 600 studies, 18 endocrine disruptors showed evidence of non-monotonic responses with low-dose health effects.

Non-monotonic dose response curves can be attributed to the presence of more than one monotonic response affecting a common endpoint, down regulation of receptors, receptor competition, differences in receptor affinity at low versus high doses, cytotoxicity at higher doses or adaptive responses through complex cell signalling pathways and feed-back mechanisms.[1,7,24] Cytotoxicity at high doses are

© University of Pretoria

the most common mode of action responsible for non-monotonic dose responses.[43] Non-monotonic dose responses should be considered when interpreting results from toxicological studies as simply extrapolating from the results of high dose studies will not give accurate estimates of potential hazards at lower doses.[43]

1.2.5.3. The mixture effect

People are exposed to a multitude of chemicals simultaneously.[48] Biomonitoring studies revealed that the average human carries a footprint of several hundred chemicals, including pesticides, phthalates and other EDCs.[49] When evaluated in isolation, a chemical might not elicit measureable effects or have a very low potency, but when a number of chemicals are combined, it can produce substantial EDC effects.[1,48-51] This has been demonstrated *in vitro* using bioassays and *in vivo* using rat and fish and is called the “something from nothing” phenomenon.[1,50,52] Adverse effects resulting from exposure to a mixture of similar acting chemicals present at or below their individual no observed adverse effect levels (NOAELs) can be explained by the dose addition effect.[48,50,53,54] Furthermore, a mixture of estrogenic chemicals at low doses can jointly increase the actions of potent endogenous sex steroids.[50] Antagonist activity can also be additive. A mixture of 30 anti-androgenic chemicals at concentrations below doses that would show significant effects for the individual chemicals, revealed strong combination effects.[50] Chemicals that act through different mechanisms of action, but affect the same common adverse outcome can also act additively to enhance the risk of an adverse health effect.[6] Therefore, the more chemicals that are present in a mixture the more concern is indicated.[54]

1.2.5.4. Exposure to EDCs during critical windows

In some cases, the timing of EDC exposure (during critical developmental periods) is more important than the magnitude of the dose.[7] Chemical exposures that have little or no effect on adults may be hazardous to the growing fetus.[24,30] Besides *in utero*, other vulnerable periods of development include infancy, childhood, puberty and menopause.[7] During these life stages, massive changes in the endocrine environment result in physiological and morphological changes in the individual.[20] Exposure to EDCs during these periods can have permanent effects, can have an influence on the functioning of the endocrine system in adulthood and predispose the adult to a number of chronic diseases.[1,2,6,27,30] The effects of an exposure to EDCs

© University of Pretoria

might therefore only be detected many years later, making the link between such an exposure and the observed adverse effect very difficult.[9] An example is that developmental exposure to low doses of BPA increases hormone-dependent prostate cancer risk. Stem-progenitor cells in the normal human prostate gland are direct targets of BPA. BPA can amplify and modify the stem-progenitor cell populations, increasing the prostate cancer risk when relative estradiol levels start to rise in aging men.[55,56] Similarly, prenatal exposure to DES increases the risk for developing vaginal, uterine and breast cancer.[30] Prenatal and/or early postnatal exposure to EDCs are also associated with an increased risk for developing other chronic diseases later in life, including diabetes, obesity, cardiovascular disease, infertility and psychiatric and behavioural impairments.[57] One of the proposed mechanisms whereby fetal exposure to EDCs can cause the development of adult diseases is through epigenetic alterations.[57,58]

1.2.5.5. Vulnerable populations

Populations from some geographical areas may be exposed to higher levels of certain EDCs, for example, people living in some agricultural areas (e.g. the agricultural Salinas Valley of California) are exposed to high levels of pesticides.[1] Similarly, high exposure levels to contaminants related to e-waste were reported for workers and residents in areas surrounding e-waste disposal and recycling sites in developing countries.[1] Some populations are more susceptible to the effects of EDCs than others. Age, pre-existing disease, genetics and other factors can influence the effects of EDC exposure on an individual.[6] For example, adults can rapidly metabolize and excrete BPA, but the fetus and infants have lower hepatic expression of the UGT2B enzyme that metabolizes BPA, resulting in a greater exposure risk compared to adults.[56,59] Young children also ingest more food, drink and air per body mass compared to adults, and they also have higher object-to-mouth activity, increasing their exposure to EDCs.[7] In healthy adults, fluctuations in hormone levels induced by EDCs may be buffered by normal homeostatic corrections, but may lead to the induction of carcinoma in individuals who are predisposed to cancer.[42] A study on phthalate concentrations in urine and semen quality indicated that the ratio of the primary and secondary metabolites of di(2-ethylhexyl) phthalate (DEHP), rather than the actual concentration of the metabolites, was associated with human semen parameters.[60,61] The results suggest

© University of Pretoria

that different individuals might have different abilities to metabolize phthalates into less harmful metabolites, rendering some individuals more vulnerable to the effects of phthalates than others.[60]

1.2.6.6. Multigenerational and transgenerational effects

The effects of EDC exposure can be persistent over multiple generations due to their ubiquity in the environment, resistance to degradation and bioaccumulation of some EDCs.[7] Lipophilic EDCs that bioaccumulate in organisms can move up through the food chain, resulting in increased concentrations in organisms at the top of the food chain.[7,49]

The effects of certain EDCs can also be passed down to next generations through genotoxicity or epigenetic changes. With genotoxicity, heritable damage occurs to deoxyribonucleic acid (DNA).[7] Epigenetic changes involve alterations in DNA methylation patterns, histone modification, chromatin packaging and micro ribonucleic acid (RNA) expression, resulting in persistent, heritable changes in gene expression.[7,9,23,41,57,58,62] For example, ancestral exposure to obesogenic EDCs may alter DNA methylation patterns, resulting in an obesity phenotype that can be transmitted to subsequent generations.[33]

Both somatic and germ cells can be affected by epigenetic reprogramming leading to subtle functional changes and disease later in life and future generations.[24] Somatic epigenetic mutations may result in adult onset disease and is not heritable, but can be multigenerational if multiple generations are exposed to the toxicant, for example when a gestating female is exposed.[62] Epigenetic changes to the germ cell on the other hand are transgenerationally inherited in the absence of the initial exposure.[62] For example, exposure to a mixture of BPA, DEHP and dibutyl phthalate (DBP) during gonadal sex determination resulted in epigenetic transgenerational inheritance of adult-onset disease in the F3 generation, including pubertal abnormalities, testis disease, obesity and ovarian disease.[63]

1.3. EDCs in the aquatic environment

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

© University of Pretoria

includes a constant supply of clean, safe water.[64] The demand for the supply of clean water is increasing due to the continuing human population growth.[65] However, population growth, urbanization, industrial development, and associated changes in agricultural and other land-use practices contribute significantly to the reduction of water quality through naturally occurring and anthropogenic contamination.[66] EDCs have been reported in environmental, waste, treated waste and drinking water sources in South Africa.[67-72] Some poor rural communities in South Africa are exposed to untreated water due to a lack of services in some areas.[69]

EDCs can enter the aquatic environment via direct discharge into water, leaching (e.g. leakage from septic tanks and landfill sites), storm water runoff and accidental spills. Natural hormones, including estrogens can be released into the environment via sewage effluent and from such sources such as agricultural and pharmaceutical activities.[9,15,66,70] E_1, E_2, EE_2, BPA, nonylphenol (NP) and short chain phthalates are some of the substances that contribute to the estrogenic load in water bodies and may cause adverse effects in aquatic organisms.[73]

Some EDCs are more persistent in the environment than others and the aquatic environment conditions could have an influence on the degradation of EDCs. For example, the photodegradation of NP is influenced by pH, ferric ion concentration and humic acid concentration in the water.[74]

1.3.1. Natural and synthetic steroids in sewage effluent

Sewage is a major source of organic contaminants in the environment.[18] Studies from various countries reported estrogenic activity in municipal sewage water that can be attributed to human excretions.[9] Free estrogens are metabolized in the body to sulfate and glucuronide conjugates that are excreted in urine.[75] The conjugates do not possess significant biological activity, but are converted back to their potent free forms during water treatment processes.[75] Jobling et al.[18] screened 20 organic man-made chemicals commonly found in sewage effluent and found that half of the chemicals were able to interact with the estradiol receptor. Estrogenic potencies of natural and synthetic estrogens are three to seven orders of magnitude greater than the potencies of other EDCs identified in wastewater, making them the major contributor to estrogenic activity in wastewater.[44] The four most commonly found

© University of Pretoria

Bestpfe.com

estrogens in wastewater are E_1, E_2, E_3 and EE_2.[44] The compilation of a population would determine the estrogenic steroid input into sewage works, for example, males excrete 3.9 µg E_1, 1.6 µg E_2 and 1.5 µg E_3 in urine per day, whereas pregnant women can excrete 600 µg E_1, 259 µg E_2 and 6 000 µg E_3 per day.[76] The number of women taking oral contraceptives or that are on hormone replacement therapy would determine the concentration of EE_2 in waste water.[76] In South Africa approximately 38% of women use oral contraceptives.[67]

Although most studies focus on estrogenic hormones in wastewater, androgen (testosterone) and progesterone have also been detected.[67,77] A study at the wastewater treatment works in Pietermaritzburg, South Africa reported average concentrations of 408 ng/L progesterone, 343 ng/L testosterone, 119 ng/L E_2, 84 ng/L E_1, 30 ng/L EE_2 and 5 ng/L E_3 in the influent. The effluent contained average concentrations of 23 ng/L E_1, 20 ng/L E_2, 11 ng/L testosterone, 9 ng/L progesterone, 3 ng/L EE_2 and 1 ng/L E_3.[67]

If waste water treatment plants are not effective to remove these steroids from the wastewater, it could end up in environmental waters. Compared to E_1 and E_2, EE_2 is relatively stable under aerobic conditions of activated sludge treatment at waste water treatment plants and is more resistant to biodegradation by microorganisms in the environment, making it a substance of concern in environmental water.[73]

EDC activity is generally lower in environmental samples compared to waste water due to the dilution, adsorption and biodegradation of these compounds.[9,77] However, several studies showed strong evidence that steroidal estrogens found in waste water treatment plant effluents can have endocrine disruptive effects on fish populations in the receiving waters.[73,78] Fish in the River Ray in the United Kingdom (UK) are exposed to two to three times more estrogens in the first three km downstream of the Rodbourne sewage treatment works than fish living 7-10 km downstream.[79] Some of the effects on male fish exposed to sewage effluent include feminization of reproductive ducts, appearance of oocytes in male gonads and production of the vitellin (female egg protein) or vitellogenin (precursor of vitellin).[77,80] Risk assessment of the steroid hormones present in wastewater effluent at the Pietermaritzburg wastewater treatment works identified E_2 and EE_2 to pose the

© University of Pretoria

greatest risk to fish and human health.[67] Although natural estrogens are essential for normal development and reproduction and play critical roles in the sexual differentiation and development of the brain, breast, prostate and other organs, natural and synthetic estrogens are also known human carcinogens.[13,37,48]

Although wastewater effluent has to comply with certain legal requirements before discharge into natural water courses, there is currently no legislation in South Africa with respect to maximum allowable levels for steroid hormones in wastewater effluent.[67] The European Water Framework Directive determined annual-average environmental quality standards (AA-EQS) for surface water. It gives the concentrations of priority substances on the European monitoring list that should not be exceeded in order to protect the aquatic environment and human health. E_2 and EE_2 are two of the substances on the list, with proposed AA-EQS of 0.4 ng/L for E_2 and 0.035 ng/L for EE_2.[73]

Residential septic systems may also play a major role in the contamination of water systems, especially groundwater. These on-site wastewater treatment systems may not treat waste as effectively as municipal wastewater treatment plants. People are exposed to the contaminants if groundwater is used as a source of drinking water. A study in Cape Cod, Massachusetts found organic wastewater contaminants, including NP, E_1 and E_2 in groundwater samples. The results suggested that these contaminants originated from the residential septic system.[81]

1.3.2. Pharmaceuticals and hospital effluents

A growing environmental concern is the presence of pharmaceutical compounds in treated wastewater, surface waters, groundwater and drinking water.[82-89] Only a fraction of medication is completely absorbed by the body, the rest is excreted and land up in the sewage system.[82] Absorbed pharmaceuticals are metabolized, and the metabolites are released into the sewage system.[86] Hospital effluents contain various pharmaceuticals including antibiotics, contrast media drugs and anticancer drugs that can contaminate water sources.[87] A study published in 2008, reported 24 ng/L E_2 equivalent activity, 86 ng/L dihydrotestosterone (DHT) equivalent activity and 96 ng/L dexamethasone equivalent activity in raw hospital effluent from The

BEST PFE.COM
List of research project topics and materials

© University of Pretoria

Netherlands. The activity in hospital effluents can largely be attributed to excreted hormones and administered drugs.[90]

Sewage treatment facilities that are not required to test for pharmaceuticals are not equipped to degrade medicinal substances, resulting in the release of these substances in the aquatic environment, often unknowingly.[82,84] Some pharmaceutical metabolites can be transformed during sewage treatment into degradation by-products with similar or higher toxicity than the parent compound.[86] Pharmaceuticals can also enter the wastewater system when people dispose of unwanted or expired medicine by flushing it down the toilet or sink.[82,86,89] Medicines that are disposed of in household waste may also eventually land up in the environmental water system through leachate from landfills.[82,86] Pharmaceutical transformation products are formed through biodegradation, hydrolysis and photolysis in the environment, but there is still a huge lack of data on the occurrence and effects of these products.[83]

1.3.3. Personal care products

Personal care products, like soaps, shampoo, skin care products, sun screen, perfumes, oral hygiene products, etc., are used in large amounts all over the world.[88] Many personal care products also contain EDCs, for example oxybenzone in sun screen.[11] Parabens are used as preservatives in cosmetics, toiletries and pharmaceuticals, but some parabens (e.g. butylparaben) show estrogenic activity.[88] Triclosan is another potential endocrine disruptor that is used as an antiseptic agent in many consumer products including toothpaste, hospital handsoap and acne creams.[88] Personal care products are continuously being discharged into the environment via domestic sewage systems,[88] making them ubiquitous contaminants that are regularly detected in wastewater treatment plant effluents.[11]

1.3.4. Household products and industrial effluents

Nonionic surfactants are found in many household products, including heavy-duty laundry powders and liquid detergents, personal care products and household cleaners.[91] These surfactants can be released into the environment through wastewater. Alkylphenol polyethoxylate (APEO) surfactants are broken down in the environment to alkylphenol ethoxycarboxylates and the estrogenic alkylphenols, nonylphenol and octylphenol (OP).[91] A study in Cape Cod, Massachusetts

© University of Pretoria

suggested that residential septic tanks were a significant source of APEOs in groundwater and drinking water wells.[91]

BPA and phthalates may be present in some plastics[91] and BPA may also be found in recycled paper products.[92] When discarded through household waste, these plastic products accumulate in landfill sites and may contaminate groundwater through leaching.[18,93] Toilet paper made from recycled paper can also be an important source of BPA in wastewater.[92]

Industrial effluents also contribute to considerable amounts of endocrine activity.[9] Industrial effluents in The Netherlands showed agonist activity in various bioassays with an estrogenic activity of 3.4 ng/L E_2 equivalent, androgen activity of 81 ng/L DHT equivalent, progestagenic activity of 2.2 ng/L Org2058 equivalent and glucocorticoid activity of 243 ng/L dexamethasone equivalent.[90] Some examples of EDCs that may be present in industrial effluents include NP from cleaners used in industrial processes and BPA from plastics.[94]

1.3.5. Agricultural effluents

Estrogens were detected in streams in areas with intensive agricultural activities in the US.[9] When wastewater is used to irrigate soil or when manure is applied to the soil, hormones in the wastewater or manure can land up in the aquatic environment.[44] In 60% of cases, steroid concentrations were higher downstream of livestock farms in the UK.[95] The excretion of hormones in manure and urine from livestock feed lots[94] and the leaching of estrogens from dairy farm waste[44] are more examples of how agricultural activities can contribute to the contamination of water bodies with endocrine disruptors. Besides the natural excretion of hormones, agricultural applications of EE_2 can also contaminate environmental water, as it is used to treat reproductive disorders and control ovulation in livestock.[73] Veterinary pharmaceuticals can enter the environment via the application of manure to the soil.[83,86]

Agrochemicals containing alkylphenol and nonylphenol ethoxylate surfactants are other sources of EDCs that can be present in agricultural effluents.[94] Pesticides are used by farmers to protect crops from pests, weeds and fungal disease. Many of the

© University of Pretoria

pesticides are persistent in the environment and can enter the aquatic system through runoff from agricultural lands or disease vector control operations and may reach groundwater through percolation. The most commonly found pesticides in the aquatic environment are organochlorines, of which many are EDCs.[96] More than 3 000 pesticide products are approved for use in South Africa and a number of studies reported pesticides in surface and groundwater.[97] This is of particular concern in some communities that don't have access to treated drinking water and rely on groundwater or other untreated water sources for drinking water.[97]

1.4. Removal of EDCs through water treatment processes

Estrogenic activity was found at varying concentrations in raw and treated water in South Africa.[67,70,98,99] Waste water is treated at waste water treatment plants before it can be released into the environment, whereas drinking water treatment plants treat water in order to be fit for human consumption. The treatment methods or combination of methods might therefore differ between waste water treatment plants and drinking water treatment plants, although many of the methods are used in both.

Polar compounds are readily soluble in water and more difficult to remove during water treatment processes and therefore require more advanced removal techniques.[65] Treatment conditions, such as concentrations of contaminants, dose of treatment applied, temperature, pH of the water and treatment time are some of the factors that can have an influence on the removal efficiency of different treatment methods.[100,101] Treatment options to remove EDCs include separation processes, adsorption and biological and chemical conversion.[11] Each treatment method has its own limitations and benefits to remove EDCs.[12,101] Separation techniques, such as coagulation, flocculation and precipitation processes are not effective in removing EDCs, but more advanced treatments such as membrane filtration, ion exchange, ultra-violet photolysis and adsorption using granular activated carbon demonstrate much better removal efficiencies.[11,12] Ultra-violet photolysis is however not a feasible removal option, as it requires high doses to remove EDCs.[12] Reverse osmosis is the most effective membrane filtration technique for the removal of EDCs, but its high energy consumption makes it an unfavourable method.[12,101] A disadvantage of physical methods is the post-treatment required for the absorbent material or generated waste that can increase the cost of treatment.[100] Biological processes

© University of Pretoria

include activated sludge, biofiltration and soil aquifer treatment.[11,12] Microbial organisms (bacteria, microalgae and enzymes produced by fungi) are used to degrade organic compounds present in the water.[101] Biological methods show limited EDC removal.[11,12] Chemical oxidative processes like chlorination and ozonation are effective to reduce the concentrations of several classes of EDCs,[11,12,100,102] but disinfection by-products are formed in the process.[12,100,101] These by-products may also have endocrine disruptive properties, supporting the observation that estrogenic activity could not be completely removed after ozonation and chlorination treatment.[100]

1.4.1. Removal of EDCs from wastewater

In South Africa, the activated sludge process is the most common treatment method for municipal wastewater.[103] Some steroids, like EE_2 is highly stable and persistent in activated sludge, whereas others, like E_2, are easily biodegraded.[9] The nitrification degree determines the EDC removal potential and depends on pH, oxygen and temperature.[12] Other factors that can have an effect on the efficiency of activated sludge to remove EDCs are the age of the activated sludge, organic loading, size of the catchment and sunlight.[9] Antibiotics, that might be present in wastewater, can also inhibit the ability of bacteria to degrade estrogens.[44] Some compounds are broken down to metabolites that might have higher endocrine activity than the parent compound.[9]

Studies reported that the removal of E_1 can be up to 98%, E_2 between 44-99.9%, E_3 between 18-100% and EE_2 between 34-100% using activated sludge.[44] A South African study at the Pietermaritzburg wastewater treatment works reported an averaged removal efficiency of 92% for steroid hormones using activated sludge. The removal efficiency was higher for progesterone and testosterone compared to natural and synthetic estrogens. The study also reported an averaged estrogenic potency removal of 85% after activated sludge treatment.[67] The efficiency of water treatment plants at removing NP varies between 11% and 99%, depending on the type of treatment process.[104] BPA removal was reported to be between 37% and 94%.[93]

© University of Pretoria

Wastewater effluent is discharged into the aquatic environment, and can potentially be found in drinking water if a water source downstream of the effluent discharge is used.[44]

1.4.2. EDCs in drinking/tap water

Much more research has been conducted on wastewater compared to drinking water.[102] Estrogenic activity has been reported in drinking water from The Netherlands,[105] Taiwan,[106] China,[107] Italy[108] and the US.[109] Thyroid hormone antagonist activity was reported in drinking water from eastern China.[110] E_1, E_2, EE_2, BPA, NP, and various phthalates are some of the EDCs that have been reported in drinking water from various countries.[91,102,106-108,110-124] A US study found forty organic wastewater-related contaminants in stream and raw water supplies at a drinking water treatment facility.[124] Although many contaminants were reduced to non-detectable concentrations, between 11 and 17 organic wastewater-related contaminants were detected in finished water samples, indicating that the treatment processes were not able to remove all the contaminants.[124] Compounds detected in the finished water samples included prescription and non-prescription drugs and their metabolites, fragrance compounds, flame retardants and plasticizers, cosmetic compounds and a solvent.[124] In South Africa, a study by Slabbert, et al.[70] investigated the estrogenic activity in water from selected drinking water treatment processes. 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.[70,98]

Water treatment process technology differs at different water treatment plants and various steps in the water treatment process can remove estrogenic activity to some degree.[9,70] Chlorination is often used in drinking water treatment to kill harmful microorganisms.[100] It is a powerful oxidant that oxidizes the organic matter present in the water.[100] Chlorination showed good removal efficiencies for estrogens, but residual disinfectant by-products may be present in the finished drinking water and may pose potential health risks to consumers.[100] 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.[98]

© University of Pretoria

Although water treatment processes can be effective in removing EDCs from drinking water, chemicals might also migrate from the water lines/pipes that transport water to the reservoir and to the home, thereby adding to the contamination of the drinking water.[125] NP, phthalate esters and BPA can migrate from reservoirs and pipes containing polyethylene plastic, epoxy resins or paints.[111] Romero et al.[126] showed that BPA, phthalates and NP can migrate from epoxy paint approved for use in drinking water reservoirs. However, the dynamic nature of drinking water reservoirs and presence of chlorine can help to dilute and break down BPA and other EDCs that migrate into the water.[126]

1.5. Bottled water as an alternative to tap water

Worldwide the demand for bottled water continues to increase,[127-130] even in countries with safe potable tap water.[128-130] The global average consumption of bottled water was approximately 35 L per capita in 2012.[131] Although bottled water consumption is much lower in South Africa (8.3 L per capita in 2012), a dramatic 315% growth in consumption was seen from 1999 to 2012.[128]

There are various reasons for the increasing preference for bottled water over tap water. Many people prefer bottled water for health and safety reasons.[128,129,132] Some bottled waters are supplemented with vitamins or other neutraceuticals and are therefore considered better from a nutritional point of view than tap water.[130] One of the major reasons people give for using bottled water, however, is the perceived risks of tap water.[129] They argue that bottled water is submitted to more advanced treatments, making it safer than tap water.[129,130] Bottled water consumption is often higher in communities that have serious problems with their tap water.[129] The organoleptics of tap water (taste, odour and appearance) can also have an influence on people's decision to rather drink bottled water.[129] Branding and marketing influences, convenience, as well as demographic variables (including ethnic group, age, income, occupation and gender) can also influence bottled water usage.[128-130] In addition, the choice to use bottled water may differ according to location and intended use.[129] For example, some people may use tap water at home, but prefer bottled water at work or when travelling.[129] An example of how intended use can influence the choice of bottled or tap water is that people may use bottled water to drink directly, but use tap water for preparing tea.[129]

© University of Pretoria

Contradictory to the perception that bottled water is safer than tap water, studies suggest that bottled water might also contain harmful substances. Several studies reported the presence of EDCs in bottled water.[132-136]

1.5.1. Sources of contamination of bottled water

Bottled water is primarily sold in plastic polyethylene terephthalate (PET) containers.[137,138] The inexpensive, lightweight and durable properties of plastics make them ideal for single use packaging material.[139] To optimise the properties of packaging material a variety of additives, such as stabilizers, antioxidants, coupling agents and pigments are used in the formulation.[133] Food packaging can improve food safety by preventing bacterial contamination and extending the shelf life of products.[140] However, many of the additives used in food contact material are EDCs.[54,141,142] Muncke[54] listed 50 known or potential EDCs approved for use in food contact materials. Some of these additives are known to leach out of the packaging and consequently accumulate in the foodstuff.[133] Unintentionally added substances can also be found in the final product, for example impurities in the starting material or additives and degradation products formed in the production process or during storage.[138,143] The recycling of PET is encouraged to reduce plastic waste, but is also implicated as a route to introduce unintentionally added substances to PET.[138] Presently, only a few EDCs have been examined for their migrating properties and only a small fraction of migrating substances have been evaluated for EDC activity.[140] The widespread use of EDCs with endocrine disrupting properties in food packaging may therefore present a risk which requires investigation.[54]

Endocrine disrupting activities were also observed in water from glass bottles, indicating that packaging is not the only source of EDC contamination of bottled water.[141] Besides the migration from the packaging material, Wagner and Oehlmann[133] also suggested that the water source may be contaminated or that contamination can occur through the production or bottling process (e.g. estrogenic disinfectants used to clean the filling system).

EDC activity has been reported in bottled water from various countries[132,133,141,144,145] and BPA, NP, phthalates and other plasticizers, antimony, polycyclic aromatic hydrocarbons (e.g. naphthalene), organochlorine pesticides and triazines (e.g.

© University of Pretoria

atrazine) are some of the compounds that have been detected in bottled water worldwide.[108,111,121,134,135,138,146-156]

1.5.1.1. Endocrine activity

Several studies reported estrogenic activity in bottled water from various countries. Total estrogenic activity using the recombinant yeast estrogen screen measured in PET bottles ranged from 0.027 – 23.1 ng/L estradiol equivalents, while 20% of the samples showed toxicity in a study by Pinto and Reali.[132] Wagner and Oehlmann[133] tested nine brands of PET mineral water available in Germany for estrogenic activity using the recombinant yeast estrogen screen. Seven of the nine brands tested positive and the maximum estrogenic activity was 75 ng/L. Furthermore, breeding of the molluskan model *Potamopyrgus antipodarum* in water bottles made of glass and plastic resulted in an increased reproductive output of snails cultured in PET bottles, indicating possible leaching of estrogenic compounds from PET.[133] In the E-screen (a cell proliferation bioassay to test for estrogenic activity), 61% of bottled water from France, Germany and Italy showed estrogenic activity, ranging from 1.9 to 12.2 pg/L estradiol equivalents.[145]

Besides estrogenic activity (0.113 pM average E_2 equivalent units), Real et al.[141] also reported anti-estrogenic activity (11.01 pM average ICI 182,780 equivalent units), androgenic activity (0.33 pM average methyltrienolone equivalent units) and anti-androgenic activity (0.18 nM average procymidone equivalent units) in bottled water from Southern Spain. Similarly, Plotan et al.[144] reported hormonal activity in 78% bottled water samples from England, Wales, Scotland, Italy, Ireland and France with an average estradiol equivalent concentration of 10 ng/L, 26 ng/L testosterone equivalent, 123 ng/L progesterone equivalent and 13.5 ng/L hydrocortisone equivalent.

1.5.1.2. Bisphenol A (BPA)

BPA was detected in bottled water from Greece,[147] Spain,[111,151] China,[135] Italy[108] and Japan[134] at concentrations ranging from 0.83 to 819 ng/L.

BPA is used in the production of polycarbonate plastics and epoxy resins and is added to thermal paper as a phenolic developer and to polyvinyl chloride (PVC) and

© University of Pretoria

hydraulic brake fluids for its antioxidant properties. [14,92] It is found in many products, including plastic consumer products, linings of metal food and beverage cans, water pipes, thermal paper, protective coatings, adhesives, medical devices and dental sealants.[4,93,157,158] Due to its widespread use, BPA is ubiquitously present in the environment and in humans.[92] Diet is believed to be the primary source of human exposure to BPA,[158-160] but skin contact, inhalation, dental fillings and occupational exposures are other exposure routes.[158,159] Young children have the highest exposure risk due to their more frequent hand-to-mouth contact.[158] Biomonitoring studies conducted in the US, Germany, China, Korea and Belgium all reported BPA in the urine of participants, with average concentrations ranging from 0.56 to 3.40 µg/L.[92] Calafat et al.[161] reported BPA in 92.6% of urine samples in an American reference population and found that the concentrations were significantly higher in children and adolescents compared to adults. Carwile et al.[162] showed that the use of polycarbonate bottles increase BPA exposure. The study consisted of a one week washout phase, where participating Harvard College students had to consume all cold beverages from stainless steel bottles. This was followed by the intervention week, where cold beverages had to be consumed from polycarbonate bottles. Urinary BPA concentrations increased by 69% after the use of polycarbonate bottles,[162] indicating the leaching of BPA from the containers. Temperature and contact time can have an influence on the migration of BPA from polycarbonate containers.[158] BPA has also been found in human serum, saliva, amniotic fluid,[159] maternal and fetal plasma, placental tissue at birth and in breast milk of lactating mothers.[10,163] Studies on BPA levels in human blood reported concentrations ranging from 0.1 – 4.0 ng/mL.[159] Although BPA is rapidly excreted in urine, studies have also shown that it can accumulate in fat.[164]

The endocrine disruptive effects of BPA exposure have been well documented.[32,165,166] BPA is structurally similar to DES, a synthetic estrogen known to cause cancer.[4,157] BPA is an estrogen, anti-androgen, thyroid hormone antagonist and peroxisome proliferater-activated receptor agonist.[20] *In vitro* studies also showed that BPA inhibits aromatase activity (the enzyme converting testosterone to estrogen) and the aryl hydrocarbon receptor, involved in the synthesis and metabolism of steroids.[167] In humans, higher urinary BPA concentrations were associated with an increased prevalence of cardiovascular disease, diabetes and

© University of Pretoria

liver-enzyme abnormalities.[29] BPA may play a role in the development or aggravation of inflammatory conditions by affecting the biology of immune cells.[32] Various studies also indicate that exposure to low doses of BPA (below the toxicological NOAEL) during early development may alter development of the brain, behaviours, the prostate and mammary gland.[45] Rodent studies indicate that early-life exposure to BPA below the United States Environmental Protection Agency (USEPA) oral reference dose lead to increased susceptibility to mammary and prostate cancer.[39] According to Seachrist et al.,[39] BPA can be reasonably anticipated to be a human carcinogen of the breast and prostate. BPA can readily cross the placenta.[4] Exposure during fetal and early childhood development can result in secondary sexual developmental changes, neurobehavioural alterations and immune disorders.[165]

In females, BPA exposure is linked to fertility problems, polycystic ovary syndrome and endometriosis.[22] Women with polycystic ovary syndrome had higher serum BPA concentrations compared to normal women and rodent studies suggest that prenatal exposure to BPA induces changes in mammary gland development that can predispose the tissue to cancer.[4] An *in vitro* study showed that low doses of BPA up-regulated c-Myc, which induced DNA damage and proliferation in estrogen receptor alpha (ERα)-negative mammary cells.[157] The study also showed that BPA induced DNA-damage markers in cells regardless of the ERα status.[157]

In male rodents, the effects of prenatal exposure to BPA include increased ano-genital distance, enlargement of the prostate, smaller epididymis and decreased sperm count.[4] BPA can also disrupt multiple metabolic mechanisms and is linked to cardiovascular disease, diabetes, obesity and liver dysfunction.[165] A study by Bastos Sales et al.,[168] using an *in vitro* murine preadipocyte model, suggested that BPA may contribute to the developmental programming of obesity by enhancing adipogenesis and altering global DNA methylation. A cross-sectional study showed an association between urinary BPA concentrations and general and central obesity in the general adult population in the US.[169] A significant association between urinary BPA concentrations and obesity were also reported in children and adolescents (6-19 years) in the US.[164] However, it could not be ruled out that obese children have

BEST PFE.COM
List of research project topics and materials

© University of Pretoria

higher BPA levels because they consume more BPA containing foods and have greater adipose stores of BPA.[164]

There are inconsistencies among the best evidence, industry and the Food and Drug Administration (FDA) in the US on what constitutes a safe level of exposure to BPA products.[165] The European Food Safety Authority set the tolerable daily intake for BPA as 0.05 mg/kg/day.[170] This is well above the levels associated with adverse effects in some animal studies.[171] The argument is that rodent toxicity data for BPA is not relevant to humans due to pharmacokinetic differences between the species. BPA is rapidly metabolized to the glucuronide conjugate in humans, resulting in minimal exposure to free BPA.[170,171] However, Ginsberg and Rice[171] pointed out that the conjugated BPA can be deconjugated back to free BPA in various body tissues by β-glucuronidase and arylsulfatase C. As a precautionary measure, the FDA banned BPA in baby bottles and sippy cups.[164,172] Other countries that have banned BPA from some infant products include Canada, the European Union (EU), South Africa, China, Malaysia, Argentina, Brazil and Ecuador.[172] However, BPA is still used in other food-packaging materials and consumer products to which consumers are exposed, including susceptible populations like infants and expectant mothers.[172] The Canadian Health Ministry has recently banned the manufacturing and selling of BPA-containing products.[165] France also banned BPA from all packaging, containers and utensils that come into contact with food.[172] The National Institute of Environmental Health Sciences (NIEHS) developed a consortium-based, multipronged, collaborative approach for BPA research to solve controversies and address uncertainties and data gaps.[159] This information would be invaluable for regulatory decision making.

1.5.1.3. Nonylphenol (NP)

NP was reported in bottled water from Greece,[147] Spain,[111,151] Italy,[108] Japan[134] and China[135] with concentrations ranging from below 7.9 ng/L to 2 030 ng/L.

NP is used in the manufacturing of surfactants and plastics.[173] It is found in food, food packaging materials, cleaning products, skin care products, environmental water samples and drinking water.[40,54,173] It is also used as a curing agent in epoxy paints and resins.[126] Nonylphenol ethoxylates are nonionic surfactants used as

© University of Pretoria

detergents, emulsifiers, and various other products in household and agricultural applications.[174] Nonylphenol ethoxylates are precursors of NP and can biodegrade in the environment into shorter-chain derivatives and to NP.[174] It is introduced to the environment mainly through wastewater discharges.[175] NP can accumulate in environmental matrices due to its high hydrophobicity and low solubility.[176]

One possible source of NP in bottled water is from the degradation of polyethoxylated nonylphenols used as surfactants in cleaning agents used in the bottling process.[111] Human exposure to NP occurs through inhalation, cutaneous absorption and ingestion,[176] but the main route is through the ingestion of contaminated water and food.[174] The average daily intake of NP from food was estimated at 7.5 µg/day in a German population.[175] NP is found in human amniotic fluid, urine and plasma samples, breast milk, fetal cord serum, placenta and maternal blood.[176]

NP is an EDC of concern due to its toxic and estrogenic activity.[134,177-179] NP was found to mimic the natural hormone E_2 by competing for the binding site of the ER.[40] Some NP isomers showed anti-estrogenic activity in MVLN cells.[180] It also has anti-androgenic activity, decreases aromatase activity, increase aryl hydrocarbon receptor activity[167] and binds to the human progesterone receptor to elicit an antagonistic effect.[181]

Soto et al.[179] first observed the capability of NP to induce breast tumour cell proliferation. Similarly, NP enhanced gene expression of key regulators of the cell cycle and induced proliferation in human prostate non tumorigenic epithelial cells, suggesting that NP exposure may also contribute to prostate disease and cancer.[176] In humans, a negative association was reported between maternal NP exposure and plasma luteinizing hormone levels in pregnant women from Taiwan.[174] The study will continue to follow up the birth cohort to investigate the possible association between prenatal NP exposure and neurobehavioural development of early childhood.[174]

The toxic effects of NP on aquatic life were first reported by Giger and coworkers.[182] The environmental impacts of NP include feminization of aquatic organisms and decreased male fertility.[40] Due to the harmful effects of nonylphenol ethoxylate

© University of Pretoria

degradation products in the environment, EU countries banned the use and production of such compounds.[40]

1.5.1.4. Phthalates

Phthalates are dialkyl or alkyl/aryl esters of 1,2-benzenedicarboxylic acid and are produced in high volumes for a variety of applications.[183] They are used as plasticizers, solvents and additives in various consumer products, including food and personal care products.[60] Phthalates are added to products to hold colour or fragrance, to provide a film or gloss, to provide flexibility to rigid PVC and it is added to some pharmaceuticals to ensure timed releasing.[183] Phthalates are not covalently bonded to the plastics in which they are used, and are therefore continuously being released from the products.[184,185] Humans are exposed to phthalates through ingestion, inhalation, dermal absorption[183] and via medical treatment when medical devices containing DEHP are used (e.g. from PVC containing medical tubing, blood storage bags, etc.).[15,185-187]

Although phthalates are not used as substrates or precursors in the manufacture of PET, several reports suggest that phthalates may leach from PET bottles into the contents of the bottle.[137] A study by Montuori et al.[155] reported nealy 20 times higher phthalate concentrations in water from PET bottles (3.52 µg/L) than in water from glass bottles (0.19 µg/L). Phthalates were detected in bottled water from many countries, including Greece,[147,153] Canada,[148] Spain, [111,151] Italy,[155] Portugal,[121] Hungary,[152] Iran,[149] Egypt[154] and India[156]. Phthalates were also detected in military packaged water that was filled in California and Afghanistan in PET bottles.[150] Some of the phthalates that were detected in bottled water include DEHP, DBP, diisobutyl phthalate (DIBP), diethyl phthalate (DEP), dimethyl phthalate (DMP) and benzyl butyl phthalate (BBP).[121,132,136,154]

Not all phthalates have the same EDC activity. *In vitro* studies showed that several phthalates, including BBP, DBP, diisononylphthalate (DINP) and DEHP, induce thyroid hormone dependent cell proliferation using the T-screen (a cell proliferation bioassay to test for thyroid hormone-like activity).[188] DBP, DINP and DEHP also inhibited thyroid hormone induced cell growth, indicating that these compounds can compete with the thyroid hormone.[188] DBP and DEHP also showed thyroid receptor

© University of Pretoria

antagonist activity using CV-1 cells.[189] Some phthalates are estrogenic and others anti-estrogenic *in vitro*.[188,190] DBP showed estrogenic activity and DEHP anti-estrogenic activity using MVLN cells.[188] DBP and DEHP showed androgenic as well as anti-androgenic activity in the MDA-kb2 cell line.[189] Takeuchi et al.[191] suggested that the activity of phthalates depend on the length of the alkyl side chain. Phthalates with alkyl chains ranging from C_3 to C_6 exhibited ERα-mediated estrogenic activity as well as estrogen receptor beta (ERβ)-mediated anti-estrogenic activity and human androgen receptor (hAR)-mediated anti-androgenic activity. However, phthalates with shorter or longer side chains did not show activity via the ERα, ERβ or hAR.[191] DBP, DEHP and diisodecyl phthalate (DIDP) also showed aryl hydrocarbon receptor agonistic activity.[192]

Phthalates are rapidly metabolized and excreted and are nonpersistent in the environment, but the widespread use of phthalates results in ubiquitous exposure.[15,60,185,193] Phthalates can be absorbed through human skin and because they are lipophilic, phthalates can accumulate in fatty tissues.[18] Phthalates and their metabolites have been measured in urine, serum, saliva, seminal fluid, breast milk, amniotic fluid, meconium and placenta.[194] Phthalates are associated with increased adiposity and insulin resistance, decreased levels of sex hormones and other adverse effects on the human reproductive system.[136] The fetus, infants and children are especially vulnerable to the toxic effects of phthalates.[60,136] Phthalates are associated with attention deficit hyperactivity disorder and reduced IQ scores in children.[28] Phthalate exposure of unborn and infants are high through maternal exposure via the placenta and breast milk.[60] Neonates in the neonatal intensive care units are especially at risk from exposure to DEHP released from PVC medical devices.[183,185,186] Mouthing of plastic toys,[186,195] infant formulae, baby food and personal care products can also add to the phthalate exposure of infants.[60]

In female rats, DBP exposure induces spontaneous abortion and long-term DEHP exposure is associated with anovulatory cycles and polycystic ovaries.[183] In human females, a possible association between phthalates and premature breast development were found in girls from Puerto Rico.[196] DEHP exposure is associated with endometriosis and shortening of the duration of human pregnancy.[60,183] Urinary

© University of Pretoria

concentrations of phthalate metabolites showed a significant difference between couples with infertility problems and couples with proven fertility.[197]

Several phthalates are associated with anti-androgenic effects in male rats and humans.[194,198] They appear to interfere with androgen activity by inhibiting testosterone synthesis.[6] The effects of fetal and lactational exposure to phthalates in rats are similar to those in humans with testicular dysgenesis syndrome (infertility, cryptorchidism, hypospadias and testicular cancer).[6,60] Perinatal exposure to DEHP, BBP and DINP led to altered sexual differentiation in male rats and infants displayed femalelike areolas/nipples (87, 70 and 22% for the respective chemicals) and reproductive malformations (82, 84 and 7.7%). In addition, DEHP and BBP exposure led to reduced pup mass at birth (15%), shorter anogenital distances (30%) and reduced testis mass (35%).[198] Pubertal exposure to DEHP reduced testosterone production in male rats, resulting in delayed onset of puberty, and reduced androgen-dependent tissue mass.[199] However, whether phthalate exposure poses a human health risk is still a subject of debate in the scientific literature. Kamrin[195] argued that the results from rodent exposure studies cannot be applied to humans due to differences in the mechanism of action of phthalates to induce cancer in rodents and humans. He also argued that the lowest concentrations of phthalates causing adverse effects in animal models were much higher than the doses that humans are exposed to.

Human studies showed associations between metabolites of DEHP and shorter anogenital distance and incomplete testicular descent in boys.[194] Prenatal exposure to anti-androgenic phthalates may be associated with less male-typical play behaviour in boys, suggesting that these chemicals have the potential to alter androgen-responsive brain development in humans.[200] Elevated urinary phthalate concentrations in boys from China was associated with constitutional delay of growth and puberty and appeared to be mediated by circulating testosterone level.[201] Another study showed that prenatal exposure to DEHP and DINP are associated with reproductive function of adolescent men. The study by Axelsson et al.[202] investigated the association between DEHP and DINP metabolite levels in maternal sera (collected during pregnancy and stored in a biobank), with testicular size, semen quality and reproductive hormones in their adolescent sons. Higher DEHP

© University of Pretoria

exposure levels were associated with lower semen volume, whereas higher DINP exposure levels were associated with lower total testicular volume, higher follicle-stimulating hormone levels and lower semen volume.[202] The same authors also showed that DEHP metabolite levels were negatively associated with sperm motility and maturation.[203] Phthalates are associated with increased DNA damage in sperm.[204]

The European Commission prohibited the use of DEHP, DBP and BBP in cosmetics, childcare articles and toys and food-contact applications. In the US, these chemicals may not be greater than 0.1% in childcare articles and toys and there is a temporary restriction on the use of DINP and DIDP in toys that children can put in their mouths.[193]

1.5.1.5. Di(2-ethylhexyl) adipate (DEHA)

DEHA was detected in bottled water from Spain, with concentrations ranging from 185 to 6 230 ng/L,[151] but was below the detection limit (dl) in bottled water from France,[143,205] Canada[148] and Egypt.[154] DEHA is used as an emollient or solvent in various cosmetic and personal care products and as an alternative to phthalates in flexible PVC products, including food packaging material.[188,206,207] The main source of DEHA exposure is expected to be from food.[207] DEHA is not chemically bound to the polymer and can therefore migrate from packaging material.[206,207] DEHA is a liver carcinogen in mice and induced developmental toxicity in rat offspring[188] and has been classified by the USEPA as a possible human carcinogen.[207] DEHA induced thyroid hormone dependent cell proliferation using the T-screen.[188] The WHO recommended limit for DEHA in drinking water is 0.08 mg/L.[206]

1.5.2. Factors influencing the migration of chemicals from bottles into water content

The migration of chemicals from food contact materials depend on the chemical properties of the food contact material and content as well as the physical characteristics of the food contact material, like the pore size, thickness and surface area.[138,142] Hot water and alkaline solutions can hydrolyse the carbonate linkages in polycarbonate containers, resulting in BPA migration.[93] The migration of antimony were higher in carbonated water (with a lower pH) compared to non-carbonated

© University of Pretoria

water.[138,143] Higher bottle volumes are associated with lower migration levels of antimony, indicating that the contact surface area have an influence on the migration of chemicals.[138]

A number of studies also indicated that different storage conditions (sunlight, temperature and duration of each) can contribute to the migration of chemicals from bottles to water.[132,137,156] Temperature appears to influence the leaching of formaldehyde, acetaldehyde, antimony[143] and phthalates[149] from PET, with greater leaching at higher temperatures. The leaching of BPA from PET bottles has also been demonstrated, with increased leaching at higher temperatures and storage duration up to four weeks.[208] Increased temperature during sterilization, heating or storage can cause the depolymerization and leaching of BPA from containers into food or beverages.[4] DMP, DEP, NP, DBP, BPA, BBP and DEHP concentrations increased in PET bottled water after outdoor storage for 10 weeks, exposed to temperatures up to 30°C.[111] Besides increasing the temperature, exposure to sunlight will cause the polymer (e.g. PET) to undergo photochemical aging, which may lead to the formation of by-products and the migration of substances from the material.[143] When plastic material is subjected to physical stress during the production process, modifications in its chemical structure may generate degradation products that can migrate from the packaging material.[138,143] BPA migration is higher from used polycarbonate containers due to degradation of the polymer.[93]

1.6. Testing methodologies for EDCs

Testing methodologies for EDCs allow for the testing of individual chemicals or complex mixtures, such as environmental water samples. Man-made chemicals are an integral part of modern societies.[49] Over 80 000 new chemicals have been synthesized in the last century.[20] Some of these chemicals might be hazardous, with some of the hazardous effects due to endocrine disruption.[20] ToxCast™ is a high throughput *in vitro* screening program developed by the USEPA to identify chemicals with potential endocrine activity.[209] Green chemistry aims to design new chemicals without potential hazardous effects to humans or the environment.[20] Schug et al.[20] proposed a Tiered Protocol for Endocrine Disruption (TiPED) to help chemists identify chemicals with potential endocrine disruptive effects as early as possible in the design process. For these chemicals, production can either be ceased, or the

© University of Pretoria

molecular structure can be manipulated to eliminate hazardous effects. The TiPED approach consists of five tiers, starting with the simplest and least expensive approach in Tier 1 and moving to more complex and expensive Tiers. Tier 1 consists of a computation-based assessment that utilize statistical, computer and mathematical models to predict possible EDC properties based on the structure of the molecules. Tier 2 consists of high-throughput cell-based and cell-free *in vitro* screens, mostly based on receptor binding. Tier 3 uses more sophisticated *in vitro* whole cell assays to examine the functional outputs following receptor binding and activation. Tier 4 consists of fish and amphibian whole animal assessment and Tier 5 is the mammalian whole animal assessment. Fish and amphibians have the advantage that they allow for larger sample sizes and shorter stages of development allow for partial and full life-cycle assays. However, mammalian assays are needed to study EDC effects on more complex mammalian physiological processes, *in utero* exposure and behavioural effects.[20]

The analysis of complex mixtures, such as environmental water samples, for EDCs and EDC activity, is much more challenging than testing individual chemicals. The chemical content of water samples is often unknown and many potential endocrine disruptors exist as mixtures in the environment, possibly acting synergistically.[66] Suitable analytical methods are not always available for some EDCs and some substances may exert an effect on the environment below the dl of available analytical methods.[66,73] It is therefore recommended to assess endocrine activity in environmental samples with bioassays, rather than relying on chemical analysis alone.[17,66,73,210]

1.6.1. Sample preparation

The ubiquitous nature of EDCs makes it very difficult to attain a pristine environment in which to conduct experiments.[7] For example, NP, OP and BPA were even detected in double distilled and reverse osmosis water used for laboratory blank samples.[115] DEHP was detected in various commercially available and laboratory produced ultrapure water samples.[211,212] The presence of DEHP was in agreement with anti-estrogenic activity detected in the laboratory water samples using the yeast estrogen screen (YES) bioassay.[211] Aneck-Hahn et al.[213] reported that several laboratory water sources were responsible for estrogenic contamination in the

© University of Pretoria

recombinant yeast estrogen screen. These findings indicate that EDCs could leach out from plastic materials used for membranes, resin housings and piping in water purification equipment and should be taken into account when analysing results.[211]

EDCs can be present at very low concentrations in samples and may contain compounds that can mask or interfere with the analysis of the compound of interest. A sample preparation method is therefore required to extract, concentrate and clean up samples before analysis.[17,75] By concentrating a sample during the extraction process, lower method detection limits can be achieved.[65,84] It is important that the extraction method must ensure a good recovery of the active compounds in order to achieve reliable results.[90] If the extract is going to be analysed in bioassays, the solvent used to reconstitute the extract must be compatible with the cell system. It must enable the distribution of the extracted compounds to the cells, without having an effect itself.[17]

Liquid-liquid extractions (LLE) or solid phase extractions (SPE) are usually applied when analysing for EDCs.[137,141] Solid phase microextraction (SPME), liquid phase microextraction (LPME),[11,137] stir bar sorbtive extraction with liquid desorption (SBSE-LD)[122] and cloud point extraction have also been used.[75] LLE was traditionally used in the analytical method for organic contaminants in water samples, but this method requires large quantities of organic solvents.[11,15] LPME methods have been developed to address this problem and reduce analysis time.[11] SPE is based on the adsorption of the target analytes to the solid phase sorbent. The analytes are then desorbed from the solid phase using a specific organic solvent.[11] SPE requires less solvent compared to LLE and therefore produces smaller quantities of toxic waste.[11,15,75] It is also faster and offers higher concentration factors compared to LLE [15]. In SPME, the analyte is adsorbed onto the surface of a coated silica fiber and desorbed at the injection port of the analytical instrument.[11] SBSE-LD is a solventless extraction method using stir bars coated with the sorbent polydimethylsiloxane and liquid desorption.[122]

Not all extraction protocols are equally successful in extracting EDCs and may result in false negatives.[141] Due to their different physicochemical characteristics, the optimization of an analytical protocol to simultaneously determine phenolic and

© University of Pretoria

steroid EDCs remains challenging.[214] The selection of an appropriate solid sorbent and elution solvent for the specific target analytes are crucial in SPE.[11,215] The salt concentration, pH and humic acid concentration in samples can also have an effect on the recovery of target EDCs.[215] Samples can get contaminated during the extraction and sample preparation process, resulting in high background levels and high detection limits. This is especially a problem in the analysis of phthalates, which might be present in laboratory plastics, solvents and extraction sorbents.[155] In order to limit phthalate contamination during the extraction process, Montuori et al.[155] used a solvent-free SPME technique. SBSE-LD has also successfully been used for phthalate extractions. This technique has higher sample capacity, recovery and sensitivity compared to SPME and by using liquid desorption the contamination associated with the conventional thermal desorption method is avoided.[122] It is therefore crucial to select an appropriate extraction method for the samples to be analysed in bioassays and for target chemicals.

1.6.2. Bioassays and non-cellular *in vitro* assays

Bioassays are useful to measure the cumulative effects of all substances in the sample and to avoid exhaustive chemical analysis.[44,73] Different *in vitro* bioassays have different advantages and disadvantages and measure different EDC effects (e.g. agonism/antagonism of receptors, proliferation of cells, gene expression, hormone production, etc.).[17,210] It is therefore recommended to use a suite of bioassays.[7]

Cell proliferation bioassays and reporter gene bioassays are commonly used to determine EDC activity in water samples. The E-screen is an example of a cell proliferation bioassay to test for estrogenic activity. The bioassay consists of MCF-7 human breast cancer cells that contain ERs. Chemicals that activate the ER will cause the cells to proliferate.[7,17] However, a disadvantage of this bioassay is that substances can also bind to other endogenous receptors in the MCF-7 cells (androgen, progesterone, glucocorticoid and retinoid receptors) to antagonize the estrogen-induced cell proliferation.[17]

Reporter gene bioassays are sensitive and specific bioassays to determine the total agonistic or antagonistic activity of complex mixtures in the ng/L range.[17,90]

BEST PFE.COM
List of research project topics and materials

© University of Pretoria

Bioassays are available to determine estrogenic, androgenic, progesterone and glucocorticoid activity.[13,90] The bioassays consist of cells containing a steroid receptor coupled to a reporter gene. When a substance in the water sample binds to and activates the receptor, the reporter gene encodes for an enzyme, which leads to a luminescent reaction or a colour change proportional to the steroid activity of the sample.[73] The steroid activity is expressed as equivalent concentrations compared to a reference compound, for example, E_2 is used as reference compound for estrogenic bioassays and the estrogenic activity of a sample is expressed as estradiol equivalents (EEq).[73] Antagonistic activity is measured by co-incubating sample extracts with the agonist control. The cells used for the bioassays can either be yeast-based or consist of human cell lines. The yeast-based bioassays are less sensitive compared to human cell lines, but they are easier to perform.[17,73,210] Other advantages of the yeast-based bioassays are that they do not have endogenous receptors that can interfere with the response of the cells and do not require steroid-free media. They are also very robust compared to mammalian cells and therefore ideal to test extracts from dirty sample matrices.[17]

In vitro bioassays can also be used to investigate the effects of EDCs on gene expression and steroidogenesis. The transcriptomic fingerprint profiles generated by EDCs can be determined by making use of DNA micro-array technology.[17] Steroidogenesis bioassays include the H259R adrenal steroidogenesis model and a model using primary porcine Leydig cells. The H259R cell line is a human cell line capable of full steroidogenesis that can be used to identify substances that modulate the entire steroidogenisis pathway.[17] Disadvantages of this model are that it is only stable for a limited number of passages and it is not responsive to adenocorticotropic hormone stimulation.[17] The porcine Leydig cell model is excellent for investigating the effects of EDCs on hormone production and gene expression in Leydig cells, but the collection, isolation and purification methods are resource-demanding.[17]

Non-cellular assays are also available for the detection of estrogenic EDCs and include enzyme-linked immunosorbent assays (ELISA), enzyme-linked receptor assay (ELRA), Endotect[TM], RIver ANAlyser (RIANA), electrochemical biosensors, single cell coactivator recruitment (SCCoR) and microarray relative binding assay (RBA). The advantage of non-cellular assays is that they do not require whole cells

© University of Pretoria

and therefore avoid complications related to membrane permeability, organism life stages and toxicity responses to samples.[7,11,94]

In vitro assays have several advantages: it's considered more ethical than tests using a whole animal and by measuring the total endocrine effect of samples, can account for unidentified EDCs in the mix and unknown combination effects.[7,210] *In vitro* assays also give quick results and cost less than *in vivo* assays.[17] On the other hand, bioassays do not account for the pharmacokinetics and pharmacodynamics of EDCs and the endocrine effect on a whole organism[7] or behavioural effects.[17] It may also underestimate estrogenicity as it doesn't detect estrogenic effects through indirect mechanisms.[7] Therefore, although *in vitro* assays are valuable to identify potential endocrine-active compounds and provide information on their mechanism of action, they cannot provide information regarding potential adverse health effects in an intact organism.[216,217] This can be addressed using *in vivo* assays.

1.6.3. Whole organism assays

Whole organisms used to investigate EDCs in the aquatic environment include amphibians, fish, birds and insects.[11] Deformities, reproductive deficiencies, egg and offspring development and serum protein production are some of the responses monitored in these organisms to indicate endocrine disruption.[11] Many of the whole organism assays are based on the production of vitellogenin in response to exposure to estrogens.[11] The advantage of using whole organisms is that the actual effects of EDCs on the target species can be determined as well as the cumulative effect from exposure to a mixture of EDCs in environmental samples.[11] Challenges include to account for non-monotonic dose-response relationships,[216] critical windows of development and delayed effects.[1]

1.6.4. Chemical analysis

It is not possible to detect and quantify all substances present in an environmental water sample and therefore chemical analyses are usually focussed on substances that are expected to be in the sample and known to pose a potential threat.[73] Some EDCs can cause adverse effects at very low levels, but the detection of these chemicals at those levels requires great analytical effort[73] and significant capital investment.[11] Due to the diverse physiochemical characteristics of EDCs, a single

© University of Pretoria

method is not capable of detecting trace levels of different classes of compounds in a single run, but requires a variety of analytical techniques.[12,75] High performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), HPLC-MS, liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry have been used successfully to detect and quantify EDCs.[7,75,94] HPLC and GC methods may require sample derivatization to improve the sensitivity and selectivity.[75] In general, the methods employing MS have lower detection limits compared to other methods[11] and is the best technique to detect and quantify trace levels of EDCs.[75] Development of MS technology that further improved the detection of chemicals include triple quadrupole (QqQ), quadupole time-of-flight (Q-TOF) and ion-trap (IT) tandem mass spectrometers.[84] Kuch and Ballschmiter[115] described a method using high-resolution gas chromatography with negative chemical ionization mass spectrometry (HRGC-(NCI)-MS) that can detect phenolic estrogenic active compounds in the picogram per liter range.

Chemical analysis can complement bioassays in the assessment of environmental water samples. This data can be used in combination with risk assessment methods to determine the potential adverse effects of EDCs on the aquatic environment and human health.[67]

1.7. Health risk assessment of EDCs

In order to develop policy and legislation to protect humans and the environment from EDCs, it is first necessary to determine the risk to human health and the environment.[216] Health risk assessment involves quantitative and/or qualitative processes to characterize the nature and magnitude of the public health risks associated with exposure to hazardous substances.[216]

There are still many uncertainties and unknowns in the risk assessment of EDCs and to date, there are no standardized guidelines.[7] Standardized methods for regulatory risk assessment do not always cover all relevant adverse endpoints and sensitive windows of exposure associated with EDCs.[216] EDCs can therefore not be evaluated in the same way as general toxins, but should rather be based on the fundamental principles of endocrinology.[2] Applying traditional risk assessment to evaluate EDCs

© University of Pretoria

should be done with caution until a risk assessment paradigm can be designed that allow for the unique characteristics of EDCs.[7]

Health risk assessment entails four interactive stages, namely hazard identification, toxicity (dose/response) assessment, exposure assessment and risk characterization.[2,218]

1.7.1. Hazard identification

The first step in health risk assessment is hazard identification. Chemicals with potential adverse health effects are identified based on human epidemiological studies and animal toxicology studies. Potentially hazardous chemicals are divided into three main groups according to their mode of action, namely toxic chemicals, carcinogens or endocrine disruptors.[218]

Hazard identification of endocrine disruptors can be challenging. Although various databases for EDCs are available, the vast majority of chemicals in use today haven't been tested for potential endocrine disruptive activity.[7] A complicating factor is that EDCs comprise of a very heterogeneous group of chemicals and the structure of a chemical cannot always predict potential endocrine disruptive activity.[7] Furthermore, chemicals that cause acute toxicity are easy to classify as hazardous substances, but chronic exposure to low doses of endocrine disruptors will result in more subtle effects.[9,56] Some effects may only manifest later in life, making hazard identification even more challenging. Laboratory studies showed that EDCs can, for example, have an effect on organ mass, can cause precancerous growths and changes in the activity of genes and proteins. Although these changes may lead to adverse health effects in an organism, traditional toxicologists don't consider these endpoints as real health effects in risk assessment.[56] However, subtle changes, like changes in epigenetic programming, can ultimately lead to highly adverse effects, like cancer in adulthood.[20,41] Advances in the fields of toxicogenomics, transcriptomics, proteomics, metabolomics and bioinformatics can give valuable information for the hazard identification of EDCs.[41]

© University of Pretoria

1.7.2. Dose response assessment

Dose response assessment examines the relationship between the dose of a hazardous agent and the incidence of an adverse effect in the exposed population.[218] Although it is known that endocrine disruptors adversely affect human health, the levels at which these effects occur is uncertain.[98] In conventional risk assessment, a threshold level or maximum acceptable level is calculated for chemicals classified as "toxic". It is assumed that no adverse effects will occur below those doses and are therefore considered safe.[56,218] Carcinogens have no threshold value and a potency factor is determined based on available mathematical extrapolation models.[218]

EDCs can have complex modes of action and characteristics that do not fit perfectly into traditional risk assessment frameworks.[7] Some EDCs have non-monotonic dose-response relationships, making quantitative dose response assessment difficult.[218,219] Great efforts are being made to develop an approach for the risk assessment of compounds with non-monotonic dose-responses, but it remains a subject of debate.[219]

EDC exposure usually involves multiple chemicals that can interact with each other and have additive, synergistic or attenuative potential that can complicate the dose response assessment.[7] In isolation, an EDC might not pose any health risks, but the cumulative effect of a mixture of chemicals could result in significant health risks.[7] Risk assessment on a chemical-by-chemical basis is therefore not adequate for EDCs and mixture effects should be taken into account.[48,51]

1.7.3. Exposure assessment

Humans can be exposed to chemicals via different routes (ingestion, inhalation and dermal absorption) and media (e.g. air, food, soil). In exposure assessment the concentrations of identified hazardous chemicals are used to calculate the total dose a person may be exposed to.[218]

EDC activity is very complex and it is difficult to assess the combined effect of exposure to a mixture of EDCs.[1,7] Risks specific to longer-term exposure, vulnerable populations, sensitive windows and transgenerational effects are other complicating

© University of Pretoria

factors in accurate exposure assessment of EDCs.[7] Furthermore, human exposure data for most EDCs are limited, making accurate health risk assessment very difficult.[1]

1.7.4. Risk characterization

Risk characterisation calculates the incidence of health effects based on the exposure levels from the exposure assessment and the identified dose-response relationship.[218] For toxic chemicals a Hazard Quotient (HQ) is calculated by comparing the estimated exposure to a reference dose (RfD) assumed to be safe.[220] A HQ less than 1 is considered to be safe for a lifetime exposure.[218] For carcinogenic chemicals the excess cancer risk can be calculated using the oral potency factor (β) reported by the USEPA[220] and the lifetime average daily dose. The WHO acceptable risk level is 10^{-5}, meaning that the excess risk of developing cancer is deemed acceptable if ingestion of the substance results in one additional cancer case per hundred thousand of the population or less.[218] To quantify the uncertainty of risk assessment, probabilistic risk assessment techniques can be used, for example Monte Carlo modelling. These models enable good prediction of human variability.[85]

The accurate risk assessment of EDCs is restricted by uncertainties and knowledge gaps that exist, and is therefore not a reliable indicator of absolute risk posed by EDCs.[7] However, risk assessment can be very useful for the comparison of relative risk from EDCs in different sources and can be compared to what is considered tolerable. Relative risk can be used to guide researchers and decision makers in the risk management of priority sources.[7]

From the literature review it is clear that EDCs from various sources may be present in drinking and bottled water and may pose potential human health risks. There is very limited information on EDCs in drinking water and bottled water from South Africa and should be investigated.

© University of Pretoria

Chapter 2: Aims and objectives of the study

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.

This study consisted of three phases. Phase 1 included the identification of sampling points and analysis of drinking water at various distribution points/reservoirs in Pretoria (City of Tshwane) and Cape Town. Phase 2 included the identification and analysis of different brands of bottled water. Phase 3 comprised a scenario based health risk assessment, based on the data collected from phase 1 and 2.

2.1. Hypothesis

The hypothesis of this study was that potential human health risks associated with EDCs in water from municipal distribution points would be lower compared to bottled water stored at different storage conditions. EDCs present in plastic bottles will migrate into the water content at a higher storage temperature, resulting in higher estrogenic activity and increased human health risk.

2.2. Aims and objectives

2.2.1. Phase 1 – Distribution point water

Aim:

The aim of phase 1 was to determine the estrogenic activity and EDC status in water collected from selected distribution points in Pretoria and Cape Town

Objectives:

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 YES and the T47D-KBluc reporter gene bioassay

© University of Pretoria

3. To determine the concentrations of BPA, NP, DEHA, DBP, DEHP, E_2, E_1 and EE_2 present in drinking water samples collected from the identified collection points

2.2.2. Phase 2 – Bottled water

Aim:

The aim of phase 2 was to determine the estrogenic activity and EDC status in selected South African brands of bottled water under different storage conditions

Objectives:

1. To identify different brands of bottled water available in South Africa to be included in the study
2. To assess the estrogenic activity in the selected brands of bottled water using the YES and the T47D-KBluc reporter gene bioassays
3. To determine the concentrations of BPA, NP, DEHA, DBP, DEHP, E_2, E_1 and EE_2 present in the bottled water

2.2.3. Phase 3 – Health risk assessment

Aim:

The aim of phase 3 was to do a scenario based health risk assessment, based on the data collected from the estrogenic bioassays and chemical analysis

Objectives:

1. To assess the carcinogenic and toxic human health risks posed by the chemicals found in the analysed water samples
2. To compare the human health risks associated with water from the selected distribution points and bottled water as an alternative source of drinking water

© University of Pretoria

Chapter 3: Materials and Methods

A flow diagram of the three phases of the project and the processing of the water samples is depicted in Figure 3.1.

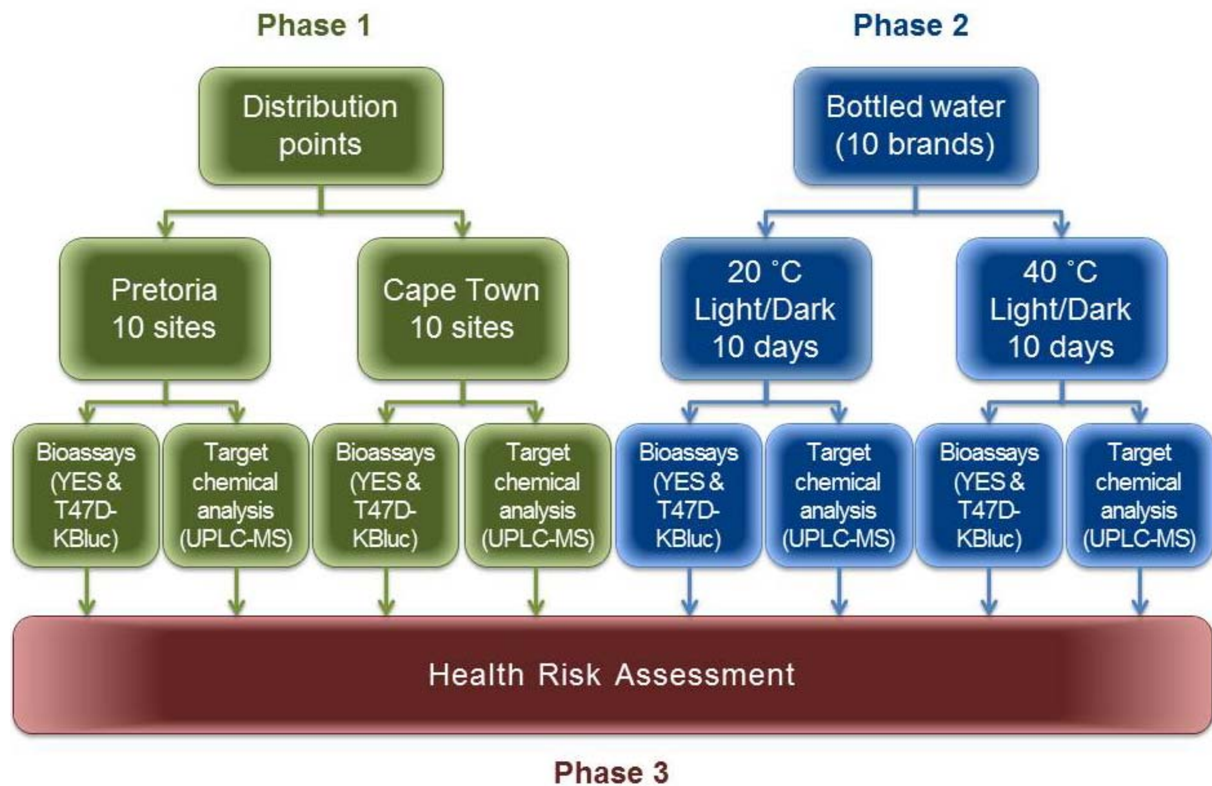


Figure 3.1: Flow diagram of the three phases of the project

© University of Pretoria

3.1. General laboratory procedures

3.1.1. Materials

Nitrile gloves (cat. no. RLAS1GL014) were purchased from Lasec (Cape Town, South Africa). The EDS-Pak Cartridge (cat. no. EDSPAK001) attached to the Milli-Q water system, HPLC grade methanol (MeOH, cat. no. 1.06007.2500) and HPLC grade ethanol (EtOH, cat. no. 1.11727.2500) were purchased from Merck (Darmstadt, Germany). Liquinox detergent was purchased from Alconox (New York, USA).

3.1.2. Methods

Nitrile (latex free) gloves were worn for all assay procedures to limit EDC contamination. All aseptic assay procedures were carried out in a Type II biological safety cabinet (Airvolution, Kya Sands, South Africa), using aseptic techniques. Double distilled water (ddH_2O) from a Millipore Milli-Q synthesis ultrapure water system (Merck Millipore, Darmstadt, Germany) was used for washing of glassware, extraction procedures, media preparation and controls. The water system is equipped with an EDS-Pak Cartridge (an activated carbon based filter) to produce EDC-free water. Glassware was prepared by washing with Liquinox detergent (1:100 dilution), rinsing ten times with tap water, five times with ddH_2O, twice with MeOH and twice with EtOH. Glassware and non-sterile equipment were sterilized in an autoclave (Rexmed Industries, Kaohsiung, Taiwan) for 20 min at 121°C and 15 psi.

3.2. Phase 1 – Distribution point water

3.2.1. 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. Estrogenic contamination of water sources in Pretoria is expected to be mostly from industrial activities. In Cape Town agricultural activities might also contribute to estrogenic contamination of 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.

BEST PFE.COM
List of research project topics and materials

© University of Pretoria

Samples were taken from selected distribution points/reservoirs and not from individual homes (i.e. not point of use). These points rather than points in private homes were selected in order to prevent the possibility of confounding factors in the form of the different types of piping used in private homes. The selected sampling points for Pretoria are listed in Table 3.1 and for Cape Town in Table 3.2.

Table 3.1: Selected sampling points for Pretoria (City of Tshwane)

Sample code	Sampling point
PTA 01	Temba - Reservoir 4
PTA 02	Roodeplaat System - Montana Reservoir
PTA 03	Fountains Combined
PTA 04	Findlay Reservoir
PTA 05	Garsfontein Outgoing
PTA 06	Garsfontein Rietvlei Supply
PTA 07	Garsfontein Ingoing Rand Water
PTA 08	Grootfontein Spring
PTA 09	Nokeng System - Refilwe pump station
PTA 10	Bronkhorstspruit System - Ekandustria

Table 3.2: Selected sampling points for Cape Town

Sample code	Sampling point
CPT01	Plattekloof Reservoir Outlet (TBW12)
CPT02	Blackheath Reservoir (OBW05)
CPT03	Faure Treated (FA TREATED)
CPT04	Steenbras Treated (SB TREATED)
CPT05	Langeberg Pump Station (OBW01)
CPT06	Kloof Nek Reservoir (GPW05)
CPT07	Constantia Nek Treated (CN TREATED)
CPT08	Pinehaven (SPW09)
CPT09	Atlantis Final Softened (ATL FNLSOFT)
CPT10	Voelvlei Treated (VV TREATED)

© University of Pretoria

3.2.2. Sample collection

3.2.2.1. Materials

Glass Schott bottles (cat. no. 218015455), pH indicator strips (cat. no. 1.09535.0001), HPLC grade MeOH (cat. no. 1.06007.2500) and 32% hydrochloric acid (HCl, cat. no. 100319) were purchased from Merck (Darmstadt, Germany).

3.2.2.2. Methods

Water samples were collected in triplicate in 1 litre glass Schott bottles, prepared by rinsing the bottles with MeOH. The lids of the bottles were lined with tin foil to prevent the samples from coming into contact with the plastic lids, which can be a possible source of EDC contamination. The pH of the water samples were adjusted to 3 using pH indicator strips and 32% HCl. The water was stored at 4°C in the dark until it was extracted, in order to minimize sample degradation. To account for seasonal variations in levels of EDCs, samples were collected from the identified sites in October 2013 (spring), January 2014 (summer), April 2014 (autumn) and July 2014 (winter). Examples of sample collection at typical distribution sampling points are shown in Figure 3.2.

© University of Pretoria

Figure 3.2: Examples of distribution points and sampling

© University of Pretoria

3.2.3. Water extraction procedure

3.2.3.1. Materials

Oasis hydrophilic-lipophilic balance (HLB) SPE cartridges (5cc, 200 mg, cat. no. 186000683) were purchased from Waters (Milford, Massachusetts, USA). Glass serological pipettes (cat. no. 7065) and disposable serological pipettes (cat. no. 4101) were purchased from Corning Incorporated (Corning, New York, USA). MeOH (HPLC grade, cat. no. 1.06007.2500) and EtOH (HPLC grade, cat. no. 1.11727.2500) were purchased from Merck (Darmstadt, Germany) and methyl tertiarybutyl ether (MtBE, cat. no. 306975) from Sigma-Aldrich (St. Louis, Missouri, USA).

3.2.3.2. Methods

All water extraction procedures were carried out at the EDC laboratory at the University of Pretoria within seven days after collection of the samples. One litre of each sample was concentrated using a SPE procedure recommended by Oasis for the extraction of EDCs in water samples.[221] The apparatus used for SPE is shown in Figure 3.3.

© University of Pretoria

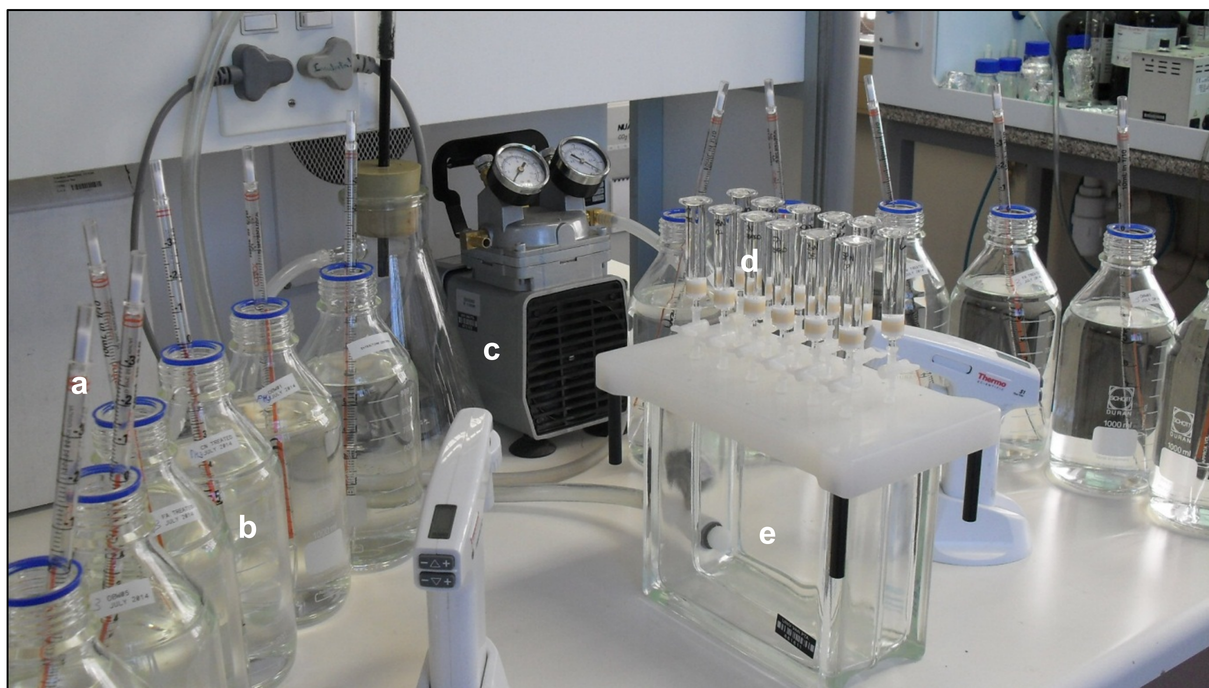


Figure 3.3: Solid phase extraction apparatus. a) Serological pipette; b) glass Schott bottles containing water samples; c) vacuum pump; d) Oasis HLB SPE cartridges; e) SPE 12-position vacuum manifold

© University of Pretoria

Oasis HLB SPE cartridges were placed on a SPE 12-position vacuum manifold (Phenomenex, Torrance, California, USA) and pre-conditioned with 5 mL 10% MeOH in MtBE, followed by 3 mL MeOH. The cartridges were equilibrated with 3 mL ddH_2O before the samples were loaded. The cartridges were washed with 3 mL 5% MeOH in ddH_2O and dried. The samples were eluted from the cartridges using 6 mL 10% MeOH in MtBE. Throughout the extraction procedure the flow rate never exceeded 10 mL/minute and care was taken not to let the cartridge run dry until the whole sample volume passed through the cartridge. A pilot study comparing glass and disposable (plastic) pipettes revealed no clear evidence of leaching from the disposable pipettes (refer to section 4.2) and therefore glass serological pipettes were used for all solvents and disposable serological pipettes were used for the samples. After elution, the solvent was evaporated to dryness at 37°C under a gentle nitrogen stream (using a Reacti-vap and Reacti-therm unit from Pierce, Rockford, Illinois, USA). The sample residue was reconstituted in 1 mL EtOH for analysis in the bioassays or derivatized for ultra-performance liquid chromatography-mass spectrophotometry (UPLC-MS) analysis. Reconstituted samples were stored at -20°C. The extraction procedure is summarised in Figure 3.4.

© University of Pretoria

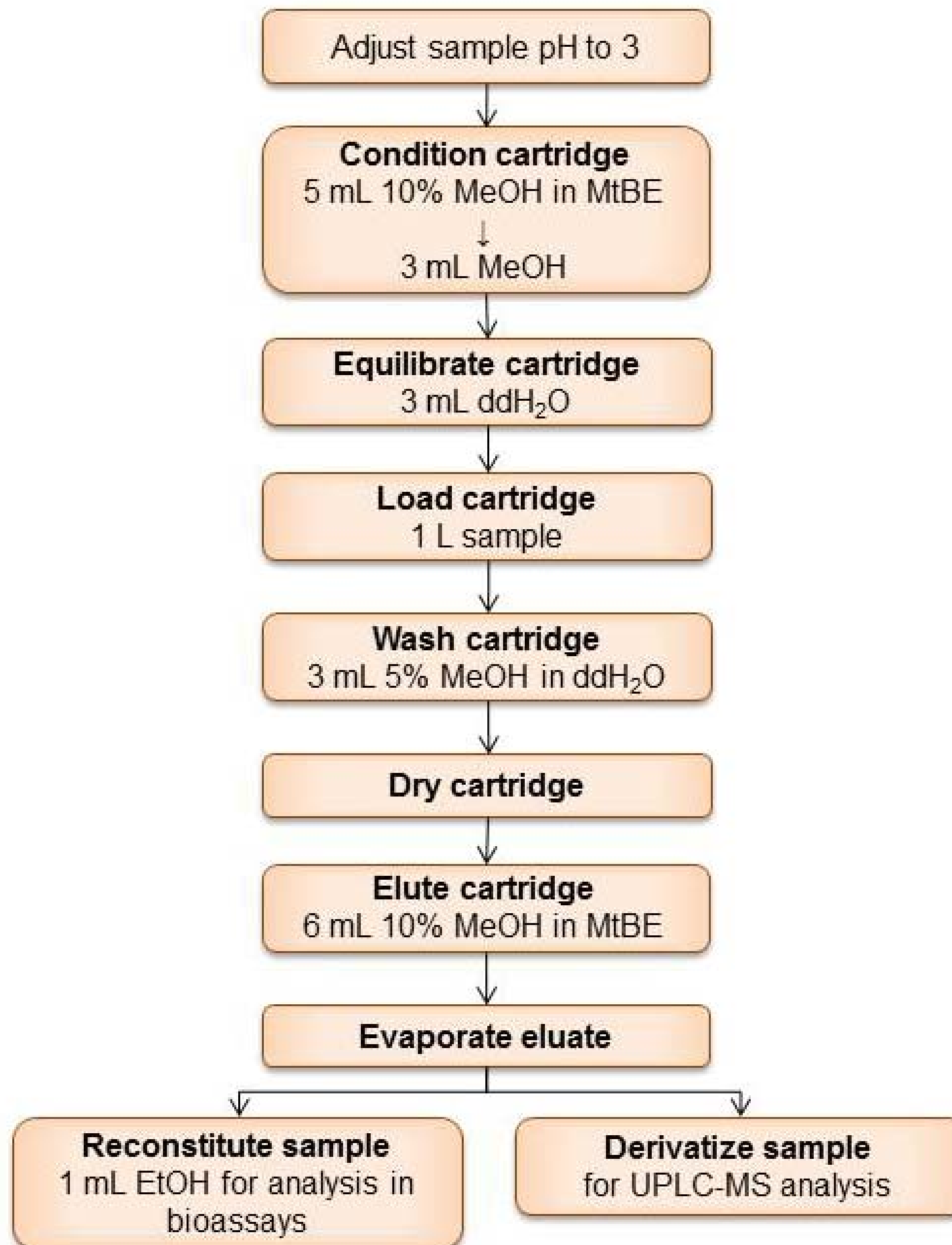


Figure 3.4: Procedural steps for solid phase extraction

© University of Pretoria

3.2.4. Bioassays for estrogenic activity

The bioassays to determine estrogenic activity in water samples were carried out in the EDC laboratory at the University of Pretoria according to the protocols described in the South African Water Research Commission (WRC) Toolbox project: K5-1816,[222] based on the original YES protocol described by Routledge and Sumpter[223] and T47D-KBluc reporter gene bioassay protocol described by Wilson et al.[224]

3.2.4.1. The recombinant yeast estrogen screen (YES) bioassay

The YES bioassay was developed in the Genetics Department at Glaxo Group Research Ltd to determine the estrogenic activity of compounds. A yeast strain (*Sacchromyces cerevisiae*) was genetically modified to contain the human estrogen receptor alpha (hERα) as well as expression plasmids carrying the reporter gene *lac-Z*, encoding the enzyme β-galactosidase. Substances that bind to and activate the ER will result in the expression of the reporter gene *Lac-Z*, resulting in the dose-dependent production of β-galactosidase. The enzyme is secreted into the medium that contains the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG). CPRG is normally yellow, but is metabolized into a red product by β-galactosidase and can be quantified by measuring absorbance.[223]

3.2.4.1.1. Materials

The yeast was obtained from Prof JP Sumpter's laboratory, in the Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex in the United Kingdom. Agar (cat. no. A9915), biotin (cat. no. B4639), E_2 (cat. no. E8875), glycerol (cat. no. G2025), potassium dihydrogen phosphate (KH_2PO_4, cat. no. P5655), ferric sulphate ($Fe_2(SO_4)_3$, cat. no. F1135), pantothenic acid (cat. no. P5155) and pyroxidine (cat. no. P5669) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Anhydrous ammonium sulphate ($(NH_4)_2SO_4$, cat. no. 106067), potassium hydroxide (KOH) pellets (cat. no. 105033), anhydrous magnesium sulphate ($MgSO_4$, cat. no. 106067), L-leucine (cat. no. 105360), L-histidine (cat. no. 104351), adenine (cat. no. 1152), L-arginine-HCl (cat. no. 101543), L-methionine (cat. no. 105707), L-tyrosine (cat. no.108371), L-isoleucine (cat. no. 105362), L-lysine-HCl (cat. no. 105700), L-phenylalanine (cat. no. 5202), L-glutamic acid (cat. no. 100291), L-serine (cat. no. 107769), L-valine (cat. no. 108495), D(+)-glucose (cat. no. 108342), L-

© University of Pretoria

aspartic acid (cat. no. 100126), L-threonine (cat. no. 108411), thiamine (cat. no. 5871), inositol (cat. no. 104507), anhydrous copper (II) sulphate ($CuSO_4$, cat. no. 102791), EtOH (HPLC grade, cat. no. 100983) and polyethersulfone (PES) membrane syringe filters (cat. no. SLGP033RS) were purchased from Merck (Darmstadt, Germany). CPRG (cat. no. 10884308001) was from Roche Diagnostics (Mannheim, Germany). Glass tubes (cat. no. 9270110) were purchased from Hirschmann (Neckartenzlingen, Germany). Cryovials (cat. no. 377267) and 96-well assay plates (cat. no. 95029780) were purchased from Thermo Fisher Scientific (Denmark) and 50 mL centrifuge tubes (cat. no. 430828) from Corning Incorporated (Corning, New York, USA). Autoclave tape was purchased from 3M Health Care (Neuss, Germany).

3.2.4.1.2. Methods: Preparation of medium and stock solutions and maintenance of stock cultures

Minimal medium was prepared by adding 13.6 g KH_2PO_4, 1.98 g $(NH_4)_2SO_4$, 4.2 g KOH pellets, 0.2 g $MgSO_4$, 1 mL $Fe_2(SO_4)_3$ solution (0.8 g/L in ddH_2O), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 375 mg L-serine and 150 mg L-valine to 1 L ddH_2O and the pH was adjusted to 7.1. The medium was sterilized by autoclaving for 20 min at 121°C and 15 psi and stored at 4°C. Stock solutions of glucose (200 g/L), L-aspartic acid (4 g/L) and L-threonine (24 g/L) was prepared in ddH_2O. The solutions were autoclaved (20 min, 121°C, 15 psi) to sterilize and stored at 4°C. Vitamin solution was prepared by adding 8 mg thiamine, 8 mg pyroxidine, 8 mg pantothenic acid, 40 mg inositol and 20 mL biotin solution (0.02 g/L in ddH_2O) to 180 mL dd H_2O. The solution was sterilized by filtering through a 0.2 µm syringe filter. Stock solutions of $CuSO_4$ (0.3192 g/L) and CPRG (10 g/L) were also filter sterilized and stored at 4°C. Growth medium was prepared by adding 45 mL minimal medium, 5 mL glucose, 1.25 mL L-aspartic acid, 0.5 mL vitamin solution, 0.4 mL L-threonine and 125 µL $CuSO_4$ together. A 54.58 µg/L stock solution of the E_2 positive control was prepared in EtOH and stored at -20°C.

Long-term yeast stock cultures were prepared on agar slopes. A 1% agar solution was prepared in minimal medium. The solution was autoclaved and the following

© University of Pretoria

growth medium components were added to 90 mL of the agar solution once it cooled down to 50°C: 10 mL glucose, 2.5 mL L-aspartic acid, 1 mL vitamin solution, 0.8 mL L-threonine and 250 µL $CuSO_4$. The solution was gently mixed, poured into sterile glass tubes, and allowed to set at an angle of approximately 45°. Approximately 2 µL of the original yeast stock was spread over the surface of the agar slopes and were incubated for 3 days at 32°C. The yeast cells were resuspended in 1 mL sterile glycerol and stored in aliquots in cryovials at -80°C.

To prepare 10x concentrated short-term stock cultures, 125 µL of the long-term yeast stock was added to 50 mL growth medium and incubated at 28°C in a rotating water bath (at 155 upm). After 24 hours incubation, 1 mL of the 24-hour yeast culture was added to two flasks containing 50 mL growth medium each. The two flasks were incubated for a further 24 hours in a water bath (28°C, 155 upm). After incubation, the yeast cultures were transferred to 50 mL centrifuge tubes and centrifuged for 10 minutes at 4°C and 2 000 x g (Sigma 4K15 centrifuge from Sigma Laborzentrifugen, Germany). The supernatant was decanted and the pellets resuspended in 5 mL minimal medium containing 15% glycerol. Aliquots of the 10x concentrated stock cultures were stored in cryovials at -20°C for a maximum of 4 months.

3.2.4.1.3. Methods: Assay procedure

Except for reading the plates, the YES bioassay was carried out in a Type II bio-hazardous safety cabinet using sterile techniques. Prior to the assay, 50 mL growth medium was inoculated with 125 µL of the 10x concentrated short-term yeast stock, and incubated overnight at 28°C in a rotating water bath (Grant OLS 200, Grant Instruments, Cambridge, UK) at 155 upm until turbid. An absorbance reading of at least 1 at 620 nm indicated sufficient yeast growth to continue with the assay. Serial dilutions of the water extracts, E_2 positive control and solvent control (EtOH) were prepared in 96-well plates. 200 µL of the extracts/controls were added to the first wells of a 96-well plate and 100 µL EtOH to wells 2-12. A 100 µL of the extracts/controls were sequentially transferred across the plate to create the serial dilutions. From the dilution plate, 10 µL volumes were transferred to triplicate 96-well assay plates and allowed to evaporate to dryness. Growth medium was prepared as described above and 0.5 mL CPRG and 0.5 mL of the 24-hour yeast culture were

BEST PFE.COM
List of research project topics and materials

© University of Pretoria

added per 50 mL of growth medium. 200 µL of the medium containing yeast and CPRG was dispensed into each well of the triplicate 96-well plates. The plates were sealed with autoclave tape and incubated for 3 to 5 days in a naturally ventilated incubator at 32°C (Scientific Series 2000 incubator from Lasec, South Africa). Each assay plate contained an E_2 standard curve ranging from 2.274 µg/L to 1.3 ng/L and at least one row with solvent control. On day 3-5, the absorbance was read on a Multiskan Spectrum 96-well plate reader (Thermo Fisher Scientific, Vantaa, Finland) at 540 nm (for colour) and 620 (for turbidity, as an indicator of yeast cell growth). Plates were read over 3 days to obtain data with the best contrast between the positive control and the solvent control and to allow for slower reacting chemicals. Figure 3.5 shows examples of dosing and reading of the plates and the bioassay procedure is summarised in Figure 3.6.

© University of Pretoria

Figure 3.5: An example of dosing and reading yeast screen bioassay plates

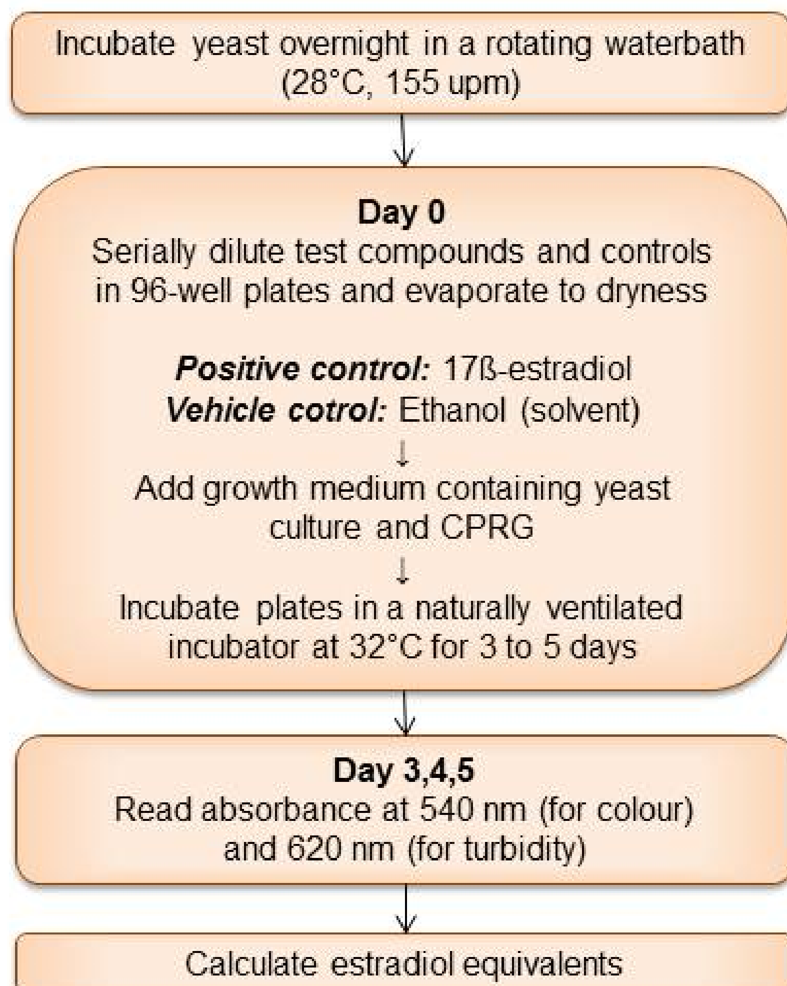


Figure 3.6: Procedural steps for the YES bioassay

© University of Pretoria

3.2.4.1.4. Methods: Calculation of results

The 620 nm turbidity readings were used to detect cytotoxicity. Samples were considered to be cytotoxic at concentrations where the 620 nm readings were below the average of the blank (at 620 nm) minus 3 times the standard deviation (SD).

Before the detection limit (dl) of the bioassay could be calculated, the 540 nm absorbance readings were corrected for turbidity with the following equation from Dhooge et al.[225]

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

Corrected absorbance values above the average of the blank plus 3 times the SD were considered above the dl of the bioassay. EEq could be calculated for samples with 3 or more points above the dl. EEq values were calculated using Graphpad Prism (version 4) software. The X-values (concentrations) were log transformed and the E_2 curve was fitted (sigmoidal function, variable slope). Sample concentrations were calculated from the E_2 standard curve as unpaired Y-values and were corrected for the appropriate dilution factors to determine the EEq value for the original sample. EEq values were reported as the average ± SD of three independent repeats.

3.2.4.2. T47D-KBluc reporter gene bioassay

The T47D-KBluc reporter gene bioassay was developed by the USEPA to screen environmental samples and chemicals for estrogenic and anti-estrogenic activities. T47D human breast cancer cells naturally express the hERα and human estrogen receptor beta (hERβ) and were transfected with an estrogen-responsive element (ERE) luciferase reporter gene construct. Substances that bind to and activate the ER (agonists) will result in the activation of the luciferase reporter gene and dose dependent production of the luciferase enzyme. The enzyme can be quantified by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. Anti-estrogenic substances (antagonists) will block E_2-induced luciferase expression.[224]

© University of Pretoria

3.2.4.2.1. Materials

The T47D-KBluc cells (cat. no. CRL-2865) were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). RPMI 1640 powder (cat. no. R8755), sodium bicarbonate ($NaHCO_3$, cat. no. S5761), glycylglycine (1 M, cat. no. G7278)), adenosine 5'-triphosphate (ATP, cat. no. A7699), bovine serum albumin (BSA, cat. no. A7906), magnesium chloride ($MgCl_2$) solution (1 M, cat. no. M1028) and E_2 (cat. no. E8875) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and D(+)-glucose (cat. no. 108342) and EtOH (HPLC grade, cat. no. 100983) from Merck (Darmstadt, Germany). 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer solution (1 M, cat. no. 15630-056), sodium pyruvate (100 mM, cat. no. 11360-039), antibiotic/antimycotic solution (cat. no. 15240-062), Hank's buffered salt solution (HBSS, 10x, cat. no. 14185-045), trypsin (0.5% EDTA, 10x, cat. no. 15400-054) phosphate buffered saline (PBS, 10x, cat. no. 14080-048) and recovery cell culture freezing media (cat. no. 12648010) was from Gibco (Life Technologies Corporation, Paisley, UK). Fetal bovine serum (FBS, characterized, cat. no. SH30071.03) and charcoal/dextran treated FBS (c/d FBS, cat. no. SH30068.03) were purchased from Hyclone Laboratories (Logan, Utah, USA). Reporter lysis buffer (5x, cat. no. PRE3971) and beetle luciferin (cat. no. PRE1603) were purchased from Promega (Madison, Wisconsin, USA) and ICI 182,780 (from here on referred to as ICI, cat. no. 1047) from Tocris biosciences (Ellisville, Missouri, USA). 25 cm^2 and 75 cm^2 tissue culture flasks (cat. no. 430372 and 430641 respectively), 96-well luminometer plates (cat. no. 3610) and 50 mL centrifuge tubes (cat. no. 430828) were purchased from Corning Incorporated (Corning, New York, USA) and cryovials (cat. no. 377267) from Thermo Fisher Scientific (Denmark). CoolCell freezing containers were from BioCision (Mill Valley, California, USA).

3.2.4.2.2. Methods: Preparation of medium and stock solutions and general cell culture procedures

RPMI medium was prepared by dissolving RPMI 1640 powder in 1 L ddH_2O and adding 2.5 g D(+)-glucose, 1.5 g $NaHCO_3$, 10 mL HEPES (1 M solution) and 10 mL sodium pyruvate (100 mM). The pH was adjusted to 7.3 and the medium was filter sterilized using a 0.22 µm bottle top filter. The medium was stored at 4°C. For maintenance medium, RPMI medium was supplemented with 10% FBS and 100 units/mL penicillin, 100 units/mL streptomycin and 0.25 µg/mL amphotericin B

© University of Pretoria

(antibiotic/antimycotic solution). Medium to withdraw the cells from steroids one week before the assay consisted of RPMI medium supplemented with 10% c/d FBS. Dosing medium consisted of RPMI medium with 5% c/d FBS. HBSS was prepared by diluting a 10x concentrated HBSS solution in sterile ddH_2O (sterilized by autoclaving). Trypsin was prepared from a 10x concentrated solution in HBSS. Concentrated PBS and lysis buffer were diluted in ddH_2O and all the solutions were stored at 4°C. Reaction buffer was prepared by adding 2 mL glycylglycine (1 M), 5 mL ATP (0.1 M), 1 mL BSA (50 g/L) and 1.5 mL $MgCl_2$ (1 M) to 90 mL ddH_2O. The pH of the solution was adjusted to 7.8 and the reaction buffer was stored in aliquots at 4°C. A 1 mM luciferin solution was prepared in ddH_2O and stored in aliquots at -80°C. E_2 (10 mM) and ICI (10 mM) stock solutions were prepared in EtOH and stored at -20°C.

All cell culture procedures were carried out in a Type II bio-hazardous safety cabinet using sterile techniques. Cells were grown in maintenance medium (RPMI medium with 10% FCS and antibiotic/antimycotic solution) in 25 cm^2 or 75 cm^2 tissue culture flasks in a water-jacketed incubator (Nuaire, Plymouth, Minnesota, USA) at 37°C and 5% CO_2. The cells were trypsinized and subcultured when confluent (3-4 day intervals). To tripsinize, cells were rinsed twice with 5-10 mL HBSS. The HBSS was discarded, 5 mL trypsin was added to each flask and the flasks were incubated for 2 min at 37°C. The trypsin was removed from the flasks and the cells detached from the surface by gently tapping the flask against the hand. Cells were resuspended in 10 mL maintenance medium and divided 1/3 into subcultures.

Cell stock cultures were kept at -80°C. Fresh stock cultures were prepared every 6-12 months. Stock cultures were thawed in a water bath at 37°C. The cells were transferred to a 25 cm^2 tissue culture flask with 10 mL maintenance medium and placed in an incubator (37°C, 5% CO_2). The medium was replaced with fresh medium after 24h and then at 3-4 day intervals until the cells were confluent. When confluent, the cells were tripsinized, transferred to a 75 cm^2 flask and subcultured as described above. To make new stock cultures, cells were trypsinized when confluent and resuspended in 10 mL maintenance medium. The cell suspension was transferred to sterile 50 mL centrifuge tubes and centrifuged at 15°C, 172 x g for 10 min. The medium was discarded and the pellet resuspended in 3 mL cell freezing

© University of Pretoria

medium. The cell suspension was aliquoted into cryovials and frozen at -80°C using a CoolCel freezing container to keep the cooling rate at 1°C/min for optimal cell recovery.

3.2.4.2.3. Methods: Assay procedure

One week prior to the assay, cells were trypsinized and resuspended and grown in RPMI medium supplemented with 10% c/d FBS to withdraw the cells from steroids. The medium was replaced with fresh steroid-free medium after 3-4 days. Cells were seeded in 96-well luminometer plates (5 x 10^4 cells/well) in dosing medium (RPMI medium with 5% c/d FBS) and allowed to attach overnight. A dilution series of the samples and controls were prepared in EtOH and 2 µL of the concentrations in EtOH was added to 1 000 µL dosing medium. The solvent (EtOH) never exceeded 0.2% in the final dosing solutions. The medium in the 96-well luminometer plates was replaced with 100 µL of the dosing solutions. In addition to the samples, each plate contained an E_2 positive control standard curve (ranging from 0.1 nM to 0.3 pM), solvent control (0.2 % EtOH), antagonist control (10 nM ICI plus 0.1 nM E_2) and background control (10 nM ICI). Each sample was tested alone, as well as in the presence of E_2 (0.1 nM) and ICI (10 nM ICI) respectively. An ICI standard curve (ranging from 10 nM to 10 pM), co-incubated with 0.1 nM E_2 was used to determine the anti-estrogenic activity of antagonists. The exposed plates were returned to the incubator (37°C, 5% CO_2) for 24 hours. After 24 hours incubation the plates were assessed under a microscope for any signs of cytotoxicity (e.g. condensed cell contents or "weathered" cells) before the dosing solutions were removed by gently shaking the plates over a waste tray (from this point forward aseptic conditions were not necessary). The cells were washed with 200 µL PBS, at room temperature. The PBS was discarded and 25 µL lysis buffer was added to each well. The plates were placed in a -80°C freezer overnight, as the lysis buffer is activated by one freeze/thaw cycle. The plates were thawed on a Thermostar plate warmer/shaker (BMG Labtech, Offenburg, Germany) and all reagents were allowed to reach room temperature before the luciferase activity was determined using a LUMIstar OPTIMA luminometer (BMG Labtech, Offenburg, Germany) with two dispensers. The luminometer was programmed to inject 25 µL reaction buffer, followed by 25 µL luciferin 5 seconds later, into each well. Luciferase activity is quantified as relative light units (RLU). The bioassay procedure is summarised in Figure 3.7.

© University of Pretoria

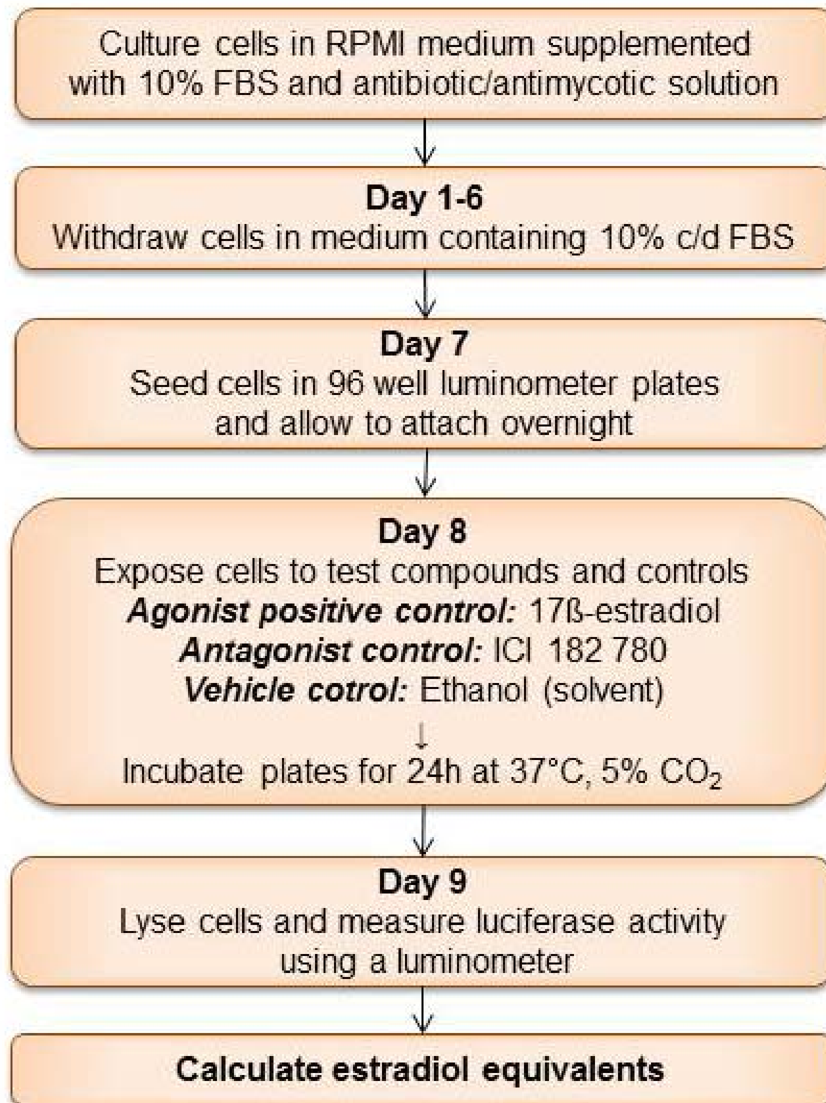


Figure 3.7: Procedural steps for the T47D-KBluc reporter gene bioassay

© University of Pretoria

3.2.4.2.4. Methods: Calculation of results

The RLU readings were converted to fold induction relative to the solvent control (RLU/average RLU reading of solvent control). The fold induction for 0.1 nM E_2 should be at least 6 and 10 nM ICI should inhibit the activity of 0.1 nM E_2. Samples are considered to be estrogenic if it induces dose dependent luciferase activity, which could be specifically inhibited by the anti-estrogen ICI.[224]

EEq values were calculated for samples with at least a twofold induction above the solvent control using Graphpad Prism (version 4) software. Fold inductions were expressed as a percentage of the maximum (0.1 nM E_2) response. The X-values (concentrations) were log transformed and the E_2 curve was fitted (sigmoidal function, variable slope). Sample concentrations were calculated from the E_2 standard curve as unpaired Y-values and were corrected for the appropriate dilution factors to determine the EEq value for the original sample. For anti-estrogenic samples the ICI curve (co-incubated with 0.1 nM E_2) was fitted to quantify anti-estrogenic activity. EEq values were reported as the average ± SD of three independent repeats.

3.2.5. Target chemical analyses

UPLC-MS was used for the target chemical analysis. The analysis was done at the Central Analytical Facility (CAF) laboratory at the University of Stellenbosch.

3.2.5.1. Materials

Acetone (cat. no. 34850), dichloromethane (DCM, cat. no. 34856), E_2 (cat. no. 75263), EE_2 (cat. no. E4876), E_1 (cat. no. E9750), DEHA (cat. no. 525197), DEHP (cat. no. 36735), DBP (cat. no. 36736), NP (4-nonylphenol, cat. no. 46405), BPA (cat. no. 239658) and dansyl chloride (cat. no. 39220) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Deuterated BPA (dBPA, cat. no. DLM-1839-0.1) was purchased from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, USA). MeOH (cat. no. H409) and acetonitrile (cat. no. H048) was from Romil (Cambridge, UK) and EtOH (HPLC grade, cat. no. 100983), sodium carbonate (Na_2CO_3, cat. no. 582200) and Contrad detergent were from Merck (Darmstadt,

© University of Pretoria

Germany). Crimp vials (cat. no. C4012-1) were purchased from National Scientific (Rockwood, Tennessee, USA).

3.2.5.2. Methods: Preparation of stock solutions

Glass bottles used for stock solutions were washed using Contrad soap, rinsed and pyrolysed (565°C for 2 hours) to prepare the bottles. Stock solutions of 10 mg/mL were prepared for all the standards. NP, BPA, dBPA (internal standard), E_2 and EE_2 were prepared in MeOH, E_1 in acetone and DEHA, DEHP and DBP in DCM. From the stock solutions a concentration range was prepared in crimp vials ranging from 250 µg/L to 5 ng/L for NP, BPA, E_1, E_2 and EE_2 and from 1 mg/L to 100 µg/L for DEHA, DEHP and DBP. For each concentration, 1 mL was transferred to a new vial and evaporated under a stream of nitrogen at 40°C.

3.2.5.3. Methods: Sample preparation and derivatization

The dried extract samples and standards (except for the DEHA and phthalate standards) were resuspended in 100 µL Na_2CO_3 (1M in H_2O). In order to improve the analytical capability, 100 µL of the derivatizing agent, dansyl chloride (2 mg/mL in acetone), was added to each vial. The vials were capped and vortexed. The samples and standards were derivatized for 2 minutes at 60°C (a colour change from yellow to colourless could be observed) and analysed using UPLC-MS.

The DEHA and phthalate standards were resuspended in 200 µL acetonitrile and were injected as is, without further preparation. Extraction controls as well as solvent controls (for all the chemicals used in the extraction procedure as well as UPLC-MS analysis) were included for quality control purposes.

© University of Pretoria

3.2.5.4. Methods: UPLC-MS analysis

Instrument and method details for UPLC-MS analyses are summarised in Table 3.3 below.

Table 3.3: Instrument and method for UPLC-MS analysis

Instrument	Waters Xevo TQ MS
Sample introduction	Waters Acquity UPLC (5 µL injection)
Solvent A	7.5% formic acid
Solvent B	Acetonitrile: MeOH: isopropanol (49:49:2)
Source	ESCI+
MS settings	
Capillary voltage	2.8 kV
Source	120°C
Desolvation temperature	400°C
Desolvation gas	600 L/h
Cone gas	60 L/h
Column	Waters BEH C18, 2.1 x 100 mm, @ 50°C, injection volume 3 µL

3.3. Phase 2 - Bottled water

3.3.1. Sample collection

A survey was done to determine which brands of still water in plastic bottles are locally available in major supermarkets in Pretoria (as representative of South Africa). Local supermarkets were visited and a list of the available brands was compiled. Information regarding the water source (e.g. natural spring, prepared water, etc.), area of origin and purification method was recorded. Based on the survey, ten of the most commonly found brands were identified for testing and included brands from different sources, areas and purification methods. Only South African brands of bottled water were considered for the study. The water source, source location, additional treatments and other information (as it appeared on the label of the bottle) for the selected brands are given in Table 3.4.

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

Table 3.4: Source, treatment and additional information of the selected brands of bottled water for Phase 2

Sample code	**BTW01**	**BTW02**	**BTW03**	**BTW04**	**BTW05**	**BTW06**	**BTW07**	**BTW08**	**BTW09**	**BTW10**
Water source	Spring water	Spring water	Mountain spring water from a quartzite aquifer	Prepared water from public or private distribution system	Mineral water	Spring water	Mineral water from quartz rock	Underground spring water	Spring water	Mineral water from deep dolomite lakes
Source location	Waveside Lagerspoort, Heidelberg	Highland Mist Farm, Waterval Boven	R102, Witelsbos, Humansdorp District	-	The Midlands	Pendio	Portion 159, Farm Houtpoort, Heidelberg, Gauteng	Ekhamanzi Springs, Silverstream Farm - Kranskop, KZN	Erf 1038/7, Wemmershoek, Franschhoek Valley	Plot 723, Doornkloof East, Gauteng
Additional treatment	Filtered and UV treated	-	-	Reverse osmosis and ozonation	-	-	-	-	Filtered	UV treatment
Additional info on product label	Bottled at source, up to 30% of the bottle is made from plants	Bottled at source, contains no *E-coli* or coliforms at source	Bottled at source	Minerals added: magnesium sulphate, potassium chloride and sodium chloride	Bottled at source	Bottled at source, BPA free	Bottled at source	Bottled at source	Bottled at source	Bottled at source
Batch number	B142	-	2-13333	134713	-	14106	079 14 11 39	28E4.4	22128	-
Expiry date	21/05/2015	-	29/11/2015	12/05/2015	20/06/2015	16/04/2016	20/03/2015	28/05/2015	08/05/2015	15/04/2016
Recycle code	1 PET	-	1 PET	1 PET	1 PET	1 PET	Bottle 1 PET, cap 2 HDPE	1 PET	1 PET	Bottle 1 PET, cap 2 HDPE
SANBWA	Yes	-	-	Yes	-	Yes	-	Yes	-	Yes
SABS approved	Yes	-	-	Yes	-	-	Yes	-	-	-

- Information not given on the bottle

Notes: Brand names are given in Appendix A

Since this project was conducted some of the bottled water suppliers changed their bottles and/or water source

© University of Pretoria

3.3.2. Sample preparation

For each brand of bottled water, 16 x 500 mL bottles from the same batch were purchased to test four different storage conditions. Four bottles from each brand were stored for ten days at 20°C or 40°C, in the dark or under lights at each temperature. The incubation period of 10 days at 40°C is in accordance with the standard European Economic Commission (EEC) migration test (82/711/EEC).[127] The samples were incubated in a Labcon low temperature incubator (Labcon Laboratory Equipment, Gauteng, South Africa), fitted with Gro-lux T8 F15W lights (Sylvania, Germany) to simulate sunlight conditions (Figure 3.8). The incubator was fitted with a minimum/maximum thermometer to monitor temperature fluctuations during the incubation periods. For the 20°C incubation period, the temperature fluctuated between 19.6 and 20.4°C and for the 40°C incubation period between 39.4 and 40.3°C.

© University of Pretoria

Figure 3.8: Incubator setup for the exposure of bottled water samples to simulated sunlight

© University of Pretoria

3.3.3. Extraction procedure

For each brand and storage condition two 500 mL bottles were extracted in duplicate, one set for bioassay analysis and the duplicate set for target chemical analysis. The water extraction procedure was done according to the method described for phase 1.

3.3.4. Bioassays for estrogenic activity

The bioassays for estrogenic activity were done according to the methods described for phase 1.

3.3.5. Target chemical analyses

Target chemical analyses were done according to the methods described for phase 1.

3.4. Phase 3 - Health Risk Assessment

A Human Health Risk Assessment was conducted in collaboration with the Council for Scientific and Industrial Research (CSIR) - Natural Resources and the Environment, Stellenbosch, to assess the potential human health impacts of the chemicals found in the tested water samples. The assessment followed the methodology outlined by the USEPA[226,227] and the WHO,[228] as described by Genthe and Steyn.[229] Health risk assessment was based on adult exposure and children and vulnerable populations were not considered for the scope of this study.

The bioassay results were compared to the trigger value of 0.7 ng/L for estrogenic activity in drinking water.[98] The trigger value was based on the acceptable daily intake (ADI) of 200 pg/kg/day (as opposed to 50 ng/kg/d suggested by the WHO[230]) to compensate for sensitive subpopulations, individual variation, percentage availability and a safety factor of 1000. If the trigger value is exceeded, possible adverse health effects are implicated and necessitate further investigation and testing of the water.[218]

The concentrations of the identified target chemicals were used to calculate the total dose a person may be exposed to. The computer programme Risk*Assistant was used for the calculations of potential exposure concentrations.[231]

© University of Pretoria

The following calculation was used to determine human exposure to the identified substances on a daily basis:

$ADD = (C_{medium} \times IR) / BW$

where:
ADD is the average daily dose (mg/kg/day)
C_{medium} is the concentration of the substance in the water (mg/kg)
IR is the ingestion rate (L/day)
BW is the body weight (kg)

For toxic chemicals a HQ was calculated by comparing the estimated exposure to a RfD assumed to be safe,[220] using the following formula:

$HQ = ADD/RfD$

A HQ less than 1 is considered to be safe for a lifetime exposure.

For carcinogenic chemicals for exposures that last less than a lifetime, the lifetime average daily dose (LADD) was calculated as:

$LADD = ADD \times ED/Lft$

where:
ED is the exposure duration (years)
Lft is lifetime (years)

The excess cancer risk was calculated as a function of oral potency factor (β) as reported by the USEPA[220] and dose:
$Risk = \beta \times LADD$

© University of Pretoria

The WHO acceptable risk level is 10^{-5}, meaning that the excess risk of developing cancer is deemed acceptable if ingestion of the substance results in one additional cancer case per hundred thousand of the population or less.

Uncertainty analysis was carried out through a Monte Carlo simulation to model the probabilistic outcome of the risks associated with the consumption of water from Pretoria and Cape Town distribution points and bottled water. This was carried out using the Excel add-in, @ Risk (Palisade). Distributions were fitted to the data (the data typically fitted an exponential distribution) and 10 000 iterations were run. The outputs of the simulations provide the values where the risk of developing cancer or HQ is expected to occur 90% of the time as well as illustrating the expected or predicted average, minimum and maximum values.

3.5. Ethical considerations

The project only entailed laboratory experiments. Water samples were analysed for estrogenic activity using the recombinant yeast screen bioassay and the T47D-KBluc reporter gene bioassay and target chemical analyses were done using UPLC-MS. No samples or information were obtained from patients or other people and no experimental animals were used. Ethical approval for the project was obtained from the Faculty of Health Sciences' Ethic committee, reference number 48/2013.

© University of Pretoria

Chapter 4: Pilot study

4.1. Assessment of the target chemicals in the YES and T47D-KBluc bioassays

The target chemicals were assessed in the YES and T47D-KBluc bioassays. DINP was not one of the initially selected target chemicals for this project, but was included after it was detected in many of the samples. BPA, NP, DBP, E_2, E_1 and EE_2 showed estrogenic activity in the YES and T47D-KBluc bioassays and DEHP only in the T47D-KBluc bioassay. DEHA and DINP did not show estrogenic activity at the tested concentrations and none of the chemicals showed anti-estrogenic activity. All the target chemicals showed estrogenic responses at much lower concentrations in the T47D-KBluc bioassay compared to the YES bioassay, making the T47D-KBluc bioassay a much more sensitive bioassay. BPA, NP, DBP and DEHP were cytotoxic at higher concentrations. Graphs of the estrogenic responses of the target chemicals in the YES and T47D-KBluc bioassays are shown in Figure 4.1 and Figure 4.2 respectively.

© University of Pretoria

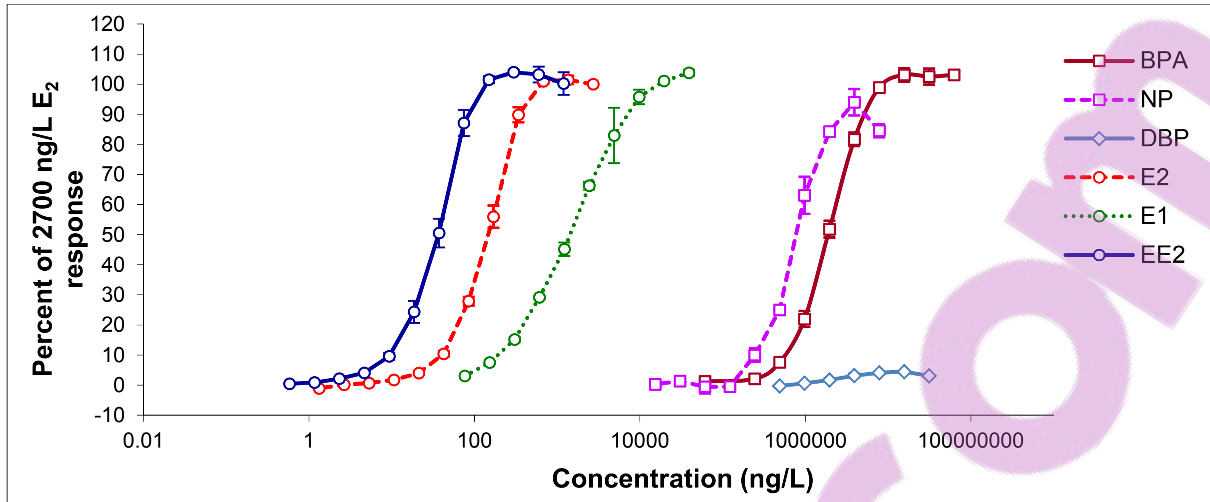


Figure 4.1: Estrogenic response of target chemicals in the YES bioassay. Data points represent the average ± SD (n=3)

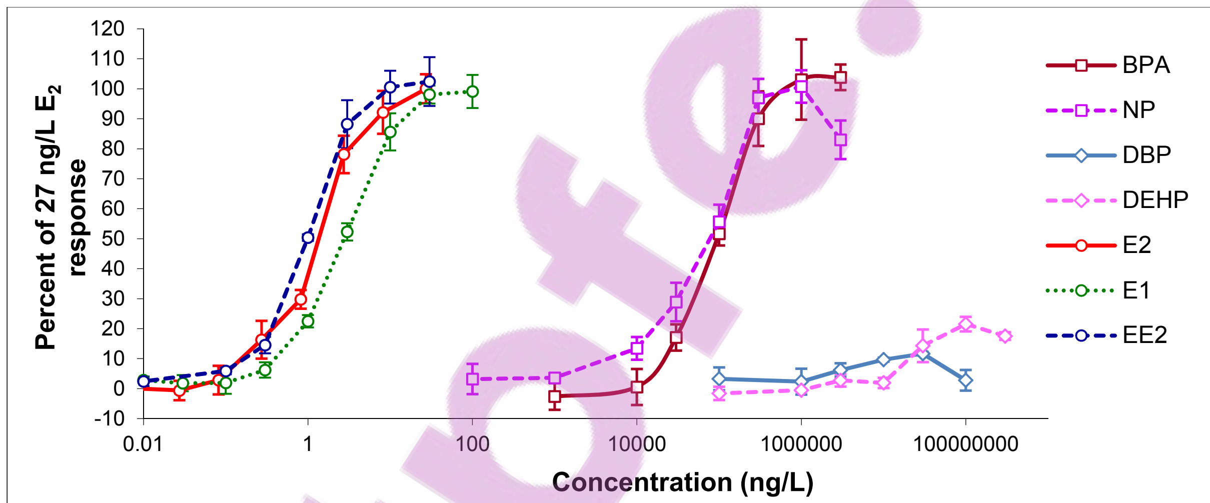


Figure 4.2: Estrogenic response of target chemicals in the T47D-KBluc bioassay. Data points represent the average ± SD (n=3)

© University of Pretoria

The relative potencies of the target chemicals are given in Table 4.1.

Table 4.1: Relative potencies of target chemicals

Target chemical	Relative Potency	
	YES	T47D-KBluc
BPA	7.42E-05	1.39E-05
NP	2.10E-04	1.93E-05
DEHA	-	-
DBP	7.49E-05	3.07E-07
DEHP	-	6.12E-08
DINP	-	-
E_2	1	1
E_1	0.09	0.46
EE_2	4.02	1.26

Relative potency = $EC50_{E2}/EC50_{chemical}$

- No estrogenic activity at the tested concentrations

DBP and DEHP were not able to reach the maximum estrogenic response obtained by E_2. DBP only reached 5% of the maximum E_2 response in the YES and 12% in the T47D-KBluc bioassay. DEHP reached 21% of the maximum E_2 response before cytotoxicity was observed. Chemicals do not always react the same in different *in vitro* bioassays. Although it was not observed in this study, DEHP showed anti-estrogenic activity in MVLN cells.[188] A different study reported weak estrogenic activity for DINP in a YES screen.[211] Similar to this study, weak estrogenic activity was observed for DBP in MVLN[188] and CV-1 cells.[189] The relative potencies obtained in this study are similar to relative potencies reported in the ERα CALUX bioassay (EE_2: 1.86; BPA: 2.5E-05; NP: 4.6E-05), except for E_1 (0.02) that showed a higher relative potency in the T47D-KBluc cells.[105]

4.2. Validation of extraction method

In order to evaluate the suitability of the extraction method recommended by Oasis[221], it was compared to the method described in the WRC toolbox[222] and a method used by Leusch et al.[210] The three different extraction methods are compared in Table 4.2.

© University of Pretoria

Table 4.2: Comparison of extraction methods

Extraction method	Oasis[221]	WRC[222]	Leusch et al.[210]
Condition cartridge	5 mL 10% MeOH in MtBE 3 mL MeOH	5 mL ddH_2O 5 mL MeOH	5 mL acetone:hexane (1:1) 5 mL MeOH
Equilibrate cartridge	3 mL ddH_2O	5 mL ddH_2O	5 mL ddH_2O
Load cartridge	1 L sample	1 L sample	1 L sample
Wash cartridge	3 mL 5 % MeOH in ddH_2O	No	No
Elute cartridge	6 mL 10% MeOH in MtBE	3-5 mL MeOH	5 mL MeOH 5 mL acetone:hexane (1:1)

Samples were prepared by spiking 1 L of ddH_2O with 0; 2 or 20 ng/L E_2. The three extraction methods were carried out using glass and disposable (plastic) pipettes for comparison. The re-use of glass pipettes could result in the contamination of samples if the pipettes are not cleaned properly and disposable pipettes might contaminate samples if plastic constituents leach from the pipettes into the samples.

Extracts were analysed for estrogenic activity using the YES and T47D-KBluc reporter gene bioassay and were also analysed for the target chemicals using UPLC-MS. The results obtained in the bioassays and the UPLC-MS results for E_2 and BPA are summarised in Table 4.3. Low levels of BPA were detected in all of the samples, but this was attributed to contamination of one of the solvents used in the preparation of the samples, as the solvent and extraction controls also tested positive for BPA. NP, DEHA, DBP, DEHP, E_1 and EE_2 were not detected.

The low recovery seen for E_2 using UPLC-MS could be explained by the fact that the E_2 used to spike the samples (Sigma cat. no. E8875), were not from the same supplier as the E_2 (Fluka cat. no. 75262) used for the calibration curves for the analysis of the samples. Beresford et al.[232] also reported different results when testing the same chemical from different batches and suppliers (when testing estradiol-3-sulfate in the YES bioassay). They've suggested that the different results could be ascribed to a difference in the purity or composition of the chemicals and that storage conditions could also have had an influence.

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 4.3: Estrogenic activity and E_2 and BPA concentrations of spiked water samples using different extraction methods

Sample	YES EEq (ng/L)	T47D-KBluc EEq (ng/L)	UPLC-MS	
			E_2 (ng/L)	BPA (ng/L)
Oasis method				
0 ng/L E_2, glass pipette	<dl	<dl	<dl	21.4
0 ng/L E_2, disposable pipette	<dl	<dl	<dl	6.0
2 ng/L E_2, glass pipette	2.4 ± 0.3	2.0 ± 0.4	0.9	2.5
2 ng/L E_2, disposable pipette	2.3 ± 0.4	2.1 ± 0.4	1.3	4.6
20 ng/L E_2, glass pipette	24.8 ± 1.5	19.0 ± 0.8	12.0	5.6
20 ng/L E_2, disposable pipette	19.4 ± 2.0	19.2 ± 2.7	12.6	5.1
WRC method				
0 ng/L E_2, glass pipette	<dl	<dl	<dl	5.2
0 ng/L E_2, disposable pipette	<dl	<dl	<dl	3.7
2 ng/L E_2, glass pipette	1.7 ± 0.4	2.1 ± 0.9	0.8	1.2
2 ng/L E_2, disposable pipette	2.0 ± 1.1	2.8 ± 1.3	1.0	6.0
20 ng/L E_2, glass pipette	13.7 ± 1.3	5.9 ± 2.9	6.7	3.4
20 ng/L E_2, disposable pipette	22.8 ± 4.6	6.4 ± 2.7	No sample	No sample
GWRC method				
0 ng/L E_2, glass pipette	<dl	<dl	<dl	3.1
0 ng/L E_2, disposable pipette	<dl	<dl	<dl	1.9
2 ng/L E_2, glass pipette	1.7 ± 0.2	1.1 ± 0.02	0.9	1.7
2 ng/L E_2, disposable pipette	1.0 ± 0.6	2.7 ± 1.3	0.7	5.5
20 ng/L E_2, glass pipette	20.7 ± 0.5	15.8 ± 9.0	11.8	8.0
20 ng/L E_2, disposable pipette	16.2 ± 2.0	6.9 ± 3.5	No sample	No sample
Controls				
Solvent control	<dl	<dl	<dl	2.9

<dl Below the detection limit of the assay

Oasis HLB cartridges are efficient to retain a wide range of structurally diverse pollutants, as the cartridges exhibit both hydrophilic and lipophilic retention characteristics.[233] The results confirmed that the Oasis extraction method was suitable for the purposes of this project. No clear evidence of leaching from the disposable pipettes could be seen. It was therefore decided to use new glass pipettes for the solvents (one pipette dedicated to each solvent) and disposable pipettes for loading the samples onto the cartridges.

© University of Pretoria

4.3. Recoveries of target chemicals using UPLC-MS

In order to determine the recoveries of the target chemicals after the extraction process, triplicate 1L ddH_2O samples were spiked with a standard cocktail containing all the target chemicals. The final concentration of each target chemical was 200 ng/L. The spiked and unspiked (control) samples were extracted and analysed for the target chemicals using UPLC-MS. The recoveries are tabulated in Table 4.4.

Table 4.4: Recoveries of target chemicals

Target chemical	% Recovery
BPA	104
NP	64
DEHA	163
DBP	167
DEHP	102
E_2	88
E_1	84
EE_2	89

Recoveries above 100% indicate background levels of the analytes in laboratory water or solvents used for UPLC-MS. Background levels of DBP and DEHA were especially high. However DBP was not detected in any of the extraction control samples or solvents. DEHA was not detected in any of the solvents but was detected in one of the nine extraction control samples.

© University of Pretoria

5.2. Phase 1 – Distribution point water

5.2.1. Bioassays for estrogenic activity

None of the samples from the distribution points were above the dl in the YES bioassay. The results obtained with the T47D-KBluc bioassay are tabulated in Table 5.1 (Pretoria) and Table 5.2 (Cape Town).

Table 5.1: Estrogenic activity of water extracts collected from selected distribution points in Pretoria using the T47D-KBluc bioassay

Sample	EEq (ng/L)			
	October 2013	**January 2014**	**April 2014**	**July 2014**
PTA01	0.089 ± 0.006	0.015 ± 0.003	0.074 ± 0.003	0.058 ± 0.006
PTA02	<dl	<dl	<dl	0.023 ± 0.005
PTA03	0.061 ± 0.009	<dl	<dl	0.077 ± 0.031
PTA04	<dl	<dl	<dl	<dl
PTA05	<dl	<dl	<dl	0.024 ± 0.006
PTA06	0.032 ± 0.004	0.015 ± 0.005	<dl	0.077 ± 0.025
PTA07	<dl	<dl	<dl	<dl
PTA08	<dl	<dl	<dl	<dl
PTA09	0.013 ± 0.001	<dl	<dl	<dl
PTA10	<dl	<dl	<dl	<dl

<dl Below detection limit of the assay

Table 5.2: Estrogenic activity of water extracts collected from selected distribution points in Cape Town using the T47D-KBluc bioassay

Sample	EEq (ng/L)			
	October 2013	**January 2014**	**April 2014**	**July 2014**
CPT01	<dl	<dl	<dl	<dl
CPT02	<dl	<dl	<dl	<dl
CPT03	<dl	<dl	<dl	<dl
CPT04	0.004 ± 0.0003	0.004 ± 0.001	0.003 ± 0.0003	0.005 ± 0.001
CPT05	0.002 ± 0.001	<dl	<dl	0.003 ± 0.001
CPT06	0.004 ± 0.001	0.003 ± 0.0003	0.004 ± 0.0004	0.002 ± 0.0004
CPT07	0.004 ± 0.001	<dl	0.003 ± 0.001	0.041 ± 0.014
CPT08	0.006 ± 0.001	0.005 ± 0.002	0.004 ± 0.001	0.026 ± 0.004
CPT09	<dl	<dl	0.044 ± 0.015	0.020 ± 0.001
CPT10	0.005 ± 0.001	<dl	<dl	0.114 ± 0.044

<dl Below detection limit of the assay

© University of Pretoria

Six of the distribution points in Pretoria and seven of the distribution points in Cape Town showed estrogenic activity in the T47D-KBluc bioassay for at least one sampling period. Estrogenic activity was detected in all four sampling periods in PTA01, CPT04, CPT06 and CPT08. The sampling period with the highest number of positive samples was July 2014 (winter) for Pretoria and Cape Town. The EEq values ranged from below the dl to 0.089 ng/L in Pretoria and from below the dl to 0.114 ng/L in Cape Town. More samples tested positive for estrogenic activity in the Cape Town distribution points (53%) compared to the Pretoria distribution points (30%), but in general the Pretoria samples had higher estrogenic activities (average EEq = 0.014 ng/L) compared to Cape Town samples (average EEq = 0.008 ng/L). None of the samples showed anti-estrogenic activity.

5.2.2. Target chemical analyses

Although DINP was not one of the selected target chemicals, it was detected in many of the samples during UPLC-MS analysis and it was therefore decided to include DINP in the project.

The dl and quantification limit (ql) obtained for each target chemical using the UPLC-MS method are summarised in Table 5.3. Due to the fact that the samples were concentrated a 1000 times, target chemicals could be detected at concentrations a 1000 times lower than the dl. For example, for BPA the dl is 0.5 ng/L and ql is 5 ng/L, but because the samples were concentrated 1000 times, BPA could be detected at 0.0005 ng/L and quantified from 0.005 ng/L.

© University of Pretoria

Table 5.3: Detection limits (dl) and quantification limits (ql) for the target chemicals using UPLC-MS

Target chemical	dl (ng/L)	ql (ng/L)
BPA	0.5	5
NP	0.5	5
DEHA	800	1 000
DBP	10 000	40 000
DEHP	10 000	40 000
DINP	800	1 000
E_2	0.5	5
E_1	5	10
EE_2	0.5	5

© University of Pretoria

5.2.2.1. Bisphenol A (BPA)

The chromatogram for the BPA standard at 10 µg/L is shown in Figure 5.1 and the BPA calibration curve in Figure 5.2.

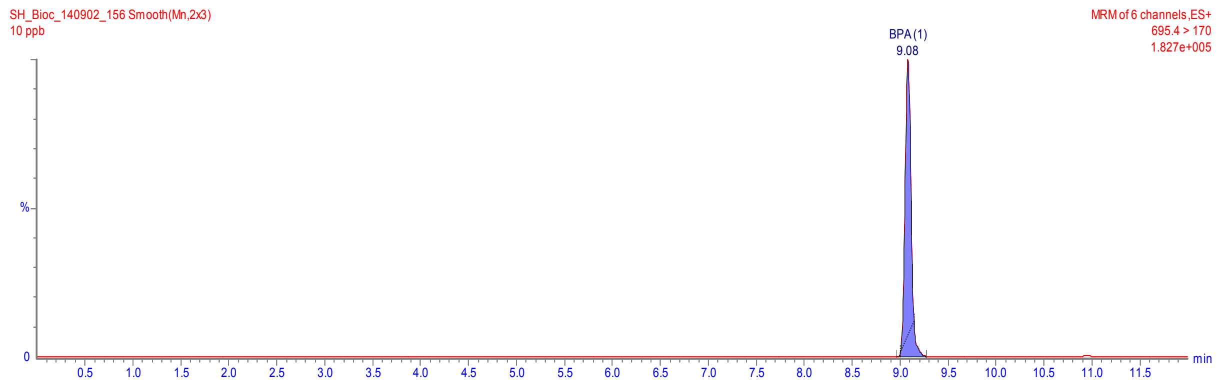


Figure 5.1: Chromatogram of BPA standard (10 µg/L)

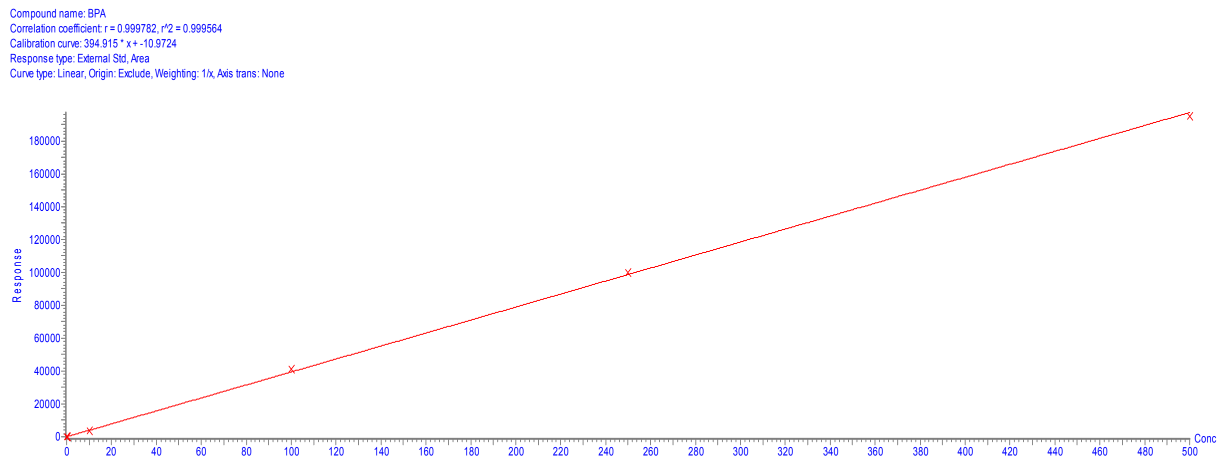


Figure 5.2: BPA calibration curve

© University of Pretoria

BPA was detected in most of the distribution point water samples from Pretoria (Table 5.4) and Cape Town (Table 5.5). The highest BPA concentration was measured in CPT05 in July 2014 (28.83 ng/L).

Table 5.4: BPA concentrations in water extracts collected from selected distribution points in Pretoria

Sample	BPA (ng/L)			
	October 2013	January 2014	April 2014	July 2014
PTA01	0.06	1.14	0.17	0.39
PTA02	0.60	<dl	<dl	0.74
PTA03	4.24	5.74	4.46	0.73
PTA04	0.01	<dl	0.07	7.80
PTA05	0.16	0.25	0.34	9.88
PTA06	0.34	<dl	0.22	1.99
PTA07	0.67	0.44	0.19	0.39
PTA08	1.81	0.33	1.41	0.79
PTA09	0.44	0.68	0.05	0.24
PTA10	0.30	0.37	0.23	0.10

<dl Below detection limit of the assay

Table 5.5: BPA concentrations in water extracts collected from selected distribution points in Cape Town

Sample	BPA (ng/L)			
	October 2013	January 2014	April 2014	July 2014
CPT01	0.12	0.16	0.16	0.25
CPT02	0.20	0.11	0.18	0.18
CPT03	4.27	1.64	1.94	0.11
CPT04	0.24	1.08	0.17	2.62
CPT05	0.36	0.26	0.28	28.83
CPT06	0.38	0.10	0.12	0.43
CPT07	0.22	1.34	0.19	<dl
CPT08	0.41	0.50	0.17	0.56
CPT09	<dl	0.04	3.47	<dl
CPT10	0.15	0.42	0.71	1.16

<dl Below detection limit of the assay

© University of Pretoria

5.2.2.2. Nonylphenol (NP)

The chromatogram for the NP standard at 500 ng/L is shown in Figure 5.3 and the calibration curve in Figure 5.4. NP was below the dl in all the samples collected from distribution points in Pretoria and Cape Town.

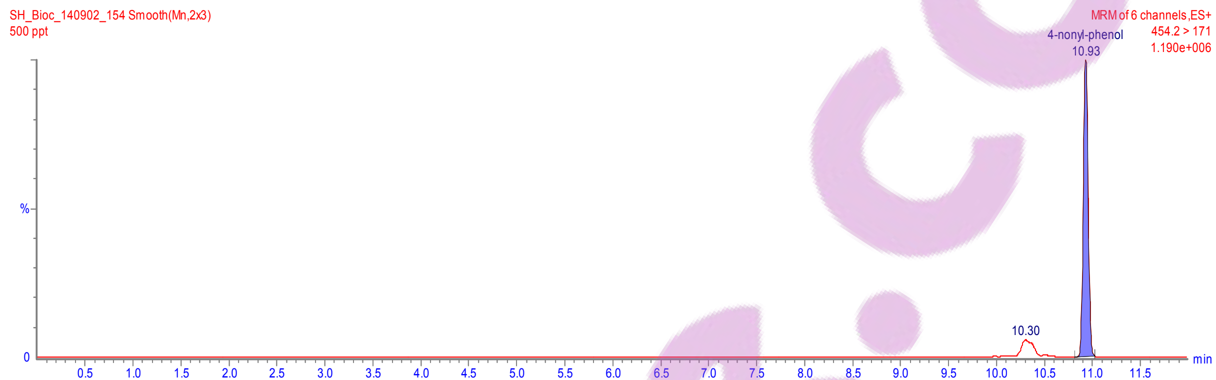


Figure 5.3: Chromatogram of NP standard (500 ng/L)

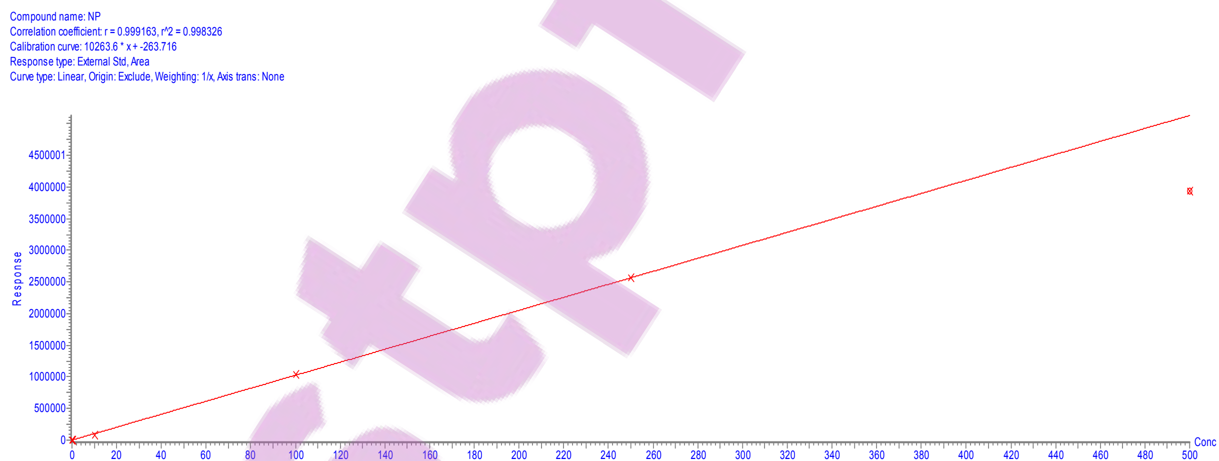


Figure 5.4: NP calibration curve

© University of Pretoria

5.2.2.3. Di(2-ethylhexyl) adipate (DEHA)

The chromatogram for the DEHA standard at 300 µg/L is shown in Figure 5.5 and the calibration curve in Figure 5.6.

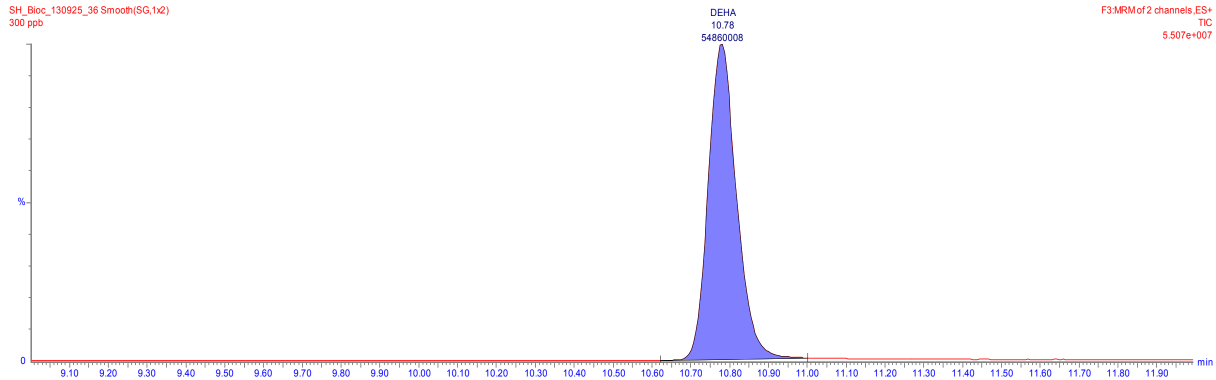


Figure 5.5: Chromatogram of DEHA standard (300 µg/L)

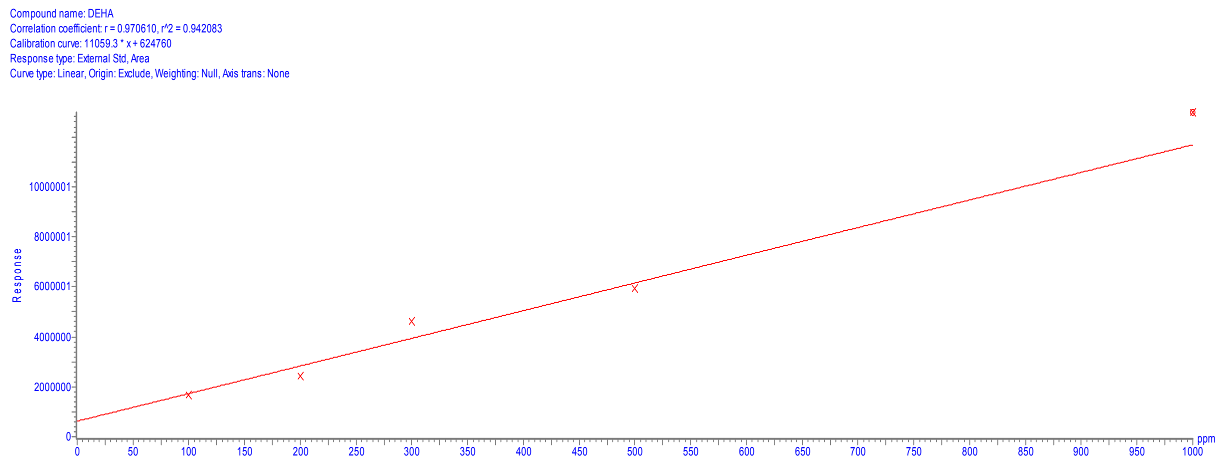


Figure 5.6: DEHA calibration curve

© University of Pretoria

DEHA was detected in all the distribution point samples and ranged from 1.07 to 4.60 ng/L in Pretoria samples (Table 5.6) and from below the quantification limit to 4.97 ng/L in the samples from Cape Town (Table 5.7). No seasonal variations in the levels of DEHA could be observed.

Table 5.6: DEHA concentrations in water extracts collected from selected distribution points in Pretoria

Sample	DEHA (ng/L)			
	October 2013	January 2014	April 2014	July 2014
PTA01	2.66	3.95	2.37	1.64
PTA02	3.15	2.97	2.36	2.21
PTA03	2.96	3.60	3.10	3.12
PTA04	1.95	3.15	2.83	2.56
PTA05	1.38	1.07	2.87	2.55
PTA06	3.65	3.94	4.60	2.66
PTA07	3.12	3.26	3.82	2.61
PTA08	3.05	1.79	2.03	2.19
PTA09	4.09	2.51	1.12	3.01
PTA10	3.28	3.77	2.27	1.24

Table 5.7: DEHA concentrations in water extracts collected from selected distribution points in Cape Town

Sample	DEHA (ng/L)			
	October 2013	January 2014	April 2014	July 2014
CPT01	3.25	4.36	3.97	4.12
CPT02	3.38	2.59	2.28	2.32
CPT03	2.82	3.31	2.64	1.41
CPT04	1.40	4.23	2.23	3.51
CPT05	4.18	4.06	3.59	2.00
CPT06	3.14	4.13	3.02	3.44
CPT07	<ql	4.61	2.62	2.56
CPT08	4.53	4.97	2.97	2.60
CPT09	1.76	3.83	4.70	3.29
CPT10	3.44	3.77	4.87	4.21

<ql Below quantification limit of the assay

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

5.2.2.4. Dibutyl phthalate (DBP)

The chromatogram for the DBP standard at 300 µg/L is shown in Figure 5.7 and the calibration curve in Figure 5.8.

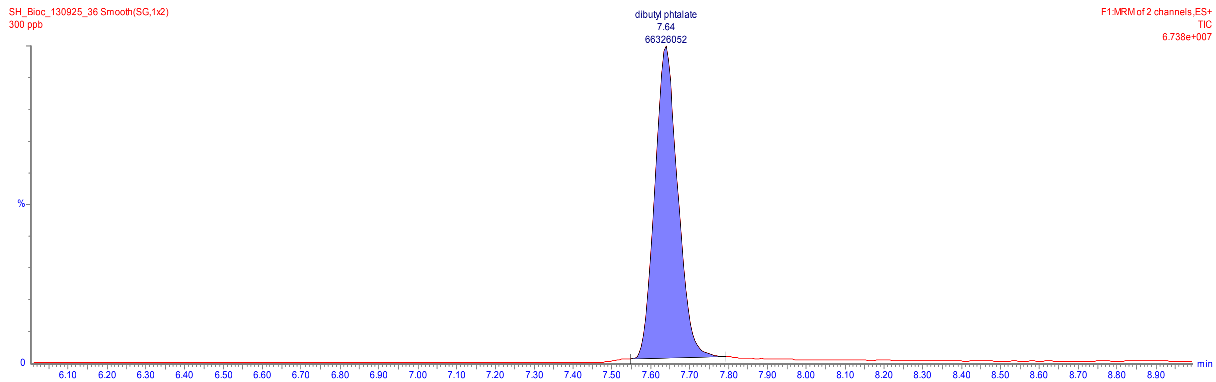


Figure 5.7: Chromatogram of DBP standard (300 µg/L)

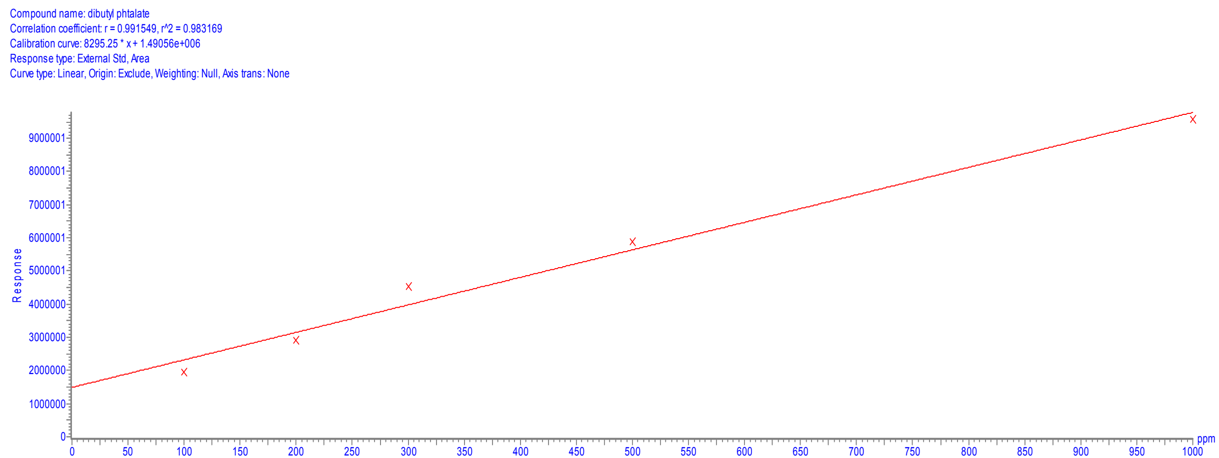


Figure 5.8: DBP calibration curve

© University of Pretoria

DBP was detected in 88% of the Pretoria distribution point samples (Table 5.8) and in all the Cape Town samples (Table 5.9). The concentrations of DBP were slightly higher in Cape Town (median = 342.62 ng/L) compared to Pretoria (median = 248.70 ng/L). No seasonal variations in DBP concentrations could be observed in the Pretoria samples, but Cape Town samples showed higher DBP concentrations in October 2013 (median = 633.90 ng/L) and lower concentrations in July 2014 (median = 214.06 ng/L).

Table 5.8: DBP concentrations in water extracts collected from selected distribution points in Pretoria

Sample	DBP (ng/L)			
	October 2013	January 2014	April 2014	July 2014
PTA01	200.34	220.34	175.56	260.18
PTA02	258.11	302.28	372.51	155.65
PTA03	291.85	402.69	347.61	235.26
PTA04	158.62	271.63	261.09	270.71
PTA05	<dl	<dl	335.51	185.39
PTA06	390.69	361.73	312.76	138.71
PTA07	248.17	128.82	148.22	179.40
PTA08	249.22	279.79	<dl	215.01
PTA09	332.50	245.57	<dl	271.79
PTA10	346.31	434.01	228.61	<dl

<dl Below detection limit of the assay

Table 5.9: DBP concentrations in water extracts collected from selected distribution points in Cape Town

Sample	DBP (ng/L)			
	October 2013	January 2014	April 2014	July 2014
CPT01	950.08	702.11	574.95	289.21
CPT02	444.76	369.50	627.53	233.15
CPT03	823.04	285.58	172.50	184.49
CPT04	343.91	656.24	310.80	222.90
CPT05	953.24	506.56	827.83	187.03
CPT06	393.55	331.26	218.33	208.46
CPT07	154.35	318.93	341.33	109.48
CPT08	393.76	576.97	180.47	123.63
CPT09	986.10	367.73	347.41	219.67
CPT10	1065.14	308.43	527.45	243.64

© University of Pretoria

5.2.2.5. Di(2-ethylhexyl) phthalate (DEHP)

The chromatogram for the DEHP standard at 300 µg/L is shown in Figure 5.9 and the calibration curve in Figure 5.10.

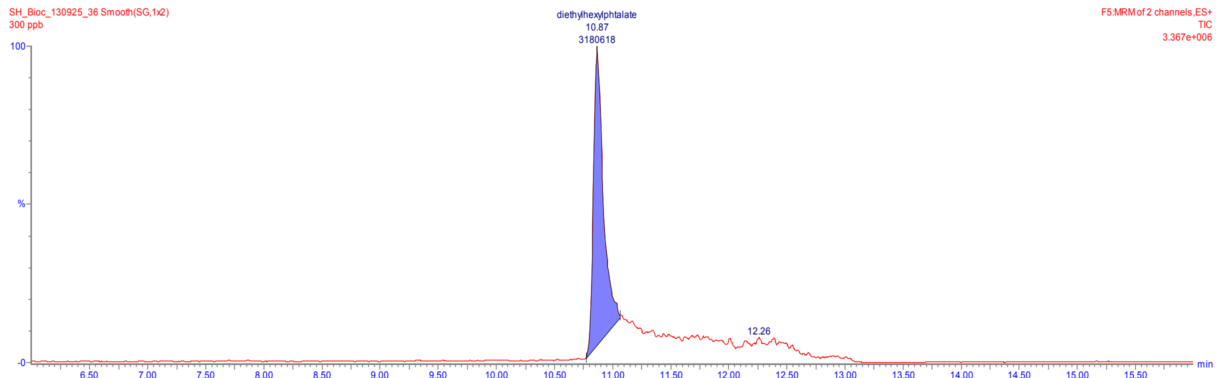


Figure 5.9: Chromatogram of DEHP standard (300 µg/L)

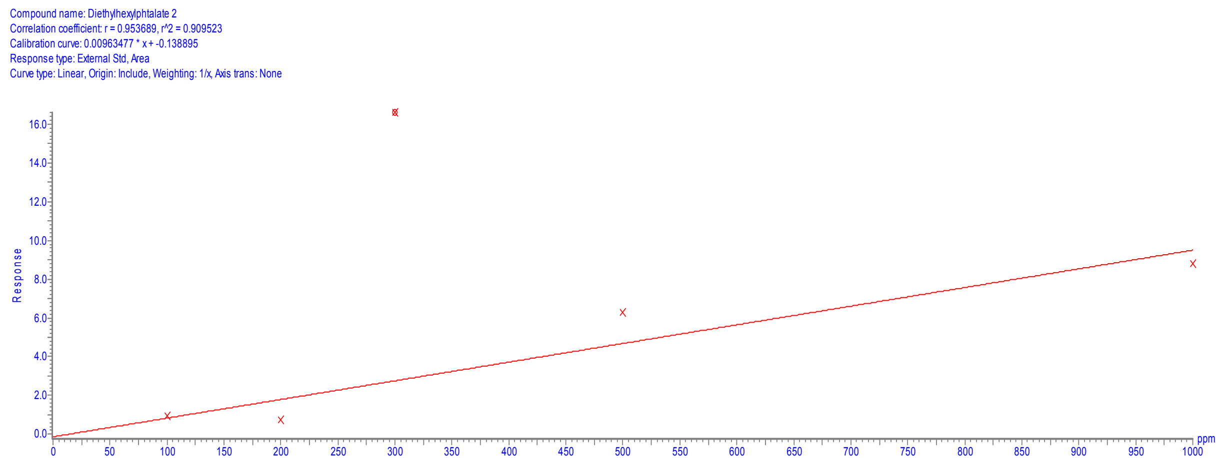


Figure 5.10: DEHP calibration curve

© University of Pretoria

DEHP were detected in 26 distribution point samples from Pretoria (Table 5.10) and in 31 samples in Cape Town (Table 5.11). Cape Town had higher levels of DEHP, ranging from below the quantification limit to 5150.76 ng/L (median = 90.01 ng/L), compared to Pretoria with levels ranging from below the dl to 247.13 ng/L (median = 58.04 ng/L). When comparing seasonal differences in DEHP concentrations, the highest concentrations were measured in January 2014 in Pretoria (average = 109.63 ng/L) and in April 2014 in Cape Town (average = 657.41 ng/L). Compared to the other samples, CPT04 had very high DEHP levels over three sapling periods (from October 2013 to April 2014), but was much lower in the last sampling period (July 2014).

Table 5.10: DEHP concentrations in water extracts collected from selected distribution points in Pretoria

Sample	DEHP (ng/L)			
	October 2013	**January 2014**	**April 2014**	**July 2014**
PTA01	55.37	92.03	118.86	46.42
PTA02	52.50	332.86	81.76	<dl
PTA03	64.05	152.04	67.56	44.30
PTA04	<dl	44.03	247.13	67.78
PTA05	<dl	<dl	146.83	51.25
PTA06	130.93	82.28	<dl	66.59
PTA07	73.20	<dl	<dl	222.77
PTA08	60.71	<dl	<dl	<dl
PTA09	175.46	<dl	<dl	74.42
PTA10	83.08	393.05	<dl	<dl

<dl Below detection limit of the assay

© University of Pretoria

Table 5.11: DEHP concentrations in water extracts collected from selected distribution points in Cape Town

Sample	DEHP (ng/L)			
	October 2013	January 2014	April 2014	July 2014
CPT01	62.36	364.47	169.06	68.07
CPT02	<dl	98.74	43.39	40.20
CPT03	42.68	196.94	74.28	<dl
CPT04	4119.94	4272.92	5150.76	117.14
CPT05	373.60	138.00	257.73	<dl
CPT06	62.50	80.87	<dl	415.01
CPT07	<dl	101.34	47.15	<ql
CPT08	99.53	125.49	62.05	<ql
CPT09	<dl	176.19	544.54	<dl
CPT10	88.67	91.35	225.12	103.25

<dl Below detection limit of the assay

<ql Below quantification limit of the assay

© University of Pretoria

5.2.2.6. Diisononyl phthalate (DINP)

The chromatogram for the DINP standard at 100 µg/L is shown in Figure 5.11 and the calibration curve in Figure 5.12.

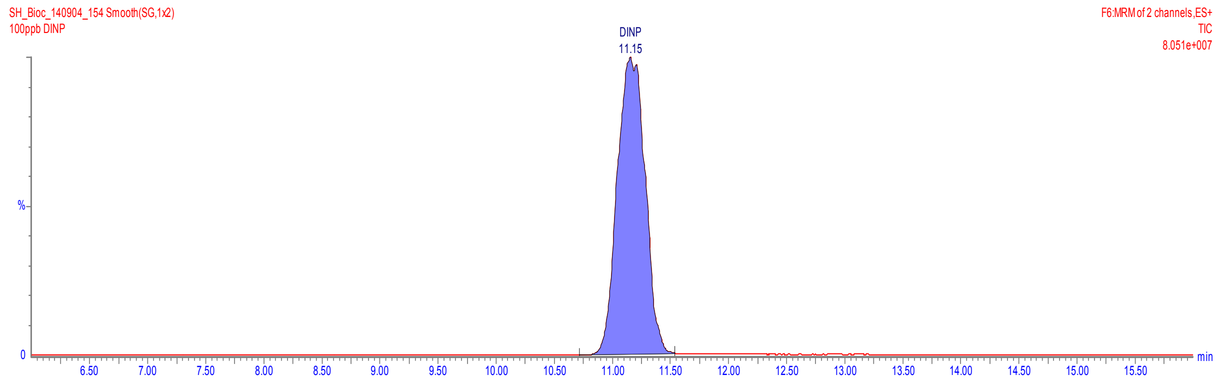


Figure 5.11: Chromatogram of DINP standard (100 µg/L)

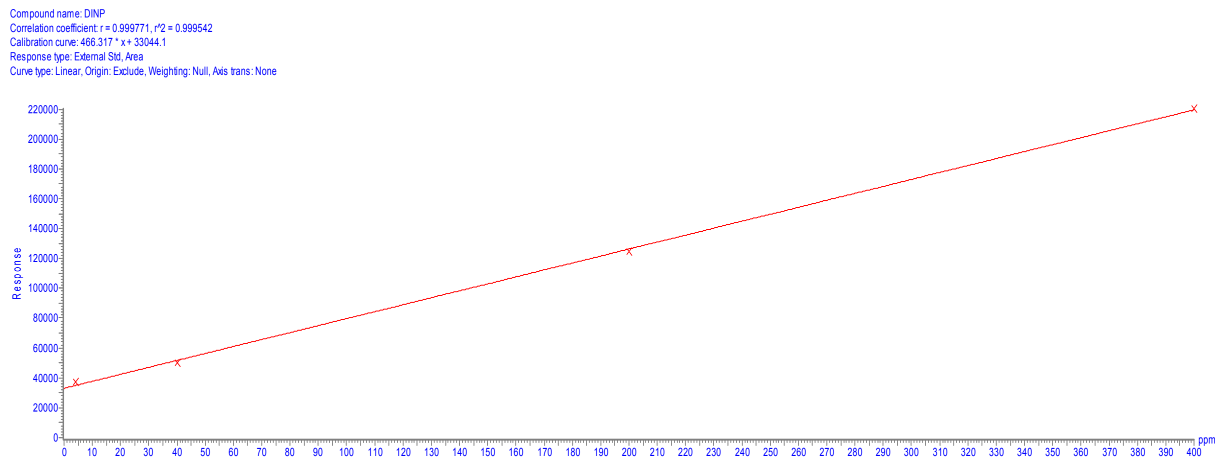


Figure 5.12: DINP calibration curve

© University of Pretoria

DINP concentrations ranged from below the dl to 187.22 ng/L (median = 16.15 ng/L) in distribution point water samples from Pretoria (Table 5.12) and from below the dl to 1250.75 ng/L (median = 51.98 ng/L) in samples from Cape Town (Table 5.13). July 2014 had the lowest median concentrations for the Pretoria (median = 3.15 ng/L) and Cape Town (median = 18.46 ng/L) samples and the highest median concentrations were measured in January 2014 in both study areas (35.14 ng/L for Pretoria and 88.93 ng/L for Cape Town).

Table 5.12: DINP concentrations in water extracts collected from selected distribution points in Pretoria

Sample	DINP (ng/L)			
	October 2013	January 2014	April 2014	July 2014
PTA01	44.90	43.90	26.67	<dl
PTA02	25.39	187.22	<dl	<dl
PTA03	10.24	56.05	11.85	<dl
PTA04	11.40	26.38	49.56	26.91
PTA05	<dl	<dl	8.34	<dl
PTA06	61.03	<dl	112.20	6.30
PTA07	77.25	117.84	140.87	85.57
PTA08	18.53	<dl	66.13	6.87
PTA09	13.76	3.02	<dl	31.09
PTA10	46.10	63.39	<dl	<dl

<dl Below detection limit of the assay

© University of Pretoria

Table 5.13: DINP concentrations in water extracts collected from selected distribution points in Cape Town

Sample	DINP (ng/L)			
	October 2013	January 2014	April 2014	July 2014
CPT01	1250.75	81.68	34.00	33.92
CPT02	54.59	69.13	<dl	<dl
CPT03	377.66	171.74	35.32	<dl
CPT04	<dl	71.06	<dl	30.44
CPT05	936.99	22.59	32.86	<dl
CPT06	54.28	122.54	57.17	29.02
CPT07	11.32	227.12	54.77	24.85
CPT08	49.68	96.18	54.84	9.66
CPT09	61.61	64.55	40.78	12.06
CPT10	762.21	134.14	58.33	46.33

<dl Below detection limit of the assay

Bestpfe.com

© University of Pretoria

5.2.2.7. 17β-Estradiol (E_2)

The chromatogram for the E_2 standard at 500 ng/L is shown in Figure 5.13 and the calibration curve in Figure 5.14.

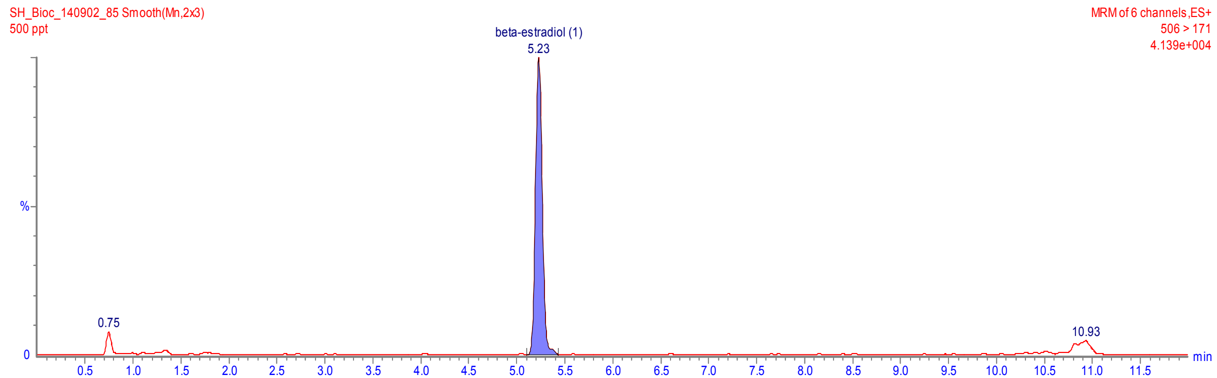


Figure 5.13: Chromatogram of E_2 standard (500 ng/L)

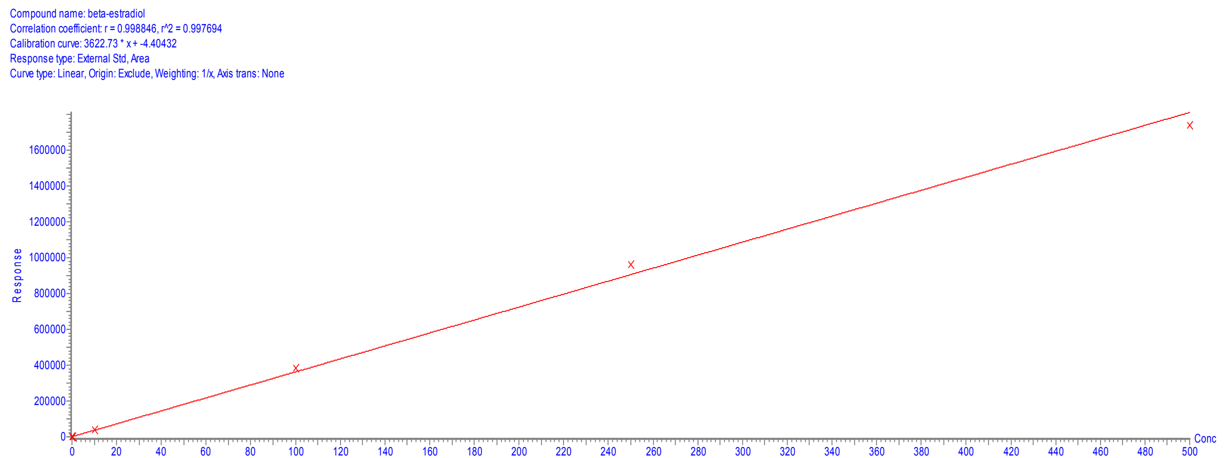


Figure 5.14: E_2 calibration curve

© University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

E_2 was detected in only one sample in Pretoria (PTA02, April 2014 – Table 5.14) and four samples in Cape Town (CPT03, October 2013; CPT06, January 2014; CPT02, July 2014 and CPT07, July 2014 – Table 5.15). E_2 concentrations ranged from below the dl to 0.05 ng/L.

Table 5.14: E_2 concentrations in water extracts collected from selected distribution points in Pretoria

Sample	E_2 (ng/L)			
	October 2013	**January 2014**	**April 2014**	**July 2014**
PTA01	<dl	<dl	<dl	<dl
PTA02	<dl	<dl	0.03	<dl
PTA03	<dl	<dl	<dl	<dl
PTA04	<dl	<dl	<dl	<dl
PTA05	<dl	<dl	<dl	<dl
PTA06	<dl	<dl	<dl	<dl
PTA07	<dl	<dl	<dl	<dl
PTA08	<dl	<dl	<dl	<dl
PTA09	<dl	<dl	<dl	<dl
PTA10	<dl	<dl	<dl	<dl

<dl Below detection limit of the assay

Table 5.15: E_2 concentrations in water extracts collected from selected distribution points in Cape Town

Sample	E_2 (ng/L)			
	October 2013	**January 2014**	**April 2014**	**July 2014**
CPT01	<dl	<dl	<dl	<dl
CPT02	<dl	<dl	<dl	0.02
CPT03	0.04	<dl	<dl	<dl
CPT04	<dl	<dl	<dl	<dl
CPT05	<dl	<dl	<dl	<dl
CPT06	<dl	0.05	<dl	<dl
CPT07	<dl	<dl	<dl	0.04
CPT08	<dl	<dl	<dl	<dl
CPT09	<dl	<dl	<dl	<dl
CPT10	<dl	<dl	<dl	<dl

<dl Below detection limit of the assay

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

5.2.2.8. Estrone (E_1)

The chromatogram for the E_1 standard at 500 ng/L is shown in Figure 5.15 and the calibration curve in Figure 5.16.

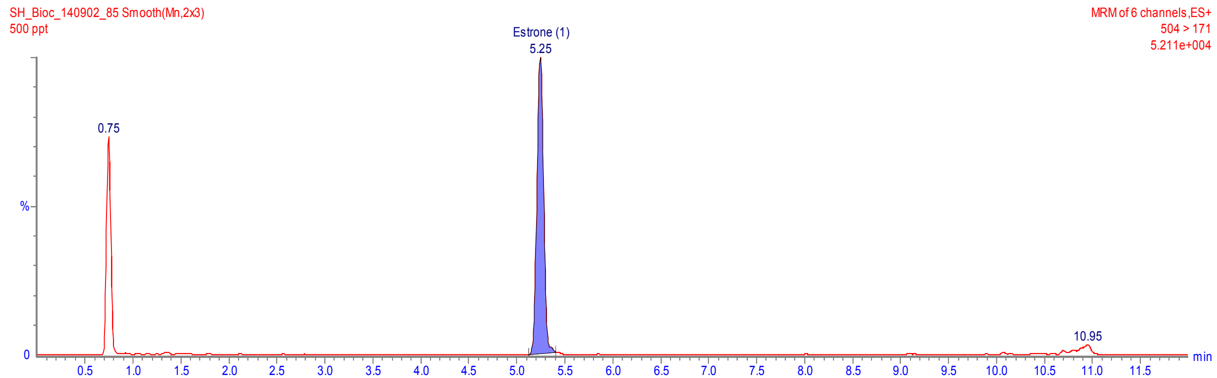


Figure 5.15: Chromatogram of E_1 standard (500 ng/L)

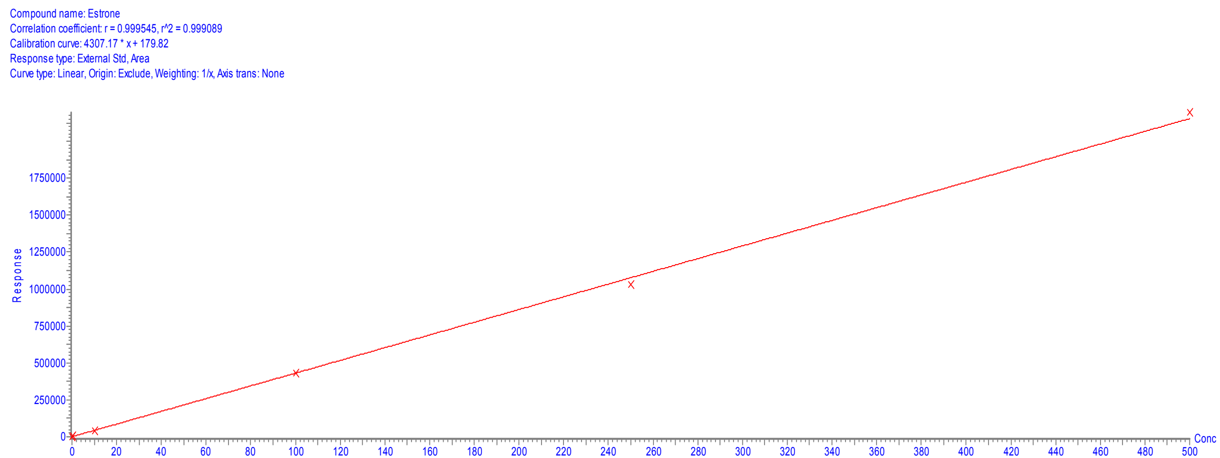


Figure 5.16: E_1 calibration curve

© University of Pretoria

E_1 was detected in four samples from Pretoria (Table 5.16) and two from Cape Town (Table 5.17), with concentrations ranging from below the dl to 4.89 ng/L. The E_1 concentrations were higher in the Pretoria samples (average = 0.32 ng/L) compared to the Cape Town samples (average = 0.04 ng/L).

Table 5.16: E_1 concentrations in water extracts collected from selected distribution points in Pretoria

Sample	E_1 (ng/L)			
	October 2013	January 2014	April 2014	July 2014
PTA01	<dl	<dl	<dl	<dl
PTA02	<dl	<dl	2.32	<dl
PTA03	3.41	<dl	<dl	<dl
PTA04	<dl	<dl	<dl	<dl
PTA05	<dl	<dl	<dl	<dl
PTA06	2.33	4.89	<dl	<dl
PTA07	<dl	<dl	<dl	<dl
PTA08	<dl	<dl	<dl	<dl
PTA09	<dl	<dl	<dl	<dl
PTA10	<dl	<dl	<dl	<dl

<dl Below detection limit of the assay

Table 5.17: E_1 concentrations in water extracts collected from selected distribution points in Cape Town

Sample	E_1 (ng/L)			
	October 2013	January 2014	April 2014	July 2014
CPT01	<dl	<dl	<dl	<dl
CPT02	<dl	<dl	<dl	0.36
CPT03	<dl	<dl	<dl	<dl
CPT04	<dl	<dl	<dl	<dl
CPT05	<dl	<dl	<dl	<dl
CPT06	<dl	<dl	<dl	<dl
CPT07	<dl	<dl	<dl	<dl
CPT08	<dl	<dl	<dl	<dl
CPT09	<dl	<dl	<dl	1.14
CPT10	<dl	<dl	<dl	<dl

<dl Below detection limit of the assay

© University of Pretoria

5.2.2.9. Ethynylestradiol (EE_2)

The chromatogram for the EE_2 standard at 500 ng/L is shown in Figure 5.17 and the calibration curve in Figure 5.18.

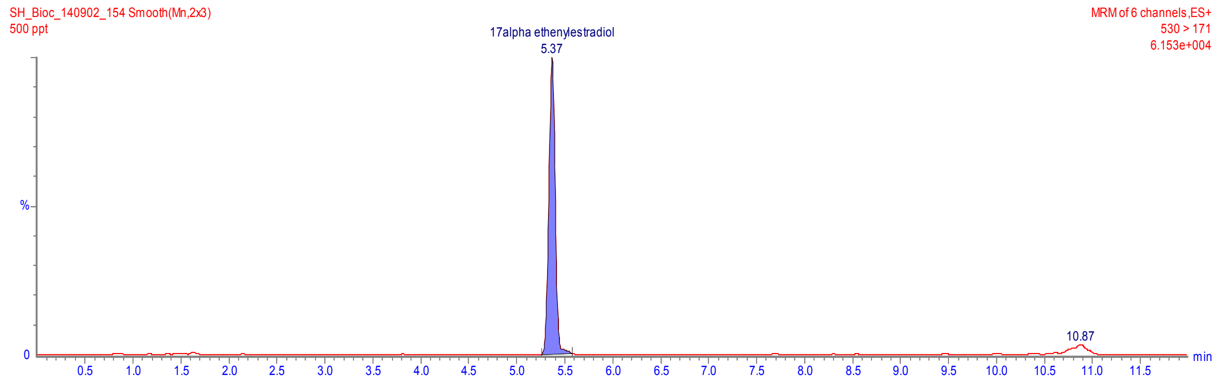


Figure 5.17: Chromatogram of EE_2 standard (500 ng/L)

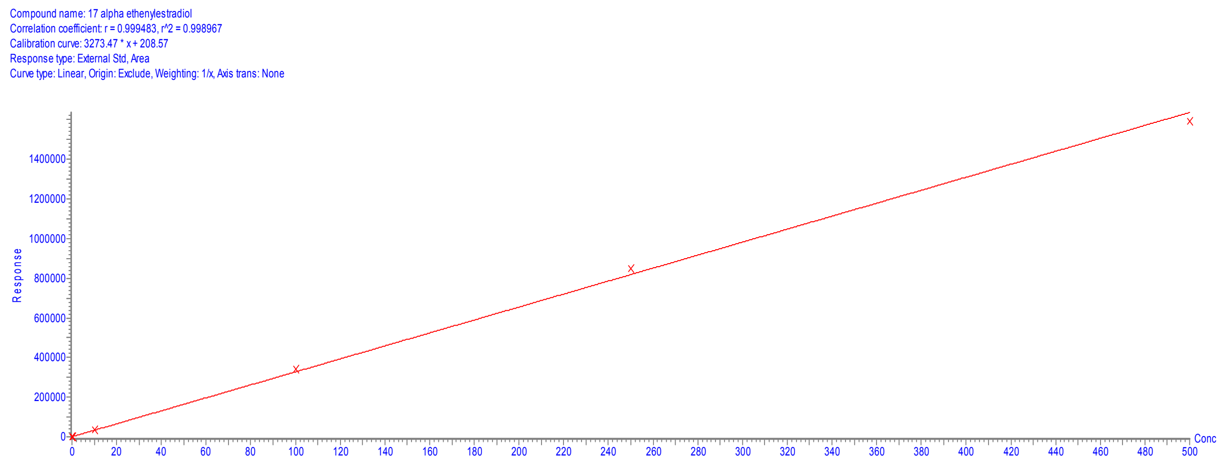


Figure 5.18: EE_2 calibration curve

© University of Pretoria

EE_2 was detected at one distribution point in Pretoria (PTA08) in all four sampling periods (Table 5.18). The highest concentration was measured in October 2013 (0.06 ng/L) and decreased to 0.003 ng/L in July 2014. None of the Cape Town samples had EE_2 concentrations above the dl.

Table 5.18: EE_2 concentrations in water extracts collected from selected distribution points in Pretoria

Sample	EE_2 (ng/L)			
	October 2013	January 2014	April 2014	July 2014
PTA01	<dl	<dl	<dl	<dl
PTA02	<dl	<dl	<dl	<dl
PTA03	<dl	<dl	<dl	<dl
PTA04	<dl	<dl	<dl	<dl
PTA05	<dl	<dl	<dl	<dl
PTA06	<dl	<dl	<dl	<dl
PTA07	<dl	<dl	<dl	<dl
PTA08	0.06	0.02	0.01	0.003
PTA09	<dl	<dl	<dl	<dl
PTA10	<dl	<dl	<dl	<dl

<dl Below detection limit of the assay

© University of Pretoria

5.3. Phase 2 - Bottled water

5.3.1. Mineral composition

The mineral composition (as it was printed on the labels of the bottles) of the ten brands of bottled water selected for this study is summarised in Table 5.19. The pH of the different brands of water varied from 4.5 to 8.

Table 5.19: Mineral composition of the selected brands of bottled water analysed in this study

Concentration (mg/L)	BTW01	BTW02	BTW03	BTW04	BTW05	BTW06	BTW07	BTW08	BTW09	BTW10
Ca	10	7	<10	0.6	5.9	2.5	11.7	2	<0.2	41
Mg	10	4.1	2	3.2	6.8	3.7	13.9	1.6	1.2	25.5
Na	3	2	16	<5	6.1	7	5.1	9.3	10.6	6.2
K	1	<1.0	<1	3.4	3.1	3	1.1	2.1	<0.2	0.7
Cl	2	<5.0	31	5.6	<5	<2	8	11	18.5	7
SO_4	4	<5.0	<5	14	1.35	<5	5	3	1	8
$CaCO_3$	65	na	3	9.8	49	26	66	<15	1	203
N	1	<0.3	<0.5	<1	na	<0.1	3.5	1.6	0.2	7
F	<0.1	0.09	<0.2	<0.1	na	<1	0.2	<0.2	<0.1	<0.2
Fe	na	<0.05	na	<0.1	0.02	na	na	na	na	<0.01
Zn	na	<0.05	na	na	na	na	na	na	na	na
Al	na	na	na	<0.1	<0.3	na	na	na	na	<0.02
TDS	83	52	65	44	110	64	124	70	58	225
pH	7.3	6.8	5.1	6.5	7.3	6.8	7.4	6	4.5	7-8

na Not available, information not given on bottle

TDS Total dissolved solids

© University of Pretoria

5.3.2. Bioassays for estrogenic activity

None of the samples were above the dl in the YES bioassay and no cytotoxicity was observed.

In the T47D-KBluc bioassay, eight samples had estrogenic activity, with EEq values ranging from below the dl to 0.011 ng/L (Table 5.20). Only one sample incubated at 20°C were above the dl of the bioassay, the other positive samples were all incubated at 40°C (four in dark and three in light conditions). The highest EEq (0.011 ng/L) were BTW05, incubated at 40°C in the dark. None of the samples had anti-estrogenic activity or cytotoxicity.

Table 5.20: Estrogenic activity of selected bottled water extracts stored at different temperatures and light conditions using the T47D-KBluc bioassay

Sample	EEq (ng/L)			
	20°C, Dark	**20°C, Light**	**40°C, Dark**	**40°C, Light**
BTW01	<dl	<dl	<dl	<dl
BTW02	<dl	<dl	<dl	<dl
BTW03	<dl	<dl	<dl	<dl
BTW04	<dl	<dl	<dl	<dl
BTW05	<dl	0.004 ± 0.002	0.011 ± 0.001	0.002 ± 0.0002
BTW06	<dl	<dl	<dl	<dl
BTW07	<dl	<dl	0.001 ± 0.0001	0.001 ± 0.0002
BTW08	<dl	<dl	0.001 ± 0.0003	0.002 ± 0.0002
BTW09	<dl	<dl	0.001 ± 0.0001	<dl
BTW10	<dl	<dl	<dl	<dl

<dl Below detection limit of the assay

© University of Pretoria

5.3.3. Target chemical analyses

5.3.3.1. Bisphenol A (BPA)

BPA was detected in all the bottled water samples (Table 5.21). The highest concentrations of BPA were measured in samples stored at 20°C and the maximum concentration measured was 47.36 ng/L in BTW10 (20°C, light).

Table 5.21: BPA concentrations in selected bottled water extracts stored at different temperatures and light conditions

Sample	BPA (ng/L)			
	20°C, Dark	20°C, Light	40°C, Dark	40°C, Light
BTW01	2.06	2.31	1.21	1.12
BTW02	1.09	1.22	1.42	1.21
BTW03	24.19	1.82	5.60	1.96
BTW04	0.55	1.23	3.78	2.01
BTW05	1.91	2.40	2.36	1.68
BTW06	1.83	15.27	3.99	1.54
BTW07	0.94	1.84	1.55	2.07
BTW08	1.87	17.09	1.23	1.93
BTW09	1.03	2.41	1.82	4.39
BTW10	1.59	47.36	1.87	1.55

5.3.3.2. Nonylphenol (NP)

None of the bottled water samples were above the dl for NP.

© University of Pretoria

5.3.3.3. Di(2-ethylhexyl) adipate (DEHA)

DEHA was detected in all the bottled water samples, with concentrations ranging from below the dl to 205.26 ng/L with a median concentration of 2.43 ng/L (Table 5.22). For sample BTW07 and BTW08, the BPA concentrations were much higher in samples stored at 40°C, in the dark.

Table 5.22: DEHA concentrations in selected bottled water extracts stored at different temperatures and light conditions

Sample	DEHA (ng/L)			
	20°C, Dark	20°C, Light	40°C, Dark	40°C, Light
BTW01	2.63	1.83	2.32	1.29
BTW02	1.70	2.31	2.43	2.46
BTW03	3.00	2.55	2.55	2.55
BTW04	1.95	2.29	2.97	1.08
BTW05	2.41	1.81	1.84	2.63
BTW06	171.21	<ql	1.64	4.02
BTW07	1.51	2.31	59.81	36.02
BTW08	1.14	2.38	205.26	8.53
BTW09	2.65	2.44	2.03	3.90
BTW10	2.52	1.03	3.74	7.89

<ql Below quantification limit of the assay

© University of Pretoria

5.3.3.4. Dibutyl phthalate (DBP)

DBP concentrations measured in the bottled water samples ranged from below the dl to 5 481.00 ng/L, with a median concentration of 201.23 ng/L (Table 5.23). Although the samples stored at 40°C had higher median concentrations (224.83 ng/L dark and 208.57 ng/L light) compared to the samples stored at 20°C (178.03 ng/L dark and 175.53 ng/L light), no connection between the storage temperature and DBP concentration could be seen when samples were looked at individually, except for BTW07 and BTW08, that had higher DBP concentrations in samples stored at 40°C compared to 20°C.

Table 5.23: DBP concentrations in selected bottled water extracts stored at different temperatures and light conditions

Sample	DBP (ng/L)			
	20°C, Dark	20°C, Light	40°C, Dark	40°C, Light
BTW01	252.51	126.35	140.93	183.86
BTW02	161.29	336.74	231.69	267.41
BTW03	175.41	194.86	219.85	194.86
BTW04	180.65	156.19	141.19	<dl
BTW05	103.51	121.92	<dl	211.47
BTW06	5481.00	99.84	229.82	205.68
BTW07	122.48	199.94	816.85	938.39
BTW08	226.26	252.64	956.00	663.67
BTW09	321.73	302.57	241.69	538.70
BTW10	124.03	<dl	185.10	202.52

<dl Below detection limit of the assay

© University of Pretoria

5.3.3.5. Di(2-ethylhexyl) phthalate (DEHP)

DEHP was detected in 16 of the bottled water samples at concentrations ranging from below the dl to 1431.74 ng/L (Table 5.24). No association between the storage temperature and DEHP concentration could be seen.

Table 5.24: DEHP concentrations in selected bottled water extracts stored at different temperatures and light conditions

Sample	DEHP (ng/L)			
	20°C, Dark	20°C, Light	40°C, Dark	40°C, Light
BTW01	<dl	<dl	<ql	<dl
BTW02	<dl	104.22	68.85	102.21
BTW03	40.95	<dl	<ql	<dl
BTW04	<dl	<dl	<dl	<dl
BTW05	47.42	1231.56	<dl	<dl
BTW06	1431.74	<dl	<dl	58.37
BTW07	<dl	66.21	63.04	49.89
BTW08	<dl	56.38	44.46	<dl
BTW09	<dl	<dl	<dl	<dl
BTW10	<dl	<dl	<dl	<ql

<dl Below detection limit of the assay

<ql Below quantification limit of the assay

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

5.3.3.6. Diisononyl phthalate (DINP)

DINP was detected in nine samples stored at 20°C and in 15 samples stored at 40°C (Table 5.25). Only in one brand (BTW02) DINP were below the dl for all the storage conditions. Most of the samples had higher DINP concentrations in bottles stored at 40°C compared to 20°C and samples stored at 40°C in light conditions had the highest median concentration (110.46 ng/L).

Table 5.25: DINP concentrations in selected bottled water extracts stored at different temperatures and light conditions

Sample	DINP (ng/L)			
	20°C, Dark	20°C, Light	40°C, Dark	40°C, Light
BTW01	43.87	<dl	<dl	<dl
BTW02	<dl	<dl	<dl	<dl
BTW03	180.78	10.87	46.14	10.87
BTW04	<dl	10.21	14.18	<dl
BTW05	<dl	<dl	34.45	67.32
BTW06	598.96	<dl	22.96	203.01
BTW07	<dl	5.49	9138.99	3045.35
BTW08	<dl	<dl	14990.85	463.20
BTW09	30.93	47.61	66.17	153.59
BTW10	55.10	<dl	192.50	858.89

<dl Below detection limit of the assay

© University of Pretoria

5.3.3.7. 17β-Estradiol (E_2)

E_2 was below the dl in all the bottled water samples.

5.3.3.8. Estrone (E_1)

E_1 was detected in six samples (Table 5.26), with concentrations ranging from below the dl to 0.98 ng/L.

Table 5.26: E_1 concentrations in selected bottled water extracts stored at different temperatures and light conditions

Sample	E_1 (ng/L)			
	20°C, Dark	20°C, Light	40°C, Dark	40°C, Light
BTW01	<dl	<dl	<dl	<dl
BTW02	<dl	<dl	<dl	<dl
BTW03	<dl	<dl	<dl	0.26
BTW04	<dl	<dl	<dl	<dl
BTW05	0.13	<dl	0.48	<dl
BTW06	<dl	<dl	<dl	0.09
BTW07	<dl	<dl	<dl	<dl
BTW08	<dl	<dl	0.26	0.98
BTW09	<dl	<dl	<dl	<dl
BTW10	<dl	<dl	<dl	<dl

<dl Below detection limit of the assay

© University of Pretoria

5.3.3.9. Ethynylestradiol (EE_2)

EE_2 was detected in all the bottled water samples (Table 5.27). The median concentration of EE_2 for the bottled water stored at 20°C was 0.03 ng/L for samples stored in dark and light conditions. However, bottled water stored at 40°C had a median EE_2 concentration of 0.09 ng/L for samples stored in dark conditions and 0.10 ng/L for samples stored in light conditions.

Table 5.27: EE_2 concentrations in selected bottled water extracts stored at different temperatures and light conditions

Sample	EE_2 (ng/L)			
	20°C, Dark	20°C, Light	40°C, Dark	40°C, Light
BTW01	0.02	0.03	0.09	0.10
BTW02	0.03	0.03	0.06	0.09
BTW03	0.04	0.03	0.08	0.11
BTW04	0.03	0.03	0.07	0.10
BTW05	0.04	0.02	0.10	0.10
BTW06	0.03	0.03	0.11	0.10
BTW07	0.02	0.03	0.09	0.09
BTW08	0.03	0.04	0.06	0.09
BTW09	0.02	0.02	0.09	0.07
BTW10	0.03	0.03	0.13	0.11

© University of Pretoria

5.4. Comparison between distribution point and bottled water

Figure 5.19 compares the estrogenic activity of distribution point and bottled water. The figure shows the average EEq at each distribution point, with error bars representing the seasonal variation in EEq concentrations. This is compared to the average EEq for the four different storage conditions for each brand of bottled water. From the figure it is clear that the estrogenic activity in bottled water was much lower than in distribution point water from Pretoria and Cape Town.

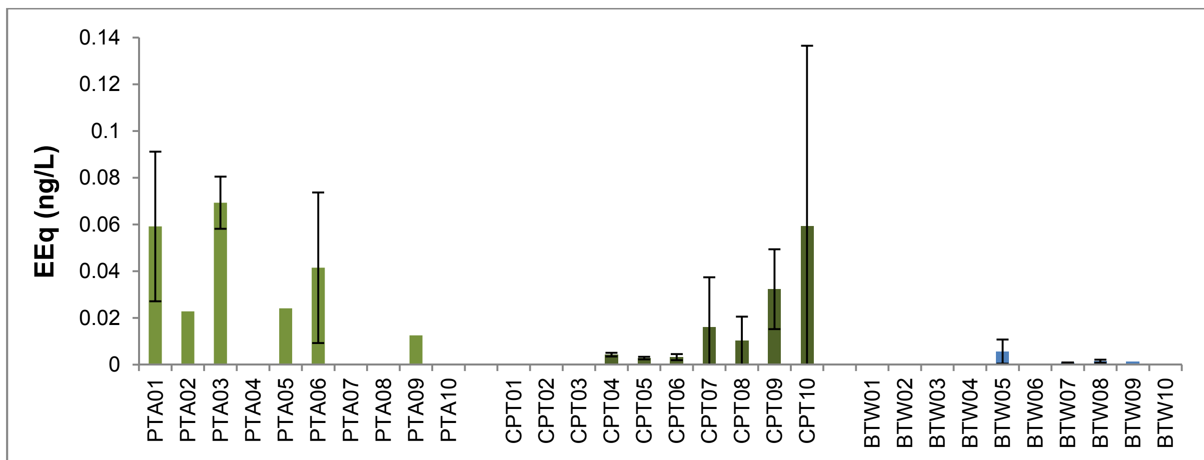


Figure 5.19: Comparison of distribution point and bottled water using the T47D-KBluc bioassay. The figure shows the average EEq ± SD

© University of Pretoria

Compared to distribution point water, bottled water had higher average BPA concentrations (Figure 5.20). The average BPA concentration for Pretoria distribution point water was 1.19 ng/L, for Cape Town distribution point water it was 1.34 ng/L, while bottled water had an average BPA concentration of 4.36 ng/L.

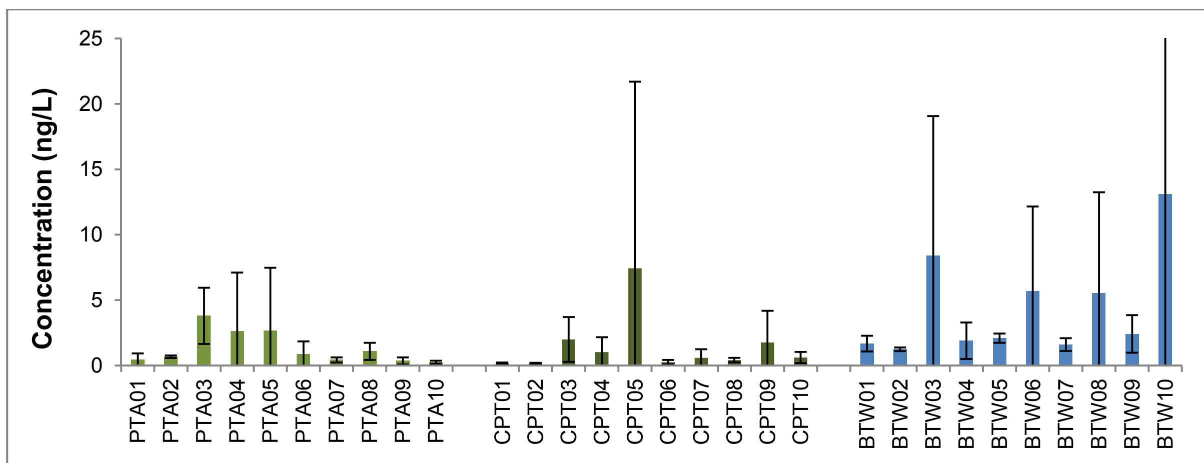


Figure 5.20: Comparison of BPA concentrations in distribution point and bottled water. The figure shows the average EEq ± SD

© University of Pretoria

Figure 5.21 shows the comparison of DEHA concentrations between distribution point and bottled water. From the figure it is clear that all the water samples had low background levels of DEHA, except for three of the bottled water samples (BTW06, BTW07 and BTW08) that had much higher concentrations of DEHA.

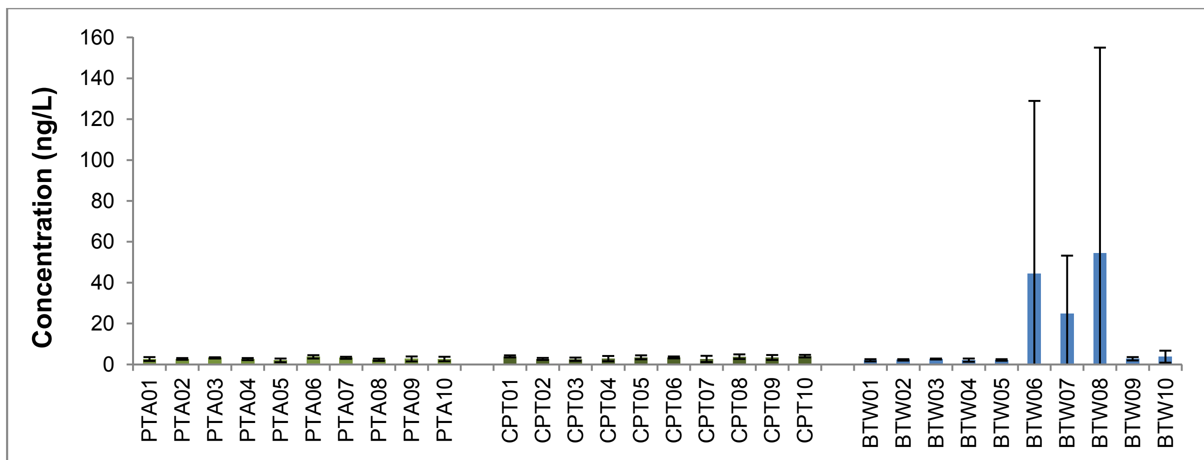


Figure 5.21: Comparison of DEHA concentrations in distribution point and bottled water. The figure shows the average EEq ± SD

© University of Pretoria

A comparison of DBP concentrations in distribution point and bottled water is shown in Figure 5.22. Although the highest DBP concentration was measured in one of the bottled water samples, Cape Town samples had the highest median concentration (342.62 ng/L), followed by Pretoria (248.70 ng/L) and bottled water (201.23 ng/L) samples.

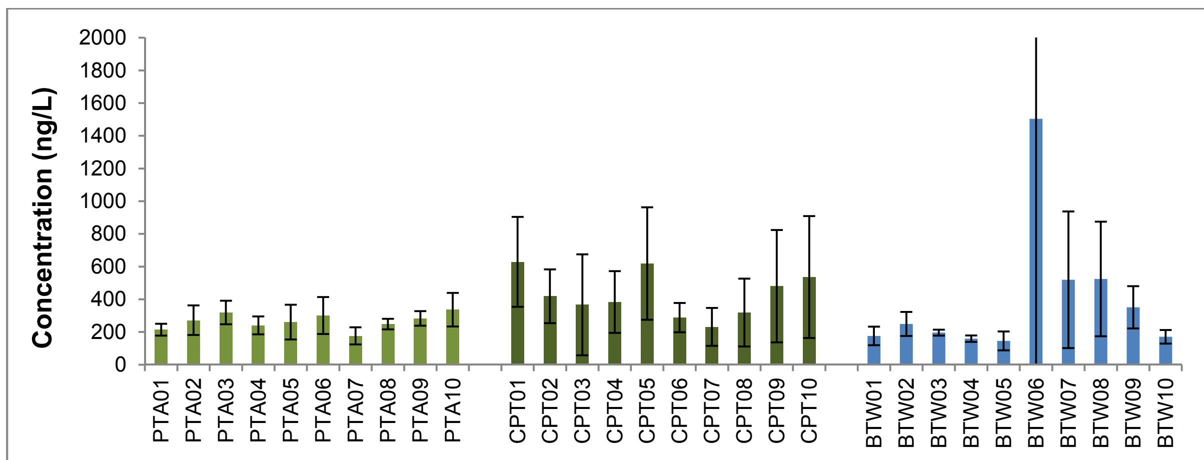


Figure 5.22: Comparison of DBP concentrations in distribution point and bottled water. The figure shows the average EEq ± SD

© University of Pretoria

Figure 5.23 compares the DEHP concentrations in distribution point and bottled water. From the figure it can be seen that the highest DEHP concentration was detected in one of the Cape Town distribution points, followed by two of the bottled water samples (BTW05 and BTW06).

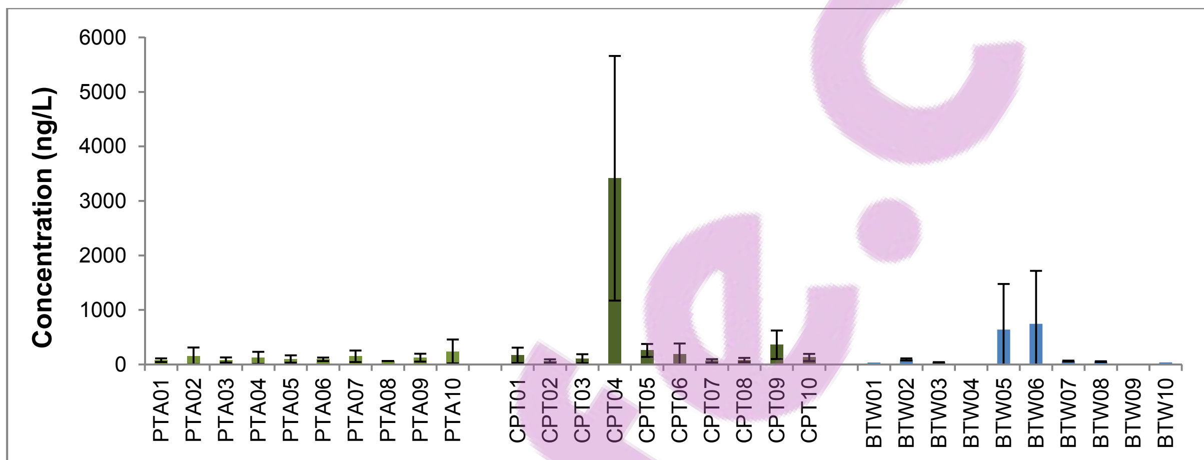


Figure 5.23: Comparison of DEHP concentrations in distribution point and bottled water. The figure shows the average EEq ± SD

© University of Pretoria

Bestpfe.com

When comparing DINP concentrations between distribution point and bottled water samples (Figure 5.24), it can be seen that all the samples had very low levels of DINP, except for two of the bottled water samples (BTW07 and BTW08).

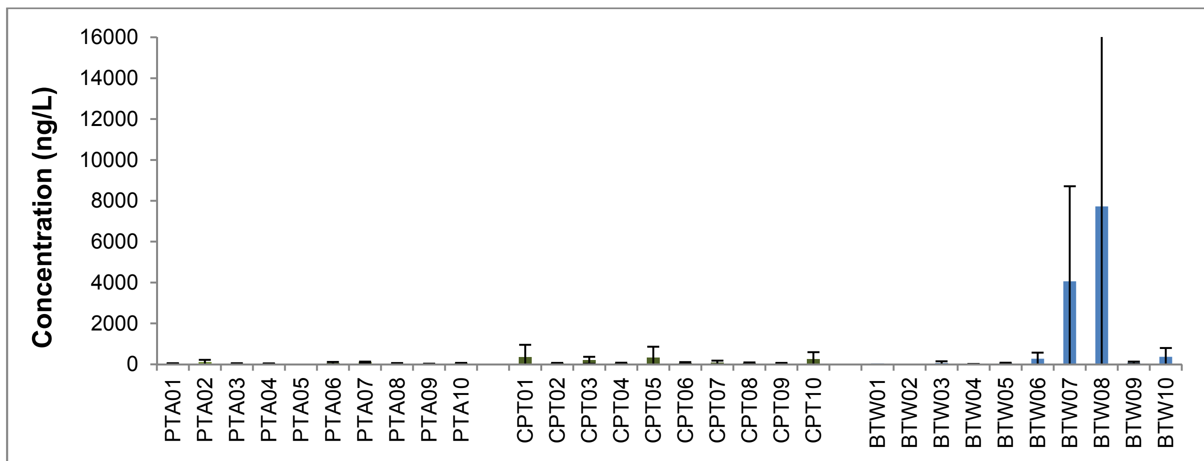


Figure 5.24: Comparison of DINP concentrations in distribution point and bottled water. The figure shows the average EEq ± SD

© University of Pretoria

E_2 was only detected in one distribution point sample from Pretoria and in four distribution point samples from Cape Town, but was below the dl in all the bottled water samples (Figure 5.25).

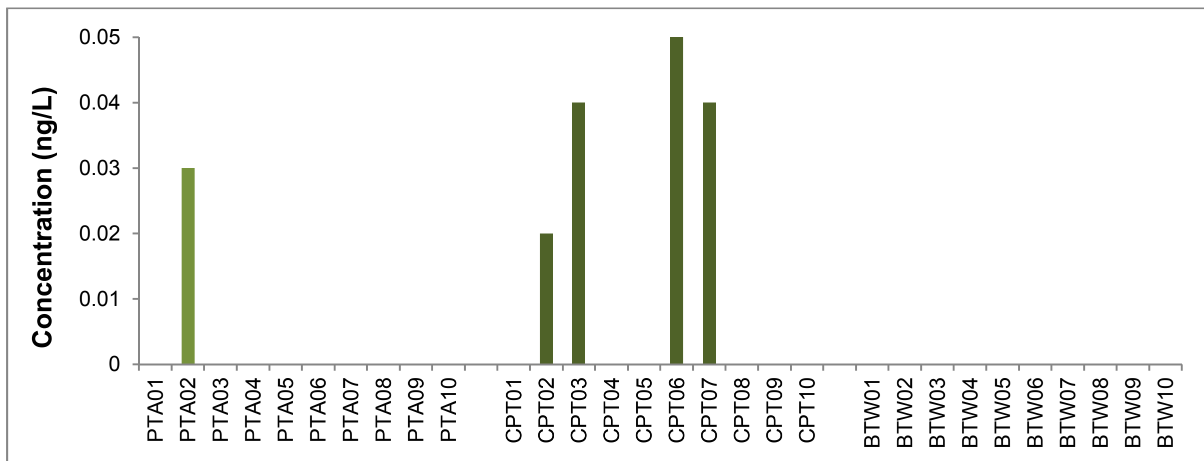


Figure 5.25: Comparison of E_2 concentrations in distribution point and bottled water

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

The highest E_1 concentrations were measured in distribution point samples from Pretoria (Figure 5.26). The average concentration for the Pretoria samples (0.32 ng/L) was eight times higher than the average concentration for Cape Town samples (0.04 ng/L) and five times the average bottled water concentration (0.06 ng/L).

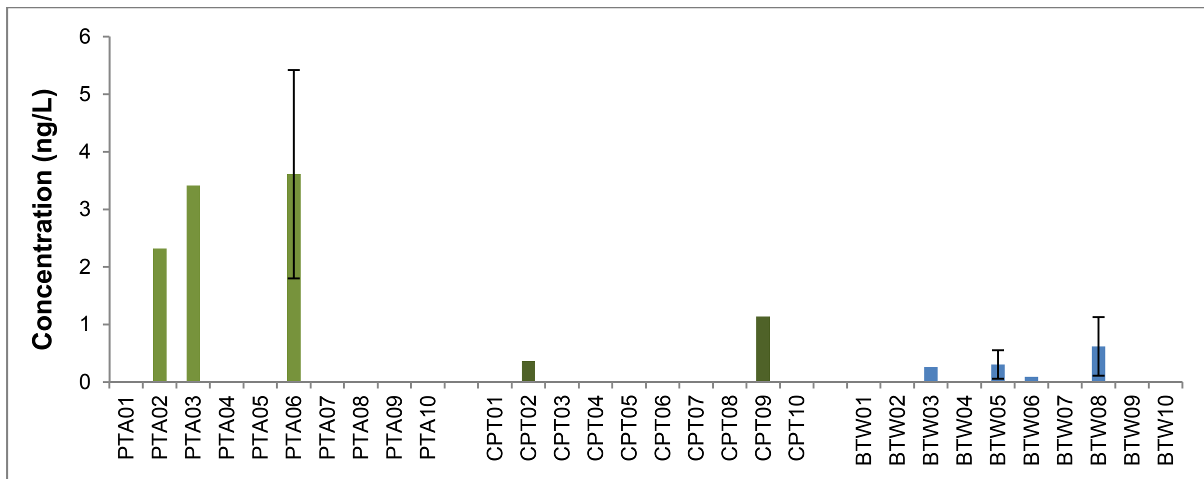


Figure 5.26: Comparison of E1 concentrations in distribution point and bottled water. The figure shows the average EEq ± SD

© University of Pretoria

Figure 5.27 compares the EE_2 concentrations found in distribution point and bottled water. EE_2 was only detected at one of the distribution points in Pretoria, but in all the bottled water samples. The average concentration of the Pretoria distribution point (PTA08) over the four sampling periods was 0.02 ng/L. Higher EE_2 concentrations were measured in the bottled water samples, with the average concentration being three times higher (0.06 ng/L) compared to the distribution point.

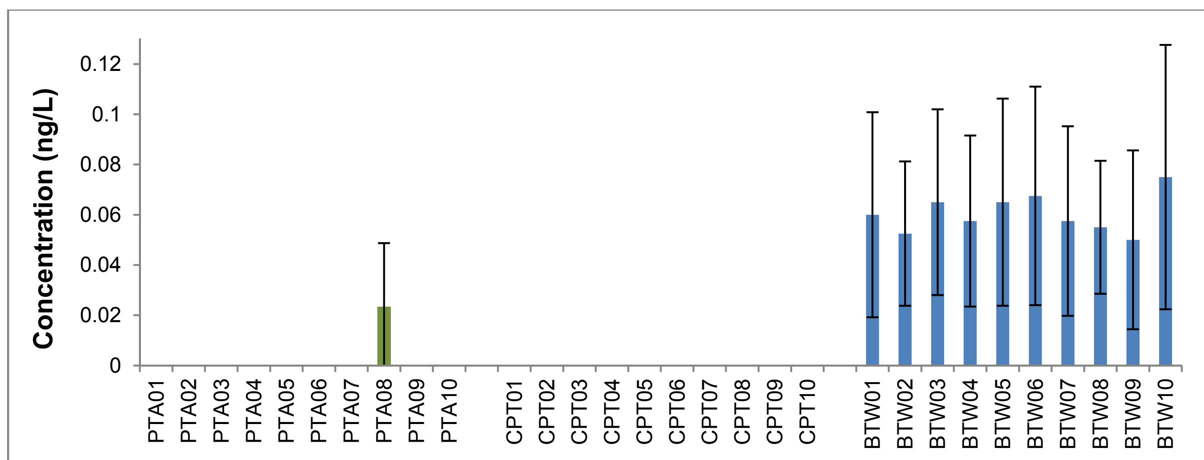


Figure 5.27: Comparison of EE2 concentrations in distribution point and bottled water. The figure shows the average EEq ± SD

© University of Pretoria

5.5. Phase 3 - Health Risk Assessment

Using the YES and T47D-KBluc bioassays, none of the samples were above the 0.7 ng/L trigger value for estrogenic activity in drinking water.

The exposure parameters used for the health risk assessment of the target chemicals found in the water samples are given in Table 5.28.

Table 5.28: Exposure parameters used in the human health risk calculations

Exposure parameter	Value used for risk calculations
Events per year	350
Body weight	70 kg
Lifetime	70 years
Ingestion rate	2 L water per day
Chronic exposure duration	30 years

For the health risk assessment 95th percentiles were used as 'reasonable maximum' values to determine the risks associated with the consumption of distribution point and bottled water. The health risk assessment is summarised in Table 5.29. Using reasonable maximum values, the HQ for E_1 in Pretoria distribution point samples were above 1, indicating potential health risk. However, only four of the Pretoria distribution point samples were above the dl for E_1. If the HQ is recalculated using the average concentration, the HQ is 0.21, indicating acceptable health risks associated with E_1 in Pretoria distribution point water. For all the other distribution points and bottled water the HQ was below one for all the target chemicals and is therefore considered safe for a lifetime exposure. The carcinogenic risks for DEHP and DEHA were also below 10^5, and therefore deemed acceptable.

© University of Pretoria

Table 5.29: Health risk assessment of distribution point and bottled water

Target Chemical	Water source	Concentration (95th percentile in ng/L)	ADD (mg/kg/d)	LADD (mg/kg/d)	RfD (mg/kg/d)	Trigger value (ng/L) #	Hazard Quotient	Slope	Risk
BPA	PTA	5.84	1.67E-07	7.15E-08	0.0125	n/a	0.00001	n/a	n/a
	CPT	2.42	6.91E-08	2.96E-08	0.0125	n/a	0.00001	n/a	n/a
	BTW	17.45	4.99E-07	2.14E-07	0.0125	n/a	0.00004	n/a	n/a
DEHP	PTA	251.42	7.18E-06	3.08E-06	0.02	n/a	0.00036	0.014	4.31E-08
	CPT	4127.59	1.18E-04	5.05E-05	0.02	n/a	0.0059	0.014	7.08E-07
	BTW	160.59	4.59E-06	1.97E-06	0.02	n/a	0.00023	0.014	2.75E-08
DEHA	PTA	3.95	1.13E-07	4.84E-08	0.6	n/a	2E-07	0.0012	5.81E-11
	CPT	4.71	1.35E-07	5.77E-08	0.6	n/a	2E-07	0.0012	6.92E-11
	BTW	65.38	1.87E-06	8.01E-07	0.6	n/a	3.1E-06	0.0012	9.61E-10
DBP	PTA	391.29	1.12E-05	4.79E-06	0.1	n/a	0.00011	n/a	n/a
	CPT	954.88	2.73E-05	1.17E-05	0.1	n/a	0.00027	n/a	n/a
	BTW	939.28	2.68E-05	1.15E-05	0.1	n/a	0.00027	n/a	n/a
DINP	PTA	118.99	3.40E-06	1.46E-06	0.115	n/a	0.00003	n/a	n/a
	CPT	770.95	2.20E-05	9.44E-06	0.115	n/a	0.00019	n/a	n/a
	BTW	3350.03	9.57E-05	4.10E-05	0.115	n/a	0.00083	n/a	n/a
E_2	PTA*	0.03	8.57E-10	3.67E-10	n/a	0.7	0.04	n/a	n/a
	CPT	0.04	1.14E-09	4.90E-10	n/a	0.7	0.06	n/a	n/a
	BTW	<dl	<dl	<dl	n/a	0.7	<dl	n/a	n/a
E_1	PTA	2.38	6.80E-08	2.91E-08	n/a	1.5	1.56	n/a	n/a
	CPT	0.02	5.71E-10	2.45E-10	n/a	1.5	0.01	n/a	n/a
	BTW	0.27	7.71E-09	3.30E-09	n/a	1.5	0.18	n/a	n/a
EE_2	PTA	0.01	2.86E-10	1.22E-10	n/a	0.6	0.02	n/a	n/a
	CPT	<dl	<dl	<dl	n/a	0.6	<dl	n/a	n/a
	BTW	0.11	3.17E-09	1.36E-09	n/a	0.6	0.1998	n/a	n/a

Trigger value suggested by Genthe et al.[98] for E_2 (0.7 ng/L) and adjusted for relative potencies (refer to Table 4.1) of E_1 (0.46) and EE_2 (1.26) in the T47D-KBluc bioassay (trigger value for E_1 or E_2 = 0.7/relative potency of E_1 or EE_2); * Only one sample above dl; n/a – not applicable; PTA - Pretoria distribution point water; CPT - Cape Town distribution point water; BTW - Bottled water; ADD - Average daily dose; LADD – Lifetime average daily dose; RfD – Reference dose reported by USEPA; Hazard quotient – Relative value expressing the assumed dose to a dose that is considered safe for a lifetime of exposure; Slope – Potency factor of a specific carcinogen; Risk – Excess risk of developing cancer

© University of Pretoria

Uncertainty analysis using Monte Carlo simulations indicated that 95th percentile or reasonable maximum EEq concentrations have a 90% chance to fall between 0.004 ng/L and 0.231 ng/L in Pretoria distribution point water (Figure 5.28) and between 0.0021 ng/L and 0.1198 ng/L in distribution point water from Cape Town (Figure 5.29). In bottled water reasonable maximum EEq concentrations are expected to fall between 0.00010 ng/L and 0.00599 ng/L, with a 90% certainty (Figure 5.30). In all cases, these values are well below the 0.7 ng/L trigger value for estrogenic activity in drinking water.

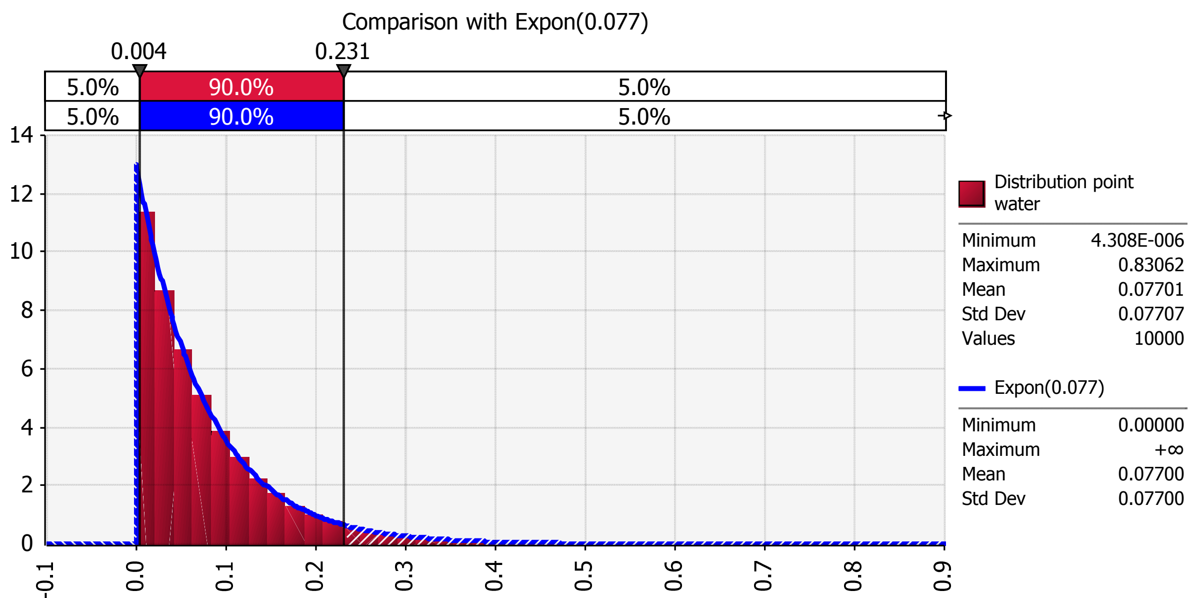


Figure 5.28: Monte Carlo simulation of Pretoria distribution point water indicating the probability distributions of reasonable maximum EEq values in ng/L

© University of Pretoria

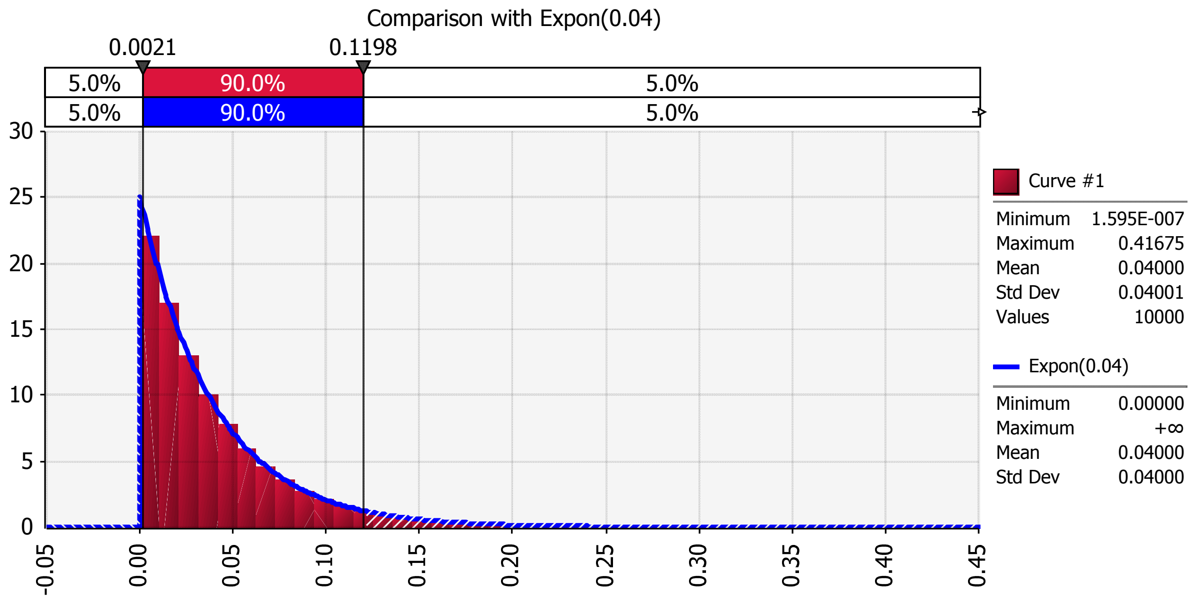


Figure 5.29: Monte Carlo simulation of Cape Town distribution point water indicating the probability distributions of reasonable maximum EEq values in ng/L

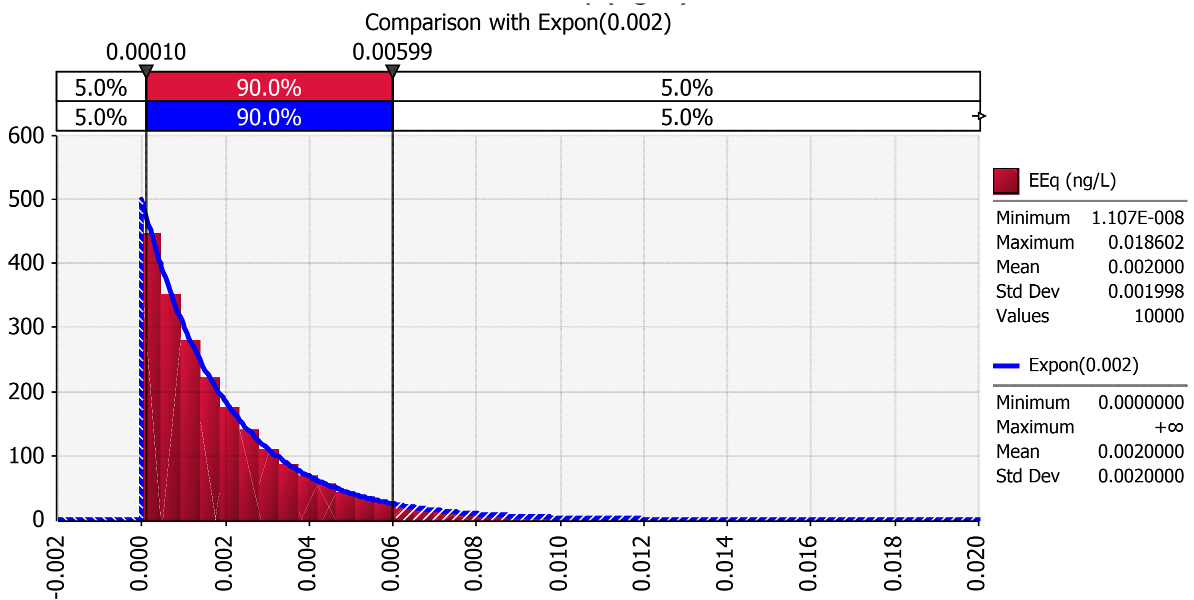


Figure 5.30: Monte Carlo simulation of bottled water indicating the probability distributions of reasonable maximum EEq values in ng/L

© University of Pretoria

For BPA the Monte Carlo simulations were run to determine the range and maximum non-cancerous HQ risks associated with the consumption of distribution point and bottled water. The results indicated a 90% certainty that the HQ would fall between 5 x 10^{-7} and 3 x 10^{-5} for Pretoria (Figure 5.31) and Cape Town (Figure 5.32) distribution point water with a maximum of 1.2 x 10^{-4} for Pretoria and a maximum of 1.3 x 10^{-4} for Cape Town water. In bottled water, the HQ is expected to fall between 2 x 10^{-6} and 1.198 x 10^{-4}, with a maximum of 4.09 x 10^{-4} (Figure 5.33). The maximum HQ values are well below one, indicating safe levels of BPA exposure from distribution point and bottled water.

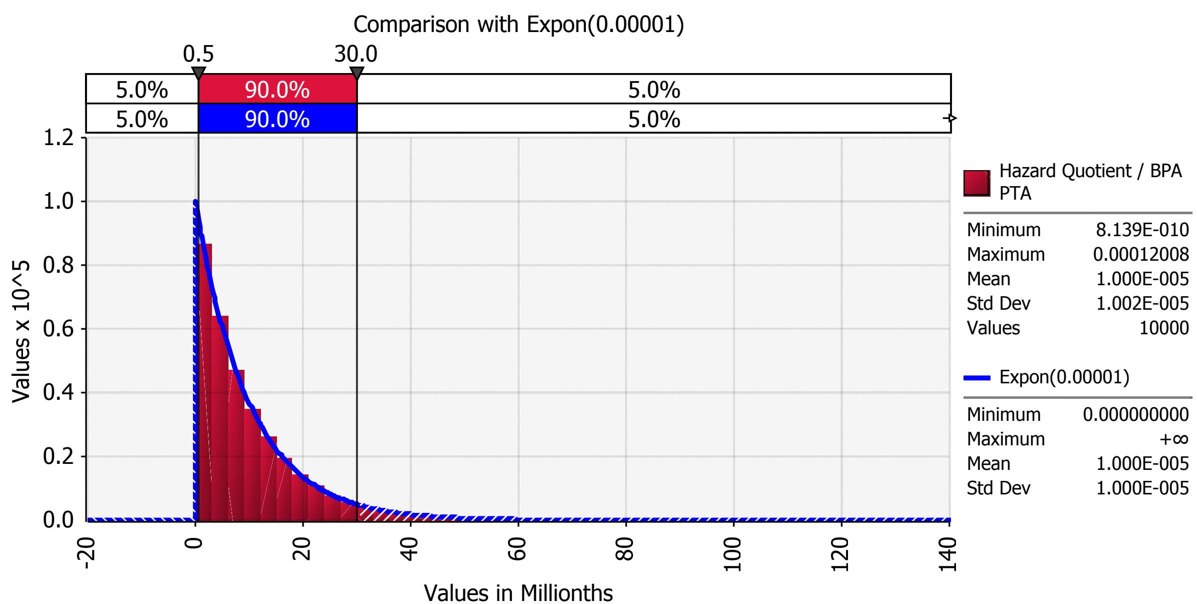


Figure 5.31: Monte Carlo simulation of HQ risks posed by BPA to people consuming distribution point water from Pretoria

© University of Pretoria

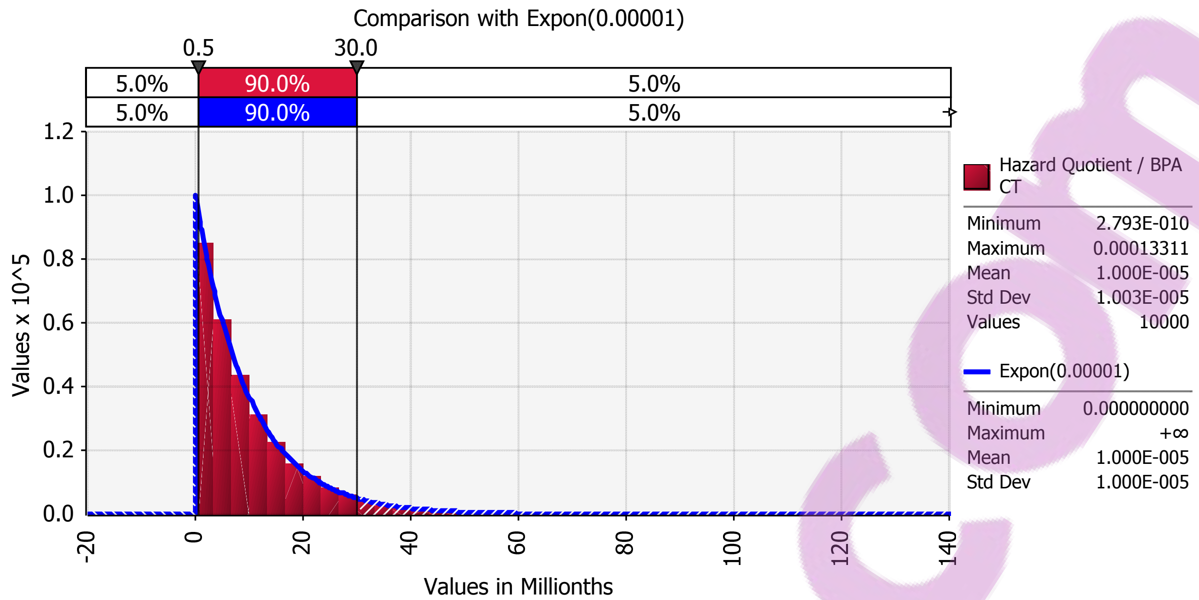


Figure 5.32: Monte Carlo simulation of HQ risks posed by BPA to people consuming distribution point water from Cape Town

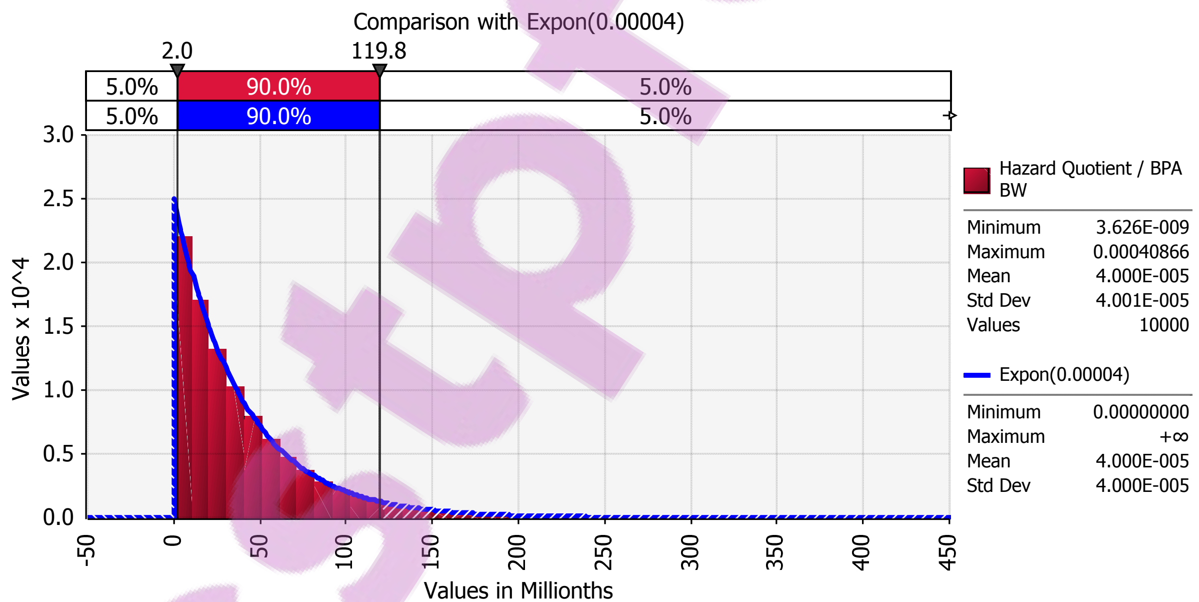


Figure 5.33: Monte Carlo simulation of HQ risks posed by BPA to people consuming bottled water

© University of Pretoria

For DEHA, uncertainty analysis revealed a 90% certainty of the HQ falling between 1×10^{-8} and 5.99×10^{-7} for Pretoria distribution point water (Figure 5.34), between 1×10^{-8} and 5.99×10^{-6} for Cape Town water (Figure 5.35) and between 1.6×10^{-7} and 9.28×10^{-6} for bottled water (Figure 5.36). For all the water samples, the simulated maximum HQ values are well below one (1.942×10^{-6} for Pretoria, 1.929×10^{-5} for Cape Town and 2.967×10^{-5} for bottled water), indicating safe levels of DEHA exposure from distribution point and bottled water.

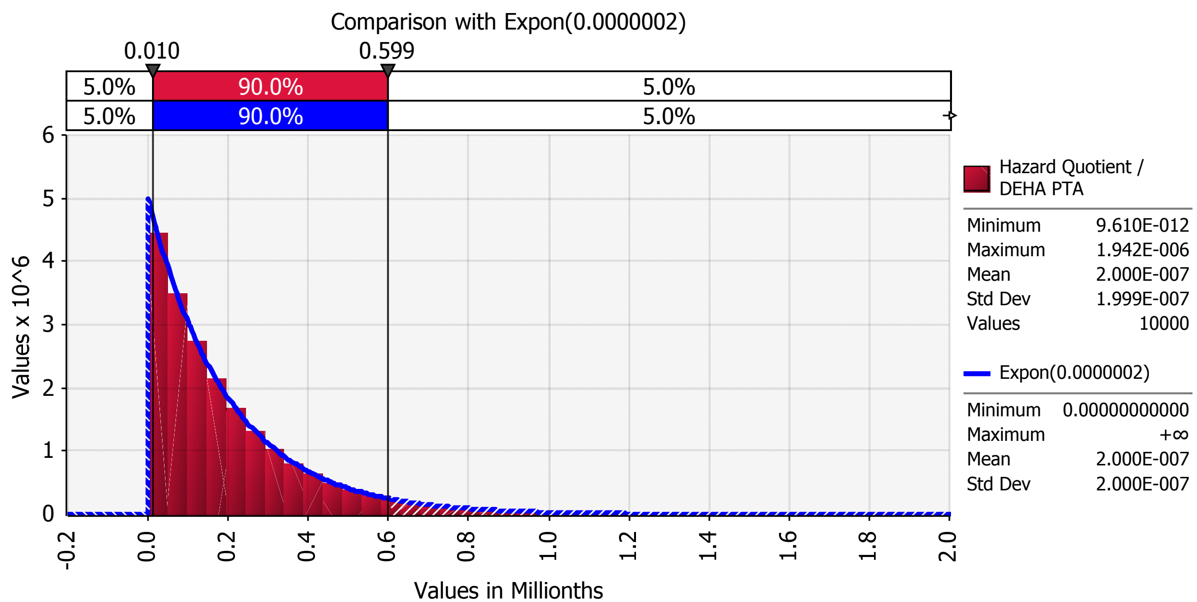


Figure 5.34: Monte Carlo simulation of HQ risks posed by DEHA to people consuming distribution point water from Pretoria

© University of Pretoria

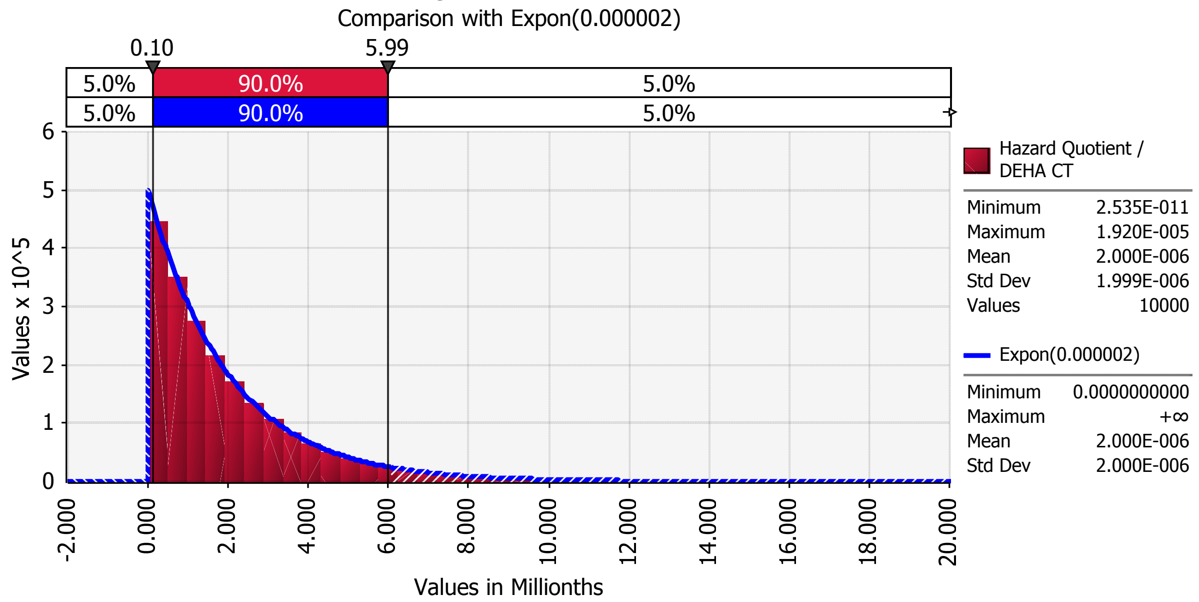


Figure 5.35: Monte Carlo simulation of HQ risks posed by DEHA to people consuming distribution point water from Cape Town

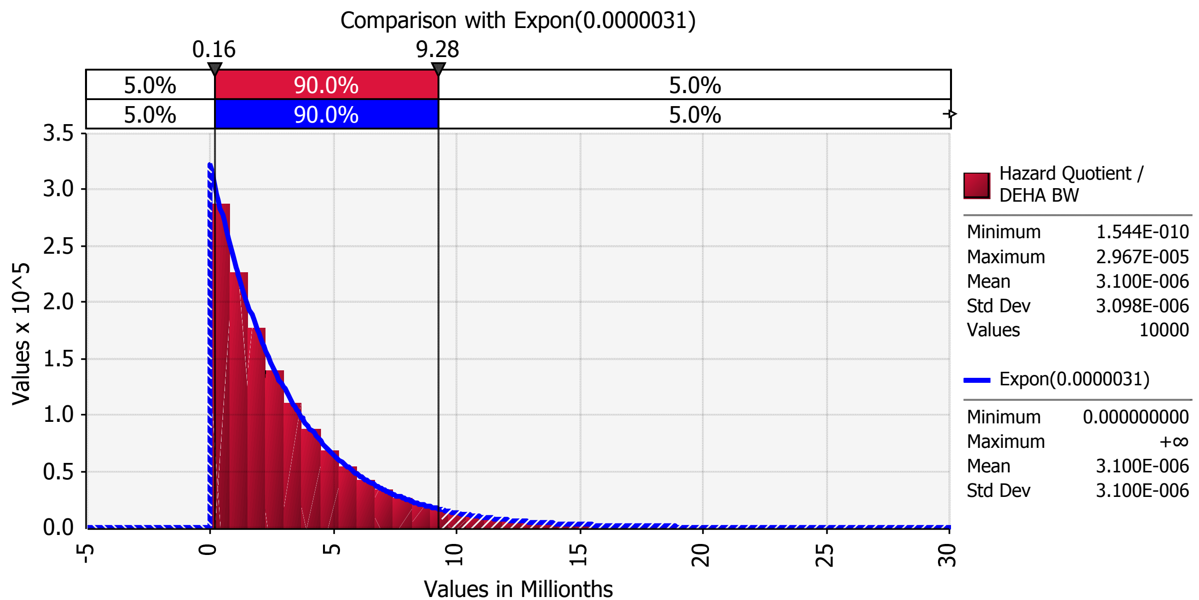


Figure 5.36: Monte Carlo simulation of HQ risks posed by DEHA to people consuming bottled water

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

In addition to the non-cancerous health risks, exposure to DEHA can also lead to the development of cancer. The predicted maximum cancer risk determined with the Monte Carlo simulation was 6.237 x 10^{-10} for distribution point water from Pretoria (Figure 5.37), 6.520 x 10^{-9} for Cape Town (Figure 5.38) and 8.850 x 10^{-9} for bottled water (Figure 5.39). Although the consumption of bottled water showed the highest risk for developing cancer, the risk is less than 1 in 100 000 000. That is 1 000 times lower than the 1 in 100 000 risk deemed acceptable by the USEPA.

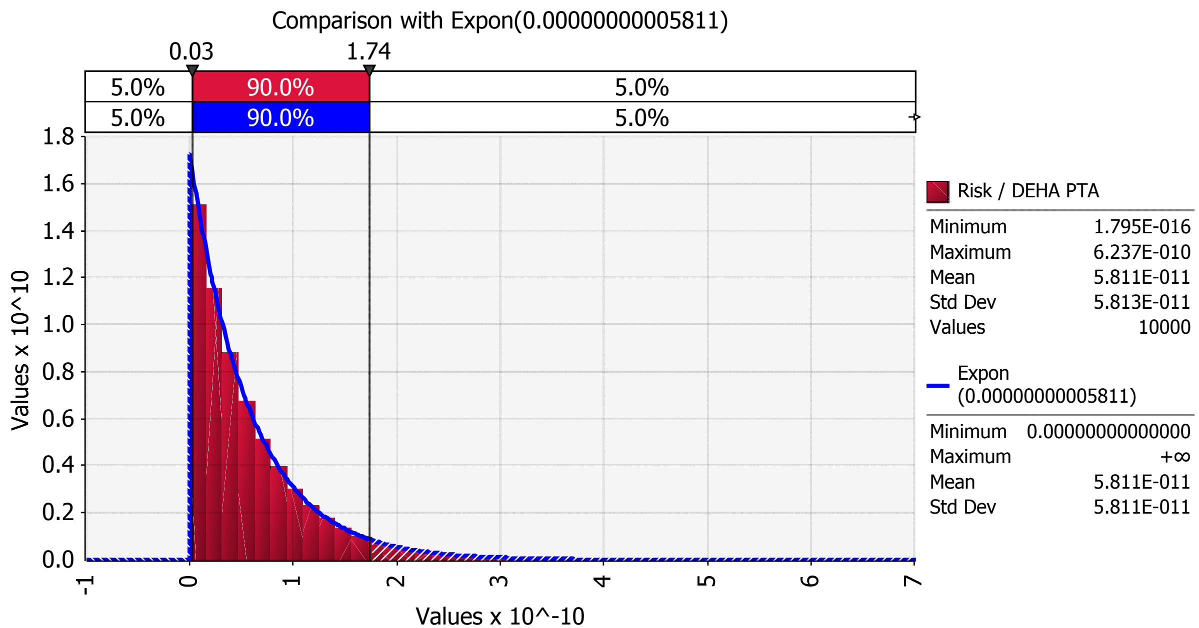


Figure 5.37: Monte Carlo simulation of cancer risks posed by DEHA to people consuming distribution point water from Pretoria

© University of Pretoria

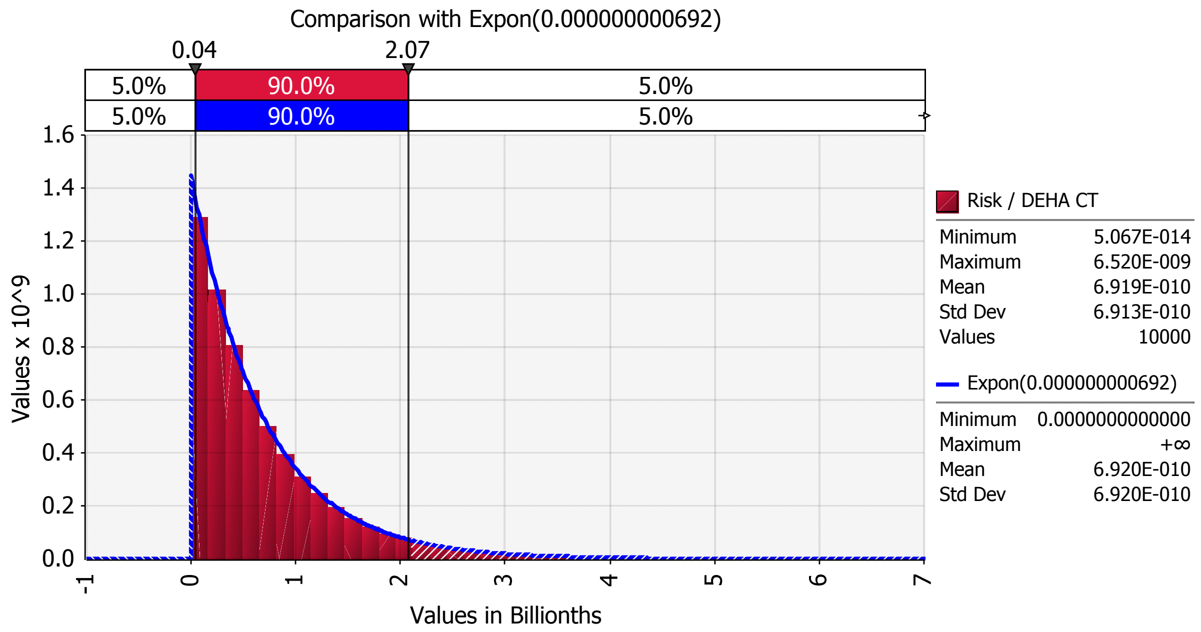


Figure 5.38: Monte Carlo simulation of cancer risks posed by DEHA to people consuming distribution point water from Cape Town

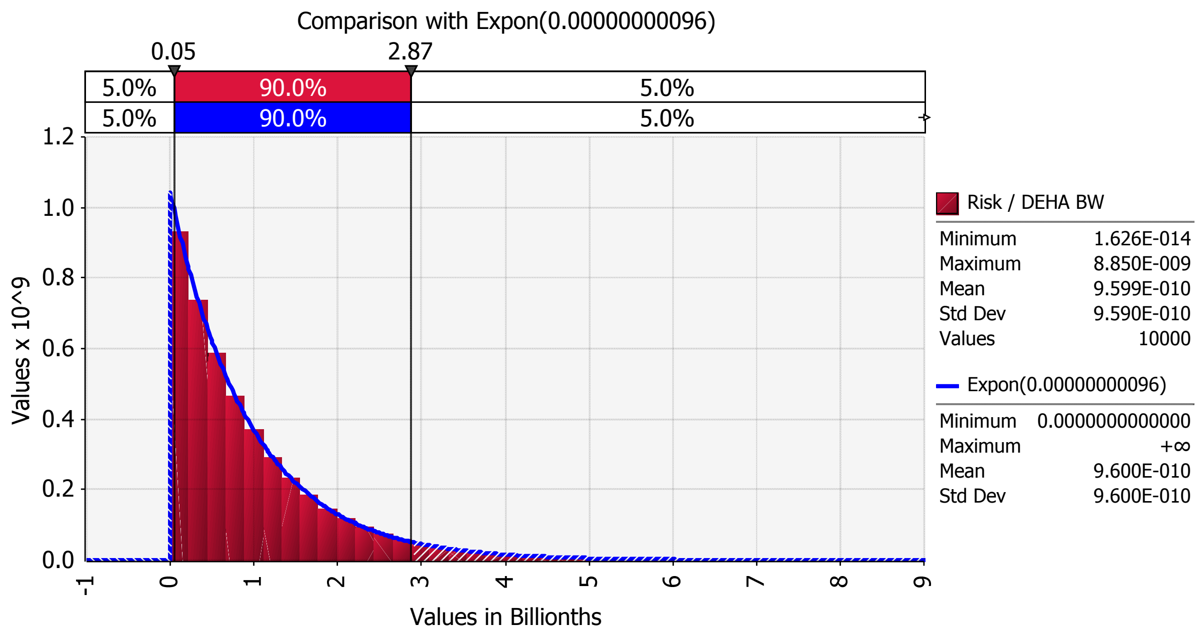


Figure 5.39: Monte Carlo simulation of cancer risks posed by DEHA to people consuming bottled water

© University of Pretoria

Uncertainty analysis revealed a 90% certainty for the HQ of DBP to fall between 6×10^{-6} and 3.29×10^{-4} in distribution point water from Pretoria (Figure 5.40), between 1.4×10^{-5} and 8.09×10^{-4} in water from Cape Town (Figure 5.41) and between 1.4×10^{-5} and 8.09×10^{-4} in bottled water (Figure 5.42). The maximum predicted HQ values were 1.04×10^{-3} for Pretoria, 2.97×10^{-3} for Cape Town and 2.90×10^{-3} for bottled water. All of the values are well below one, indicating the levels of DBP in the water are safe for a lifetime exposure.

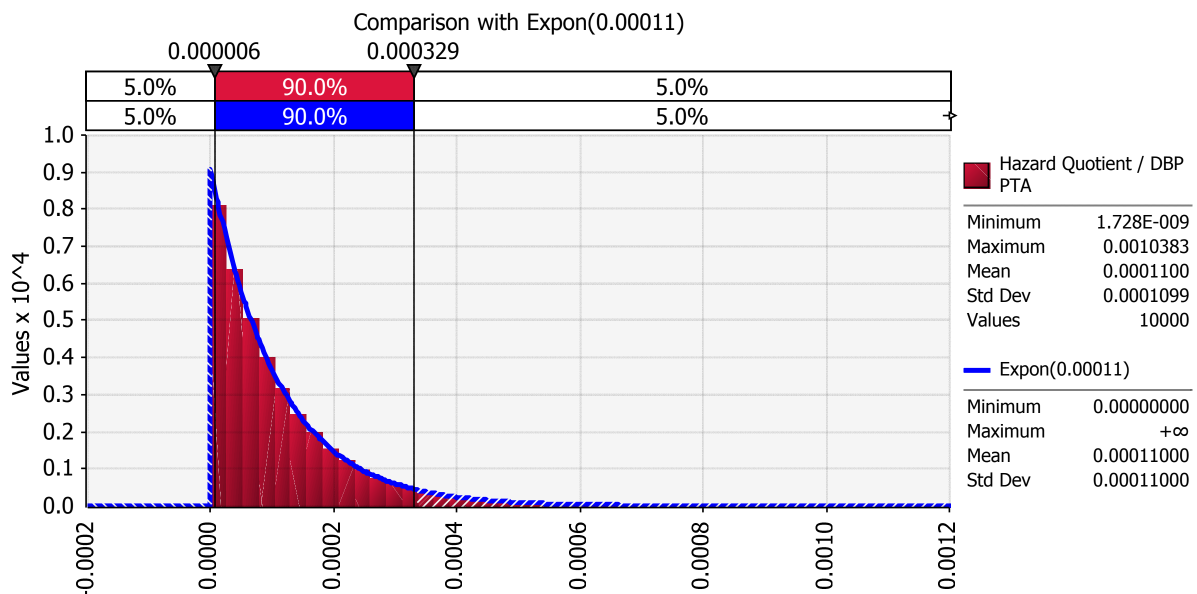


Figure 5.40: Monte Carlo simulation of HQ risks posed by DBP to people consuming distribution point water from Pretoria

© University of Pretoria

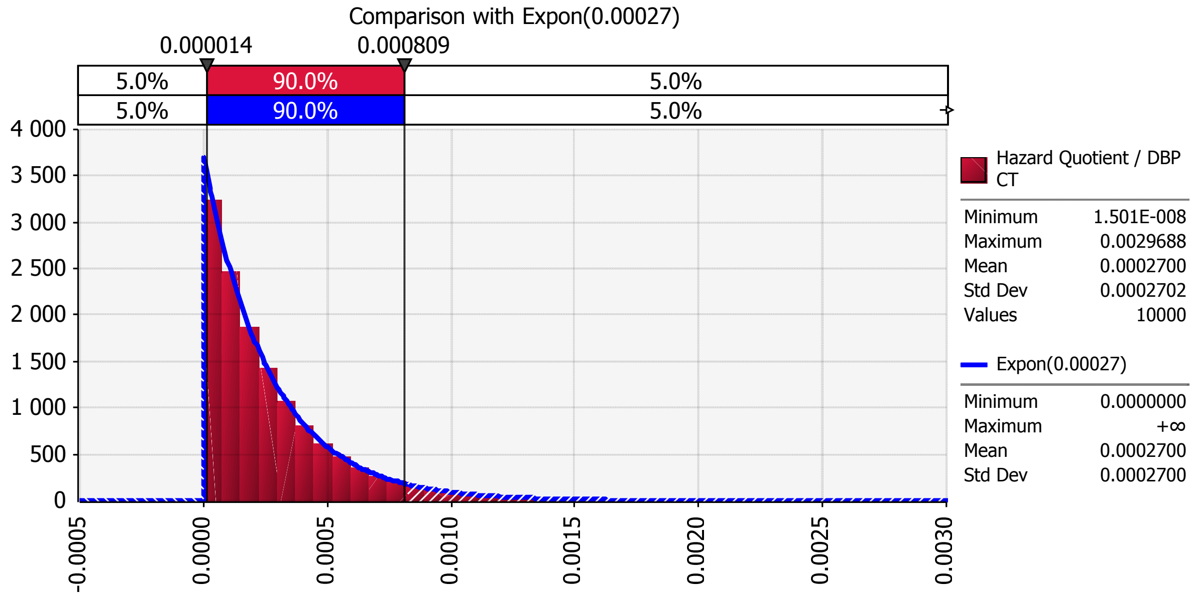


Figure 5.41: Monte Carlo simulation of HQ risks posed by DBP to people consuming distribution point water from Cape Town

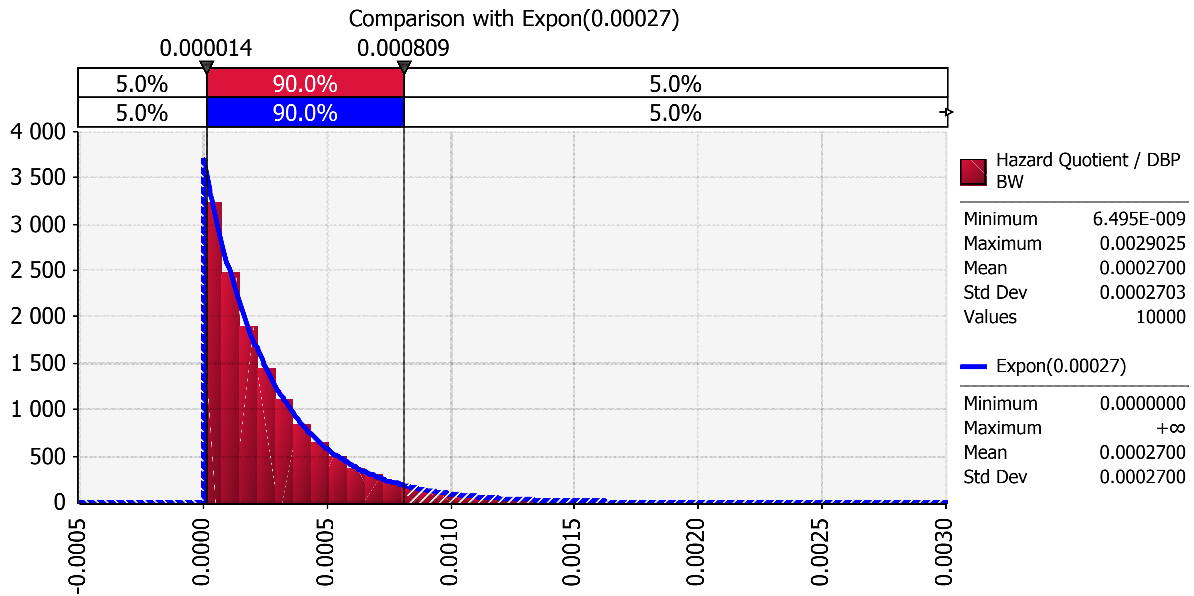


Figure 5.42: Monte Carlo simulation of HQ risks posed by DBP to people consuming bottled water

© University of Pretoria

For DEHP the HQ was expected to fall between 1.8 x 10^{-5} and 1.08 x 10^{-3} for Pretoria distribution points (Figure 5.43), between 3 x 10^{-4} and 1.77 x 10^{-2} for Cape Town distribution points (Figure 5.44) and between 1.2 x 10^{-5} and 6.89 x 10^{-4} for bottled water (Figure 5.45), with 90% certainty. The maximum HQ was 3.44 x 10^{-3} for Pretoria, 6.49 x 10^{-2} for Cape Town and 2.12 x 10^{-3} for bottled water. Although the maximum HQ was almost 20 times higher in the Cape Town distribution point water compared to the Pretoria distribution points and 30 times higher than the bottled water, it is still well below the acceptable value for a lifetime exposure.

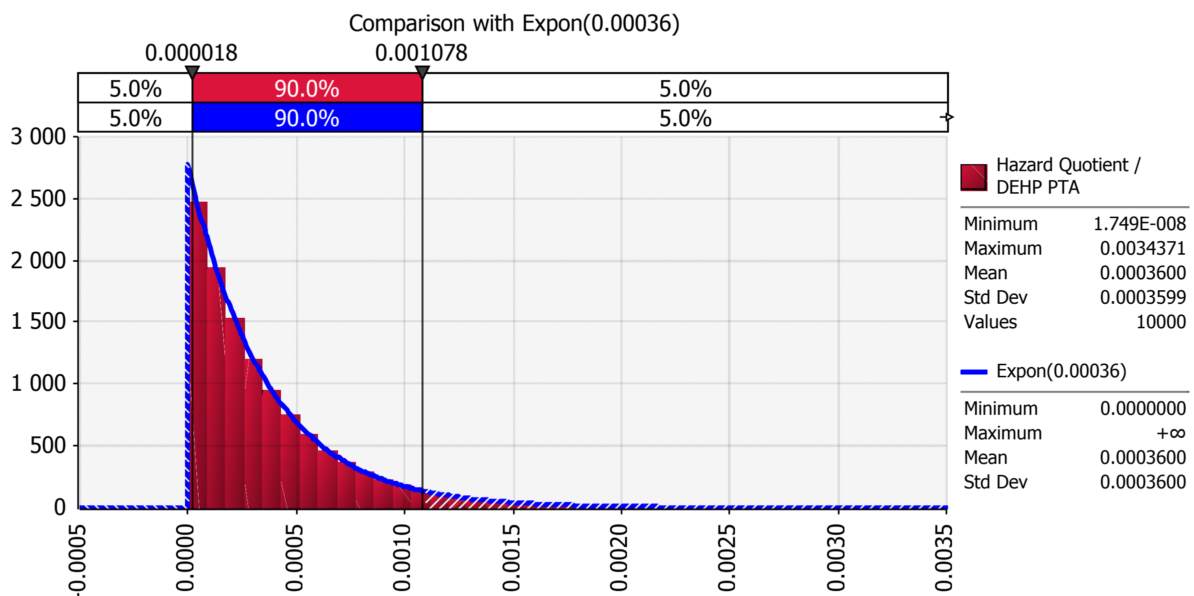


Figure 5.43: Monte Carlo simulation of HQ risks posed by DEHP to people consuming distribution point water from Pretoria

© University of Pretoria

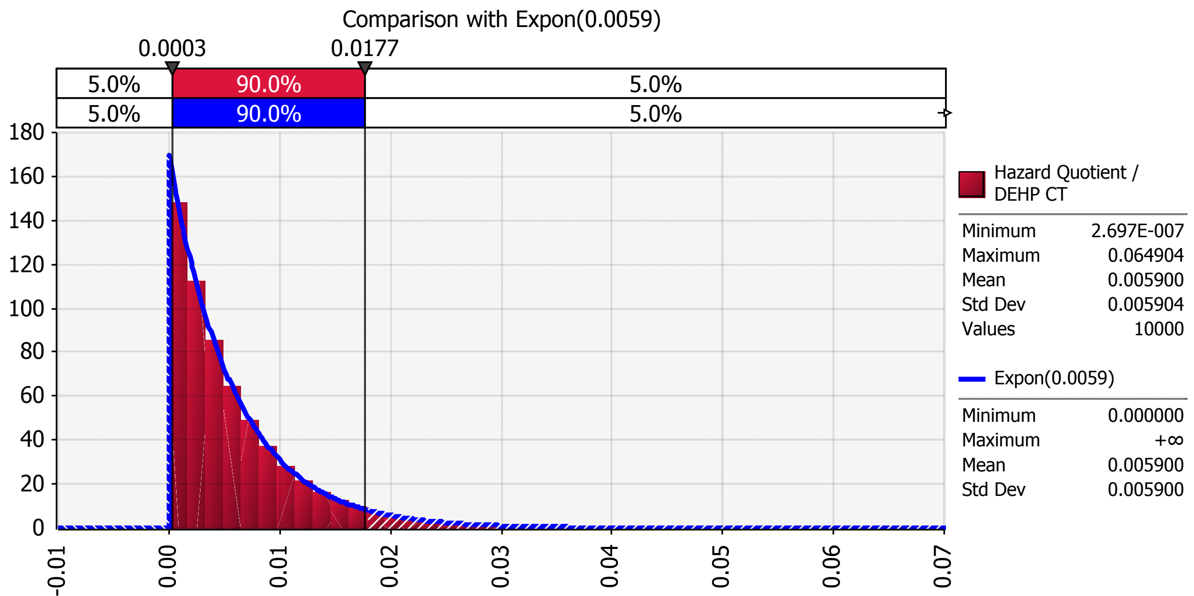


Figure 5.44: Monte Carlo simulation of HQ risks posed by DEHP to people consuming distribution point water from Cape Town

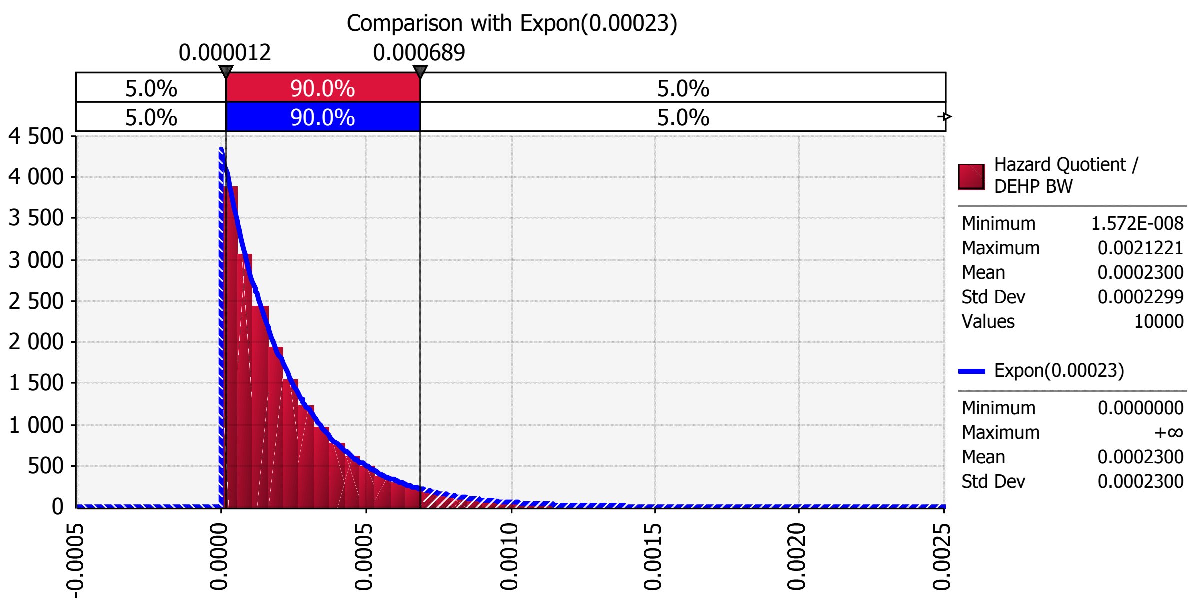


Figure 5.45: Monte Carlo simulation of HQ risks posed by DEHP to people consuming bottled water

© University of Pretoria

DEHP is also associated with the risk of developing cancer. With 90% certainty, the maximum cancer risk posed by DEHP was 4.795 x 10^{-7} for distribution point water from Pretoria (Figure 5.46), 8.292 x 10^{-6} for distribution point water from Cape Town (Figure 5.47) and 2.868 x 10^{-7} for bottled water (Figure 5.48), indicating acceptable cancer risks.

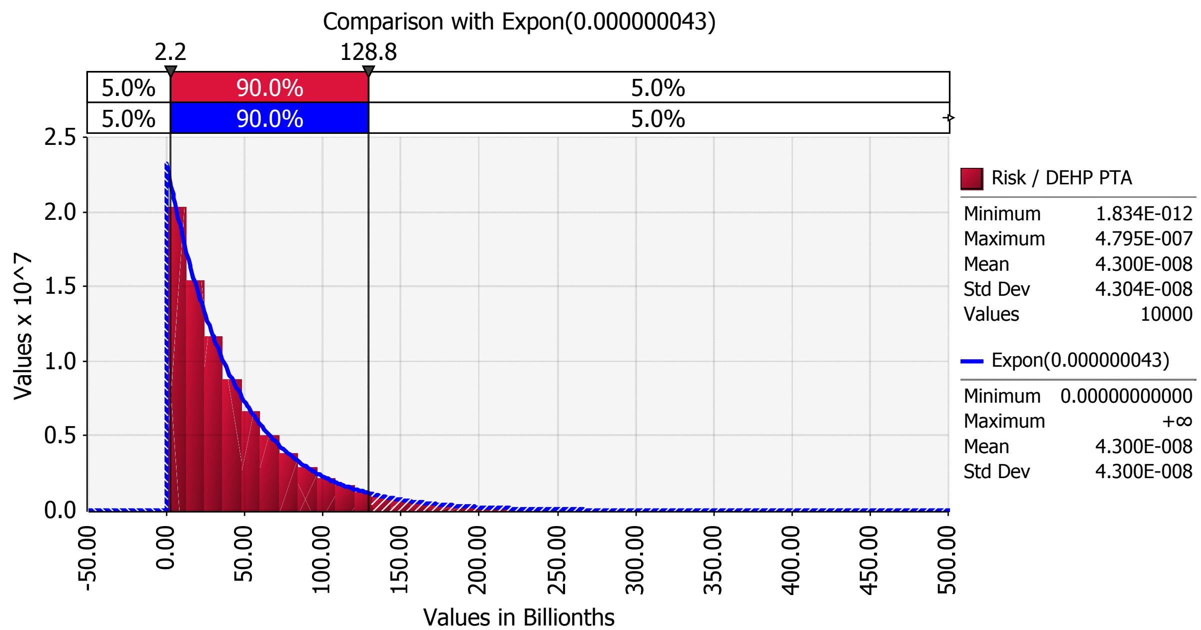


Figure 5.46: Monte Carlo simulation of cancer risks posed by DEHP to people consuming distribution point water from Pretoria

© University of Pretoria

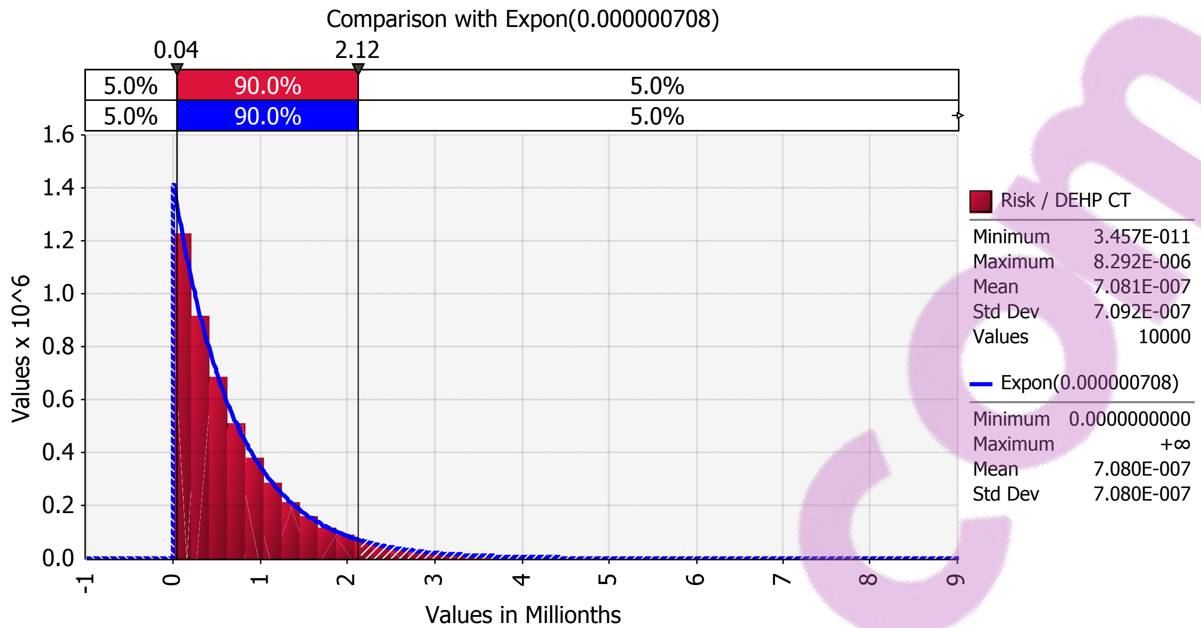


Figure 5.47: Monte Carlo simulation of cancer risks posed by DEHP to people consuming distribution point water from Cape Town

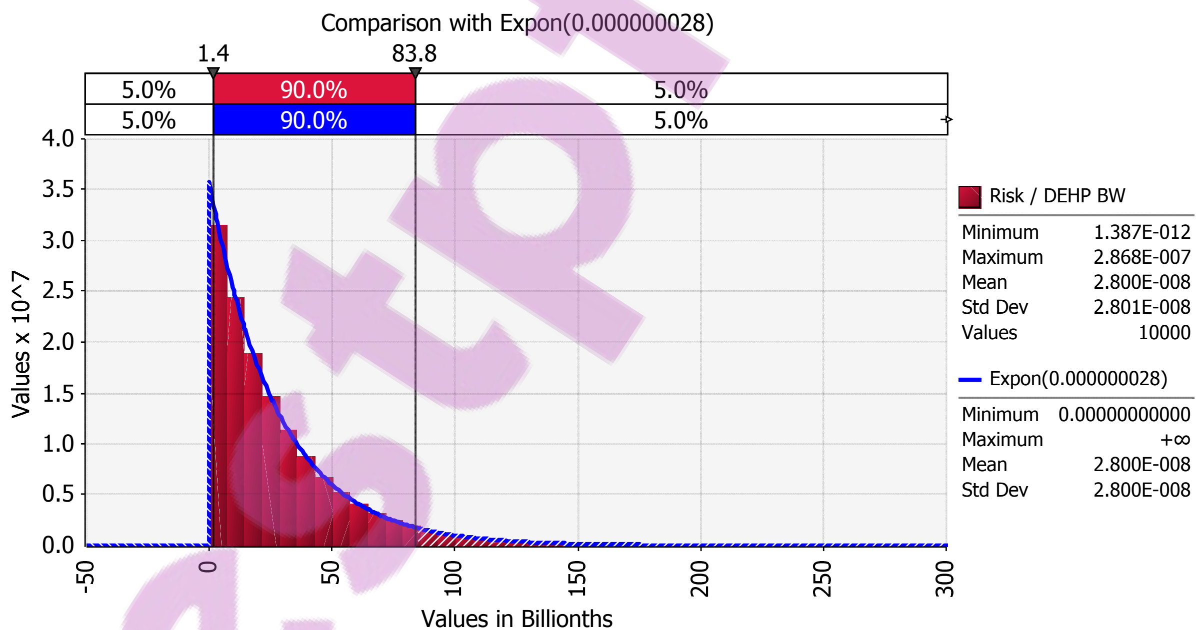


Figure 5.48: Monte Carlo simulation of cancer risks posed by DEHP to people consuming bottled water

© University of Pretoria

Uncertainty analysis revealed a 90% certainty for the HQ of DINP to fall between 1.5×10^{-5} and 8.98×10^{-4} for distribution point water from Pretoria (Figure 5.49), between 1.0×10^{-5} and 0.000569 for Cape Town distribution point water (Figure 5.50) and between 4×10^{-5} and 2.49×10^{-3} for bottled water (Figure 5.51). The predicted maximum HQ values were 2.94×10^{-3} for Pretoria, 1.82×10^{-3} for Cape Town and 8.21×10^{-3} for bottled water. All the values were well below one, indicating acceptable health risks associated with the consumption of the water.

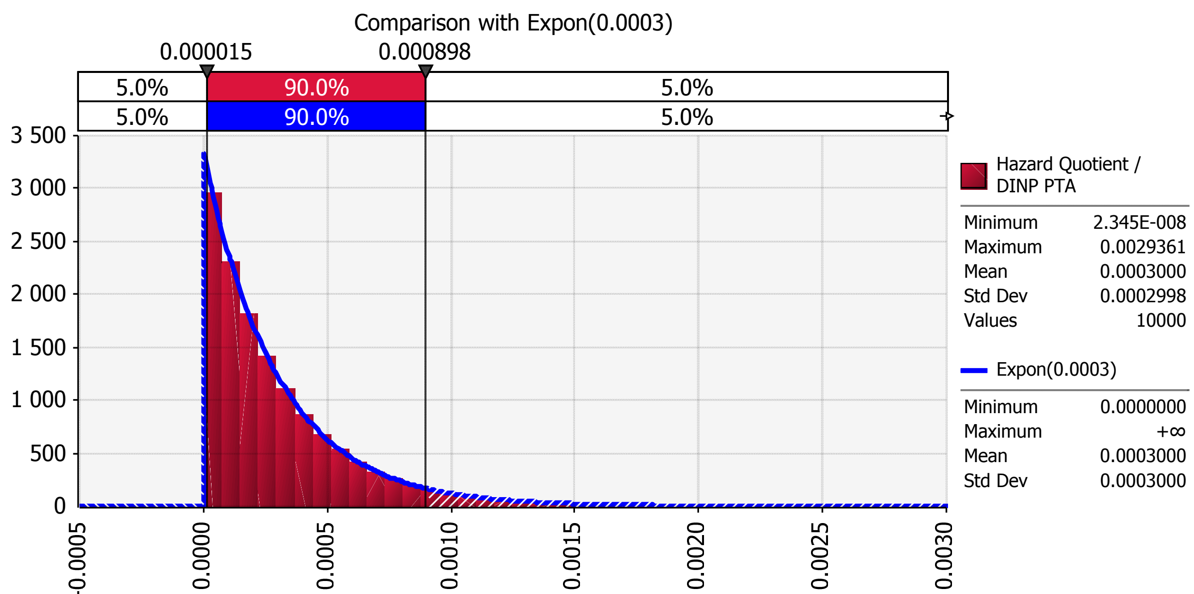


Figure 5.49: Monte Carlo simulation of HQ risks posed by DINP to people consuming distribution point water from Pretoria

© University of Pretoria

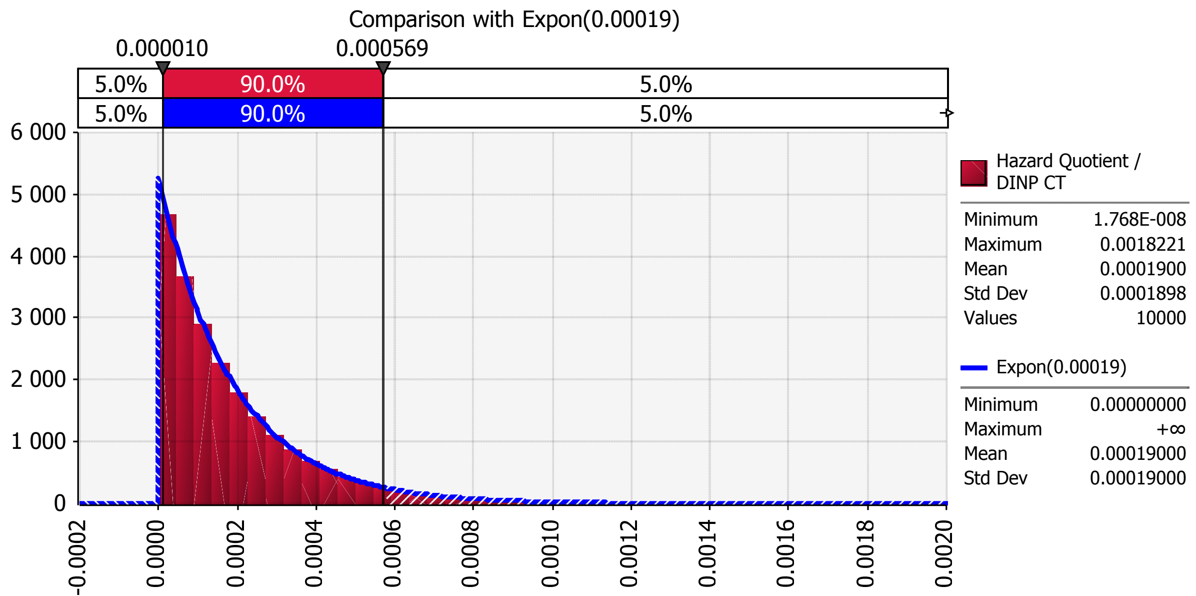


Figure 5.50: Monte Carlo simulation of HQ risks posed by DINP to people consuming distribution point water from Cape Town

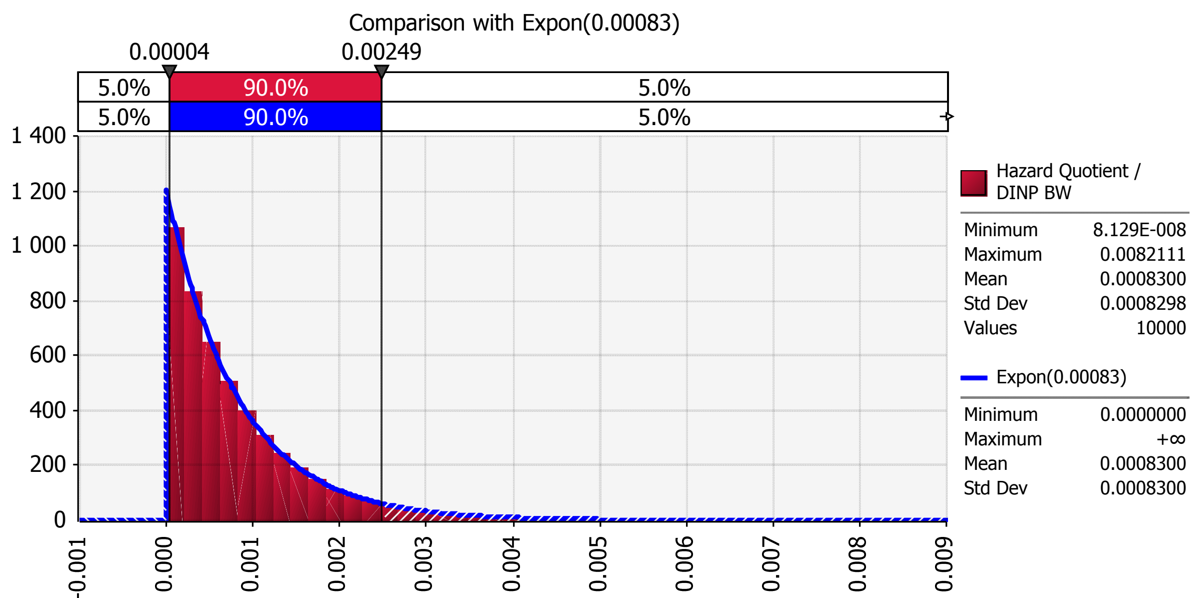


Figure 5.51: Monte Carlo simulation of HQ risks posed by DINP to people consuming bottled water

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

Uncertainty analysis for E_2 could only be done for Cape Town distribution points as only one sample from Pretoria was above the dl. The HQ for E_2 in Cape Town samples showed a 90% certainty to fall between 0.003 and 0.18, with a maximum predicted HQ of 0.89 (Figure 5.52), indicating an acceptable health risk.

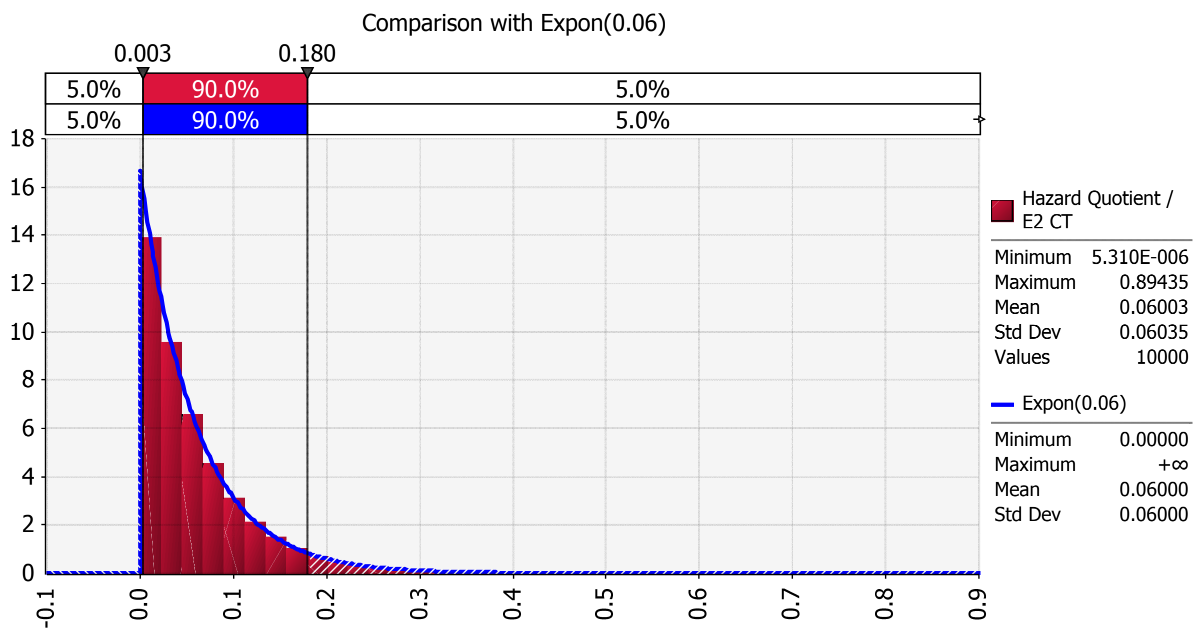


Figure 5.52: Monte Carlo simulation of HQ risks posed by E_2 to people consuming distribution point water from Cape Town

© University of Pretoria

The reasonable maximum HQ for E_1 in distribution point water from Pretoria has a 90% certainty to fall between 0.08 and 4.67 (Figure 5.53), indicating potential health risk. However, only 4 samples were above the dl. Recalculating the uncertainty analysis using average concentrations, the HQ is expected to fall between 0.011 and 0.629 with 90% certainty (Figure 5.54), indicating acceptable health risks.

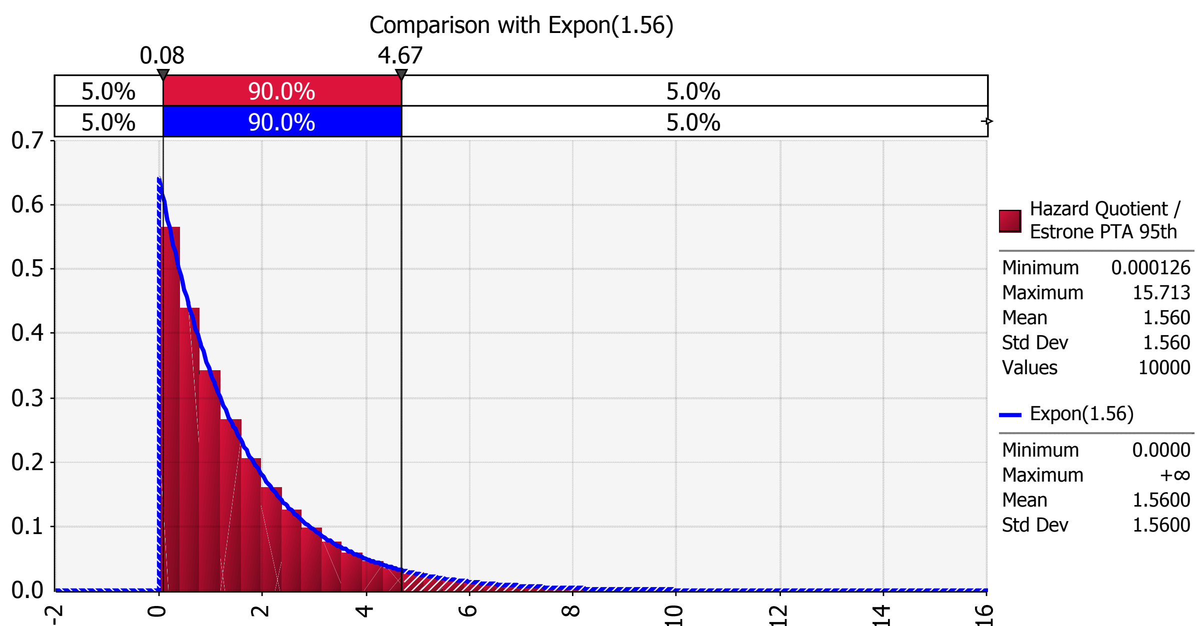


Figure 5.53: Monte Carlo simulation of HQ risks posed by E_1 to people consuming distribution point water from Pretoria (95th percentile)

© University of Pretoria

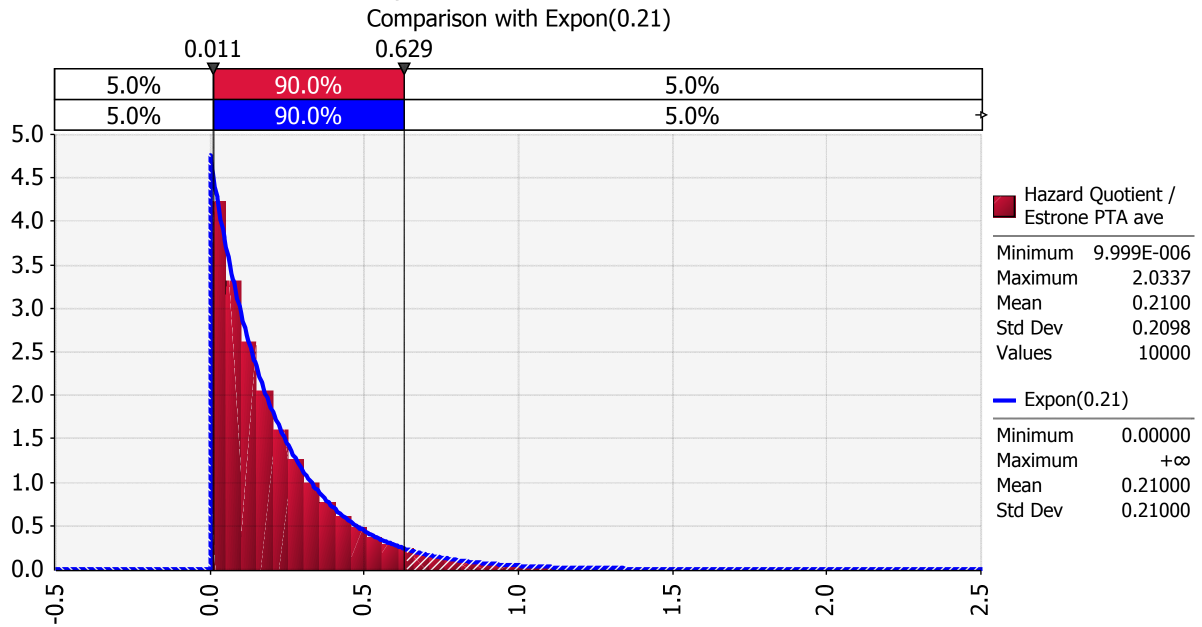


Figure 5.54: Monte Carlo simulation of HQ risks posed by E_1 to people consuming distribution point water from Pretoria (average)

© University of Pretoria

The HQ for E_1 is expected to fall between 0.0010 and 0.0599 in distribution point water from Cape Town (Figure 5.55) and between 0.017 and 0.988 in bottled water (Figure 5.56), indicating acceptable non-cancerous risk.

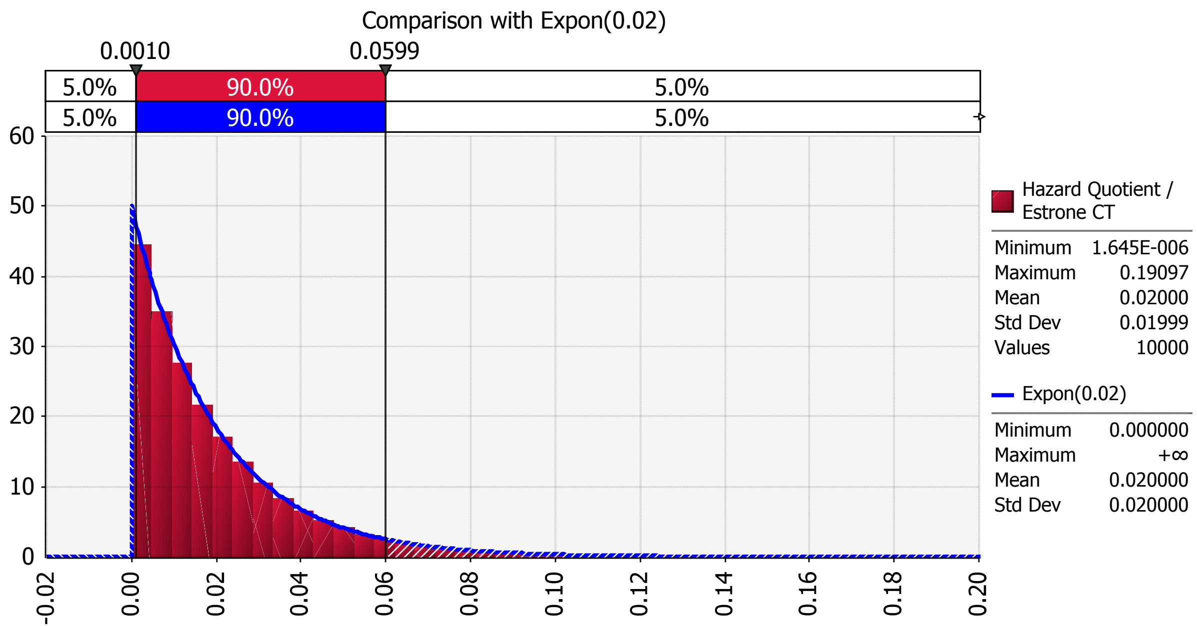


Figure 5.55: Monte Carlo simulation of HQ risks posed by E_1 to people consuming distribution point water from Cape Town

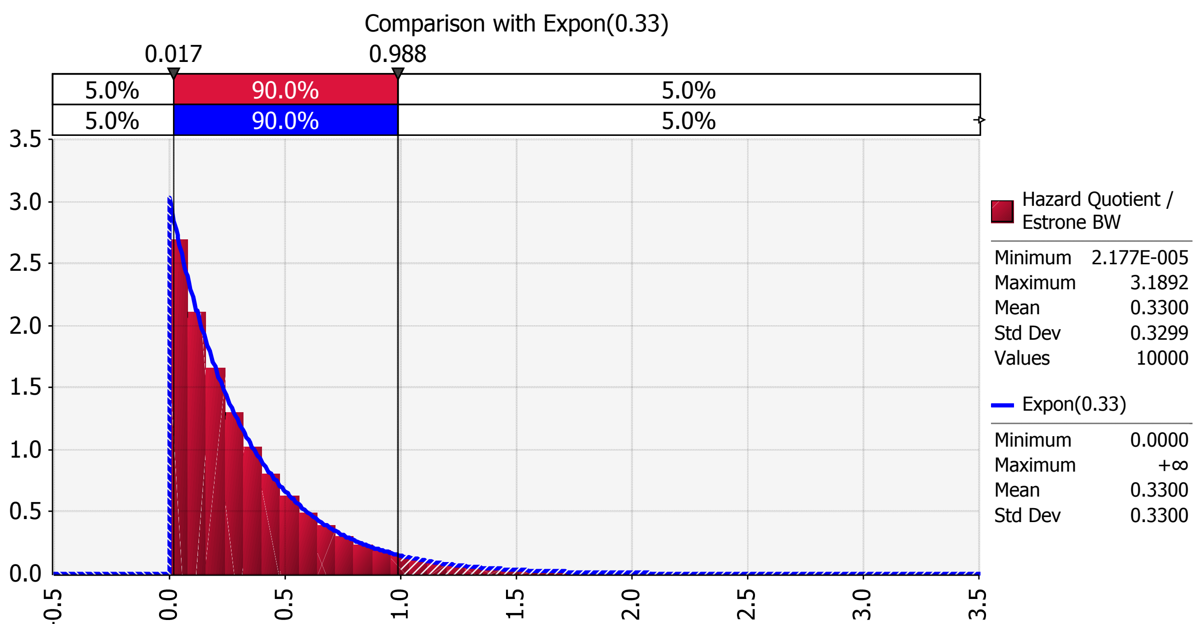


Figure 5.56: Monte Carlo simulation of HQ risks posed by E_1 to people consuming bottled water

© University of Pretoria

Uncertainty analysis of EE_2 in distribution point water from Pretoria revealed a 90% certainty for the HQ to fall between 0.0005 and 0.0300 (Figure 5.57). For bottled water the HQ is expected to fall between 0.007 and 0.389 (Figure 5.58), which is also well below 1, indicating acceptable risk for a lifetime exposure.

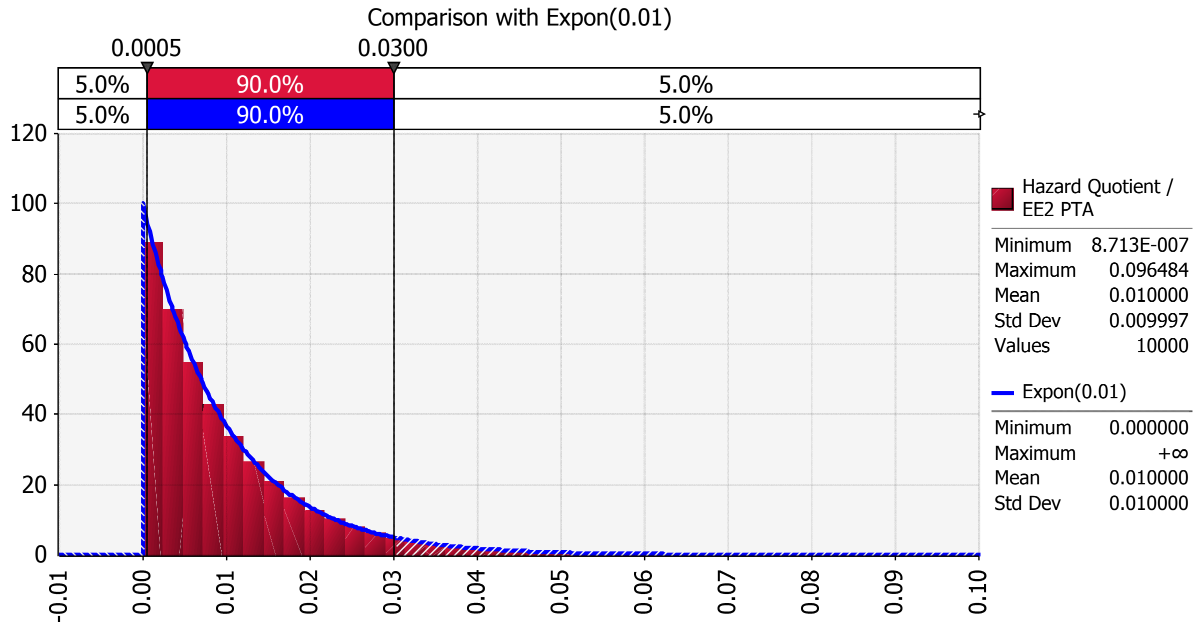


Figure 5.57: Monte Carlo simulation of HQ risks posed by EE_2 to people consuming distribution point water from Pretoria

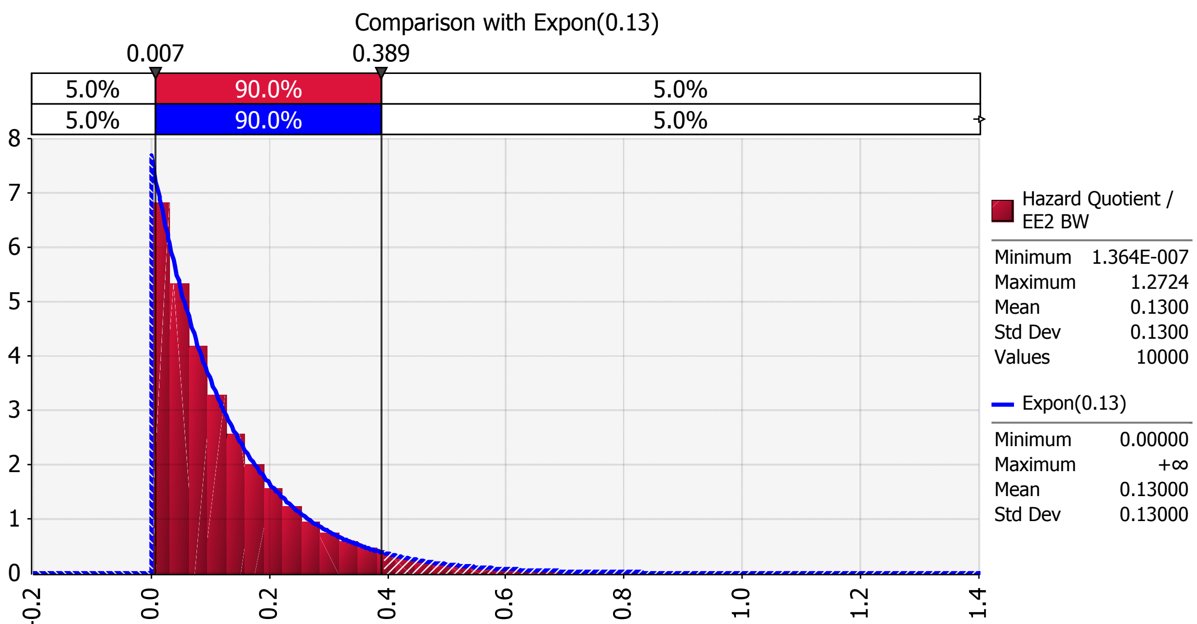


Figure 5.58: Monte Carlo simulation of HQ risks posed by EE_2 to people consuming bottled water

© University of Pretoria

Chapter 6: Discussion and Conclusions

6.1. General discussion on methods

Oasis HLB cartridges were used for SPE in this study. Although Oasis HLB cartridges show good recoveries compared to other extraction cartridges,[234] a study by Avbersek et al.[235] showed higher estrogenic activity in samples from a waste water treatment plant without sample extraction compared to the samples extracted using these cartridges. The authors postulated that water soluble endocrine agonists are removed from the sample during SPE.[235] It is therefore possible that the estrogenic activity in this study could be underestimated. However, the very low concentrations of target chemicals in tap and bottled water necessitate the use of SPE to concentrate samples in order to obtain lower detection limits.[235]

A comparison of five *in vitro* bioassays by Leusch et al.[210] showed that the ER-CALUX had the highest sensitivity and good correlation with chemical analysis. However, this bioassay is a commercially available product, and therefore not an economical option to use.[106] The T47D-KBluc bioassay also performed well in the study by Leusch et al.[210] and has been included in the South African toolbox of bioassays for estrogenic activity.[222] It was therefore a suitable bioassay to use for this study. Although the YES bioassay has several advantages, including its robustness, lack of endogenous receptors that can interfere with the response of the cells, lower cost and uncomplicated bioassay procedure, yeast-based bioassays are less sensitive compared to human cell lines.[17,73,210] The YES bioassay was not sensitive enough to detect the low levels of estrogenic activity in the drinking and bottled water in this study. Although the YES might be an excellent low cost screening bioassay for waste water samples where high levels of activity is expected, the YES is not a suitable bioassay for drinking water with low levels of estrogenic activity.

It is a challenge to detect different classes of EDCs at very low levels using a single analytical method.[75] In this study, NP was not detected in any of the samples, but NP also had the lowest recovery (64%). However, all the other target chemicals were detected in samples and good recoveries were obtained, indicating that overall the

© University of Pretoria

extraction method and UPLC-MS analysis were suitable methods to use for the purposes of this study.

Chemical analysis and bioassays can complement one another. Chemical analysis can identify and quantify specific compound that are present in a sample, whereas bioassays can assess the resultant activity of a mixture of chemicals in an environmental sample that may include unknown compounds.[236] In this study, the only target chemicals that were detected at concentrations that might elicit a response as an individual chemical in the T47D-KBluc bioassay was E_1 and EE_2. However, estrogenic activity was also detected in samples where E_1 and EE_2 were below the dl. Estrogenic activity in the samples is therefore probably due to the resultant activity of a combination of chemicals.

6.2. Distribution point water

In this study, no estrogenic activity was detected in distribution point water using the YES bioassay. Similarly, no estrogenic activity was detected in drinking water from São Paulo, Brazil[237] and Finland[238] using yeast based bioassays. However, estrogenic activity (EEq: 0.02-0.20 ng/L) was detected in drinking water from China using the YES bioassay.[107] Estrogenic activity was detected in distribution point water in this study using the T47D-KBluc bioassay, with EEq values ranging from 0.002 to 0.144 ng/L. Estrogenic activity was also detected in drinking water from Taiwan (T47D-KBluc bioassay),[106] The Netherlands (ERα CALUX),[105] Italy (HELN-ERα luciferase reporter bioassay)[108] and the US (E-screen),[109] at concentrations ranging from below the ql to 0.77 ng/L. No estrogenic activity was detected in drinking water from France using the MELN luciferase reporter gene bioassay[233] and a vitellogenin assay using adult male zebrafish.[239] Table 6.1 compares the estrogenic activity in drinking water from different countries and studies.

© University of Pretoria

Table 6.1: Comparison of estrogenic activity in drinking water from various countries

Country	Year of publication	Extraction method	Estrogenic bioassay	EEq (ng/L)	Reference
Pretoria and Cape Town, South Africa		SPE	YES T47D-KBluc	<dl 0.002-0.144	This study
Brazil (São Paulo)	2011	SPE	Bioluminescent yeast assay (BLYES)	<dl	237
China (Jiangsu province)	2016	SPE	YES	0.02-0.20	107
Finland	2015	SPE	Yeast bio-reporter assay	<dl	238
France (Paris)	2007	No extraction	Vitellogenin assay (ELISA) using adult male zebrafish	<dl	239
France (Paris)	2009	SPE	Luciferase reporter gene assay using MELN cells	<dl	233
Italy	2013	SPE	HELN-ERα luciferase reporter assay	0.0136*	108
Taiwan	2016	LLE	T47D-KBluc	<ql-1.3	106
The Netherlands	2013	Not specified	ERα CALUX	0.022-0.032	105
US	2010	SPE	E-screen	0.19-0.77	109

* Maximum concentration

<dl Below detection limit

<ql Below quantification limit

© University of Pretoria

No anti-estrogenic activity was detected in any of the water samples in this study. Van der Linden et al.[90] also reported no antagonist activity in different water sources in The Netherlands (industry, hospital and municipal sewage effluents, tap water and surface water) and ascribed it to the mixture of agonists and antagonists that is possibly masking the contribution of each individual compound.

Numerous potential EDCs have been detected in drinking water from various countries. This study analysed distribution point water from Pretoria and Cape Town for BPA, NP, DEHA, DBP, DEHP, DINP, E_2, E_1 and EE_2. Table 6.2 compares the levels of these target chemicals in this study to the concentrations found in drinking water from other countries.

© University of Pretoria

Table 6.2: Comparison of target chemicals in drinking water from various countries

Country	Year of publication	Extraction method	Analysis method#	BPA (ng/L)	NP (ng/L)	DEHA (ng/L)	DBP (ng/L)	DEHP (ng/L)	DINP (ng/L)	E_2 (ng/L)	E_1 (ng/L)	EE_2 (ng/L)	Reference
Pretoria and Cape Town, South Africa		SPE	UPLC-MS	0.01-28.83	<dl	0.91-4.97	109-1 065	39-5 150	3-1 251	0.02-0.05	0.36-4.89	0.003-0.06	Thisstudy
Brazil (Campinas)	2010	SPE	GC-MS	160**	<dl	-	-	-	-	<ql	<ql	<dl	120
Brazil (São Paulo)	2011	SPE	LC-MS/MS	<dl	-	-	-	-	-	<dl	<dl	<dl	237
Brazil (Piracicaba city)	2015	SPE	LC-ESI-MS/MS	-	-	-	-	-	-	<dl	<dl	<dl	240
Canada (Ontario)	2014	SPE	LC-MS/MS	-	-	-	-	-	-	-	0.03-1.5	-	119
China (Chongqing)	2005	SPE	NPLC-ESI-MS	-	100-2700	-	-	-	-	-	-	-	123
China	2008	Immunoaffinity monolithic column	LC-ESI/MS/MS	1	-	-	-	-	-	-	-	-	116
China	2012	SPE	TQMS	-	-	<dl-25**	1.1 - 930**	6.2 - 280**	<dl - 29**	-	-	-	110
China	2015	SPE	GC-MS	-	-	-	180*; 350**	180*; 770**	-	-	-	-	117
China (Jiangsu province)	2016	SPE	LC-MS/MS	0.17-1.22	<dl	-	-	-	-	-	-	-	107
France	2014	On-line SPE	LC-MS/MS	<9-50	<35-505	-	-	-	-	-	-	-	112
Germany	2001	SPE	HRGC-(NCI)-MS	0.50-2.0	2.50-16	-	-	-	-	0.20-2.1	0.20-0.60	0.15-0.50	115
Germany (Leipzig)	2001	SPME	GC-MS	-	-	-	380	50	-	-	-	-	118
Italy	2007	SPE	LC-MS/MS	<dl	<dl	-	-	-	-	<dl	<dl	<dl	65
Italy	2013	SPE	LC-ESI-MS/MS	0.82-102	10.30-84.00	-	-	-	-	<dl	<dl	<dl	108
Poland	2001	SPME	GC-MS	-	-	-	640	60	-	-	-	-	118

© University of Pretoria

Country	Year of publication	Extraction method	Analysis method#	BPA (ng/L)	NP (ng/L)	DEHA (ng/L)	DBP (ng/L)	DEHP (ng/L)	DINP (ng/L)	E_2 (ng/L)	E_1 (ng/L)	EE_2 (ng/L)	Reference
Portugal (Lisbon)	2006	SBSE-LD	LVI-GC-MS	-	-	90	520	60	-	-	-	-	122
Portugal	2014	SPME	IL-GC-FID(MS)	-	-	-	<dl	130-190	-	-	-	-	121
Portugal	2015	SPE	UPLC-ESI-MS/MS	<dl	<ql	-	-	-	-	<dl	<dl	<dl	241
Spain (Catalonia)	2003	SPE	GC-MS	6-25	24	-	16-32	331	-	-	-	-	111
Spain (Madrid)	2014	SPE	GC-MS	-	-	-	633 ± 255	<dl	-	-	-	-	113
Spain (Madrid)	2014	Online pre-concentration	LC-LC-MS/MS	3.7-50.3	2.5-20.5	-	-	-	-	<dl	<dl	<dl	114
Taiwan	2016	LLE	GC/MS	<dl	<dl	-	163-210**	773-1350**	<dl	-	-	-	106
US (Cape Cod, Massachusetts)	1998	LLE	GC-MS	20-44	<dl	-	-	-	-	-	-	-	91
US	2004	Continuous LLE	GC-MS	420***	<dl	-	-	-	-	-	-	-	124
US	2009	SPE	LC-MS/MS and GC-MS/MS	25	97*	-	-	<dl	-	<dl	<dl	<dl	242
US (Southeastern)	2014	SPE	LC/MS/MS	0-44.3	12.4-60.6	-	-	-	-	-	-	-	102
US	2016	SPE	LC-FTMS	-	-	-	-	-	-	<dl	<dl	<dl	236

GC-MS: gas chromatography-mass spectrophotometery; GC-MS/MS: gas chromatography-tandem mass spectrophotometery; HRGC-(NCI)-MS: high-resolution gas chromatography with negative chemical ionization mass spectrometry; IL-GC-FID(MS): ionic liquid gas chromatography associated with flame ionization detection or mass spectrometry; LC-ESI-MS/MS: liquid chromatography-electrospray ionization tandem mass spectrometry; LC-FTMS: liquid chromatography-Fourier transform mass spectrometry; LC-MS/MS: liquid chromatography-tandem mass spectrometry; LVI-GC-MS: large volume injection and capillary gas chromatography coupled to mass spectrometry; NPLC-ESI-MS: normal phase liquid chromatography electrospray ionization mass spectrometry; TQMS: triple-quadrupole mass spectrometry; UPLC-MS: ultra-performance liquid chromatography-mass spectrophotometery

*	Median concentration	**	Average concentration	***	Maximum concentration	-	Not analysed
<dl	Below detection limit	<ql	Below quantification limit				

© University of Pretoria

BPA was detected in most of the distribution point samples in this study, with quantifiable concentrations ranging from 0.01 to 28.83 ng/L. BPA was also detected in drinking water from Brazil,[120] China,[107,116] France,[112] Germany,[115] Italy,[108] Spain[111,114] and the US[91,102,124,242] at concentrations ranging from 0.17 to 420 ng/L (Table 6.2). BPA was not detected in drinking water from Portugal[241] and Taiwan.[106] Incomplete removal at drinking water treatment facilities[124] and the epoxy resin used for protective coatings in drinking water distribution pipes are possible sources of BPA in drinking water.[92]

NP was below the dl in this study. Similarly, NP was below the dl in drinking water from Brazil,[120] China[107] and Taiwan[106] and was below the ql in drinking water from Portugal.[241] NP was detected in drinking water samples from reservoirs from a water supply system in Portugal, but was below the quantification limit.[241] NP was detected in drinking water from China,[123] France,[112] Germany,[115] Italy,[108] Spain[111,114] and the US.[102,242] Concentrations varied from 2.5 to a maximum concentration of 2 700 ng/L in drinking water from Chongqing, China.[123] Drinking water might contain residual amounts of chlorine from the disinfection procedure that reacts with NP to form diverse chlorinated byproducts.[175] This could explain why NP was not detected in the samples from this project.

DEHA concentrations above the ql ranged from 0.91 to 4.97 ng/L in distribution point water from Pretoria and Cape Town. Higher DEHA concentrations were detected in drinking water from eastern China, with average concentrations ranging from 0.15 to 25 ng/L[110] and in Portugal where 90 ng/L DEHA was detected in tap water.[122]

The average DBP concentrations for this study were 230.42 ng/L for Pretoria and 427.04 ng/L for Cape Town water, but the concentrations ranged from 109 to 1 065 ng/L. Similar concentrations were detected in drinking water from China,[110,117] Poland,[118] Germany,[118] Madrid, Spain[113] and Taiwan.[106] Lower DBP concentrations were detected in water from drinking water fountains in Catalonia, Spain (16-32 ng/L).[111] Tap water from Lisbon, Portugal showed varied results. Serôdio et al.[122] reported 520 ng/L DBP in tap water from Lisbon (published in 2006), but DBP was below the dl in a study by Santana et al.[121] published in 2014.

© University of Pretoria

In this study, the average DEHP concentration was 75.68 ng/L for Pretoria and 445.83 ng/L for Cape Town. The higher average DEHP concentration of the Cape Town samples can be attributed to the high DEHP concentrations (4 120 ng/L to 5 151 ng/L) measured between October 2013 and April 2014 at one of the sampling points (CPT04). The July 2014 sample for CPT04 (117 ng/L) was comparable to the other Cape Town samples, indicating that the source of DEHP contamination in the October 2013 to April 2014 samples was no longer present. The maximum DEHP concentration (5 151 ng/L) measured in this study was still below the maximum contaminant level of 6 000 ng/L for DEHP suggested by the USEPA.[243] The median DEHP concentrations were 58.04 ng/L for Pretoria and 90.01 ng/L for Cape Town. Similar results were obtained in drinking water from China. DEHP was detected in 94.2% of drinking water samples from China, with an average concentration of 770 ng/L and a 180 ng/L median and 5 510 ng/L maximum concentration.[117] Concentrations were lower for cities in eastern China, with average concentrations ranging from 6.2 to 280 ng/L.[110] Average DEHP concentrations from drinking water treatment plants in Taiwan ranged from 773 to 1350 ng/L, with a maximum concentration of 2880 ng/L.[106] In comparison, 331 ng/L DEHP was detected in one drinking water fountain sample from Catalonia, Spain,[111] but was below the dl in drinking water from the region of Madrid in Central Spain.[113] The average DEHP concentrations were 60 ng/L[122] or 160 ng/L[121] in tap water from Portugal. Lower DEHP concentrations were also detected in drinking water from Katowice, Poland (60 ng/L) and Leipzig, Germany (50 ng/L)[118] and was below the dl in tap water from US drinking water treatment plants.[242]

DINP concentrations quantified in distribution point water in this study ranged from 3.02 to 1 250.75 ng/L, but the median concentration was 33.39 ng/L. Very few studies assessed drinking water for DINP. DINP was below the level of detection in drinking water from treatment plants in Taiwan,[106] but was detected in drinking water from eastern China, with concentrations ranging from 0.7 to 29 ng/L.[110]

From the discussion above, it is clear that phthalates are present in drinking water worldwide at a wide range of concentrations. The main contributor to phthalates in drinking water is the source water as traditional waterworks are not efficient to

© University of Pretoria

remove phthalates, but plastic pipes and slates used in waterworks can also release phthalates into treated drinking water.[117]

In this study, E_1 was detected in 7.5% of the distribution point samples, E_2 in 6% samples and EE_2 in 5% of the samples. The average concentrations for samples above the ql were 2.41 ng/L for E_1, 0.04 ng/L for E_2 and 0.02 ng/L for EE_2. In comparison, average E_2 and EE_2 concentrations (0.70 ng/L and 0.35 ng/L respectively) were higher in tap water samples from southern Germany, but E_1 was lower (0.40 ng/L).[115] E_1 (0.03-1.5 ng/L) was also detected in treated water from drinking water treatment plants in Ontario, Canada.[119] E_1, E_2 and EE_2 were below the dl or ql in drinking water from Brazil.[120,237,240] Although present in the source water, E_1, E_2 and EE_2 were below the dl in tap water from various drinking water treatment plants in the US.[236,242] E_1, E_2 and EE_2 were also below the dl in tap water from Central Spain,[114] drinking water from Italy[108] and drinking water reservoirs in Portugal.[241] In the distribution point samples from this study, E_1 concentrations were at least twenty times higher than the E_2 concentrations. Compared to other estrogens, E_1 is excreted by humans and livestock at relatively high concentrations.[236] Furthermore, E_2 is biodegradable to E_1 in surface waters and during sewage treatment plant processes, rendering E_1 one of the most frequently detected estrogens.[9,236]

The fact that target chemicals were detected in distribution point water samples, indicate that the water treatment processes were not effective in removing all EDCs from the drinking water. More advanced treatment systems might be more effective at removing EDCs from drinking water, however, it is not always a viable option due to much higher operating costs.[44]

6.3. Bottled water

The migration of chemicals from food contact materials depend on the chemical properties of the food contact material and content.[138,142] Alkaline solutions is associated with the migration of BPA from polycarbonate containers[93] and the migration of antimony is higher at a lower pH.[138,143] The pH values of the bottled water samples tested in this study varied from 4.5 to 7-8. However, no link between the physicochemical parameters of the bottled water samples (pH, total dissolved

© University of Pretoria

solids and mineral composition data given on the label and presented in Table 5.19) and the levels of target chemicals were found. Similarly, Montiuori et al.[155] and Jeddi et al.[149] also reported no correlation between physicochemical properties of bottled water samples and phthalate content and concluded that these parameters are not relevant in controlling the leaching of phthalates from bottles into water content.

No estrogenic activity was detected in bottled water samples from this study using the YES bioassay. Bottled water from Finland was also below the dl using a yeast bio-reporter assay,[238] but estrogenic activity was detected in bottled water from Germany (EEq: 2.65-75.2 ng/L)[133] and Italy (EEq: 0.027-23.1 ng/L)[132] using the YES bioassay.

In the T47D-KBluc bioassay in this study, estrogenic activity ranged from 0.001 to 0.004 ng/L. Although estrogenic activity was much lower in bottled water compared to distribution point water, EEq concentrations were three times higher in bottled water stored at 40°C compared to bottled water stored at 20°C (Table 5.20), indicating the leaching of estrogenic chemicals from the bottles into the water content at increased temperatures. Compared to this study, Wagner and Oehlmann[145] reported similar levels of estrogenic activity (EEq: 0.00096-0.0122 ng/L) in bottled water from France, Germany and Italy using the E-Screen. Real et al.[141] reported slightly higher estrogenic activity in bottled water from Spain (EEq: 0.011-0.078 ng/L) using the E-Screen. Plotan et al.[144] reported estrogenic activity (EEq: 1-34 ng/L) in bottled water from various countries with a reporter gene bioassay using MCF-7 cells. No estrogenic activity was detected in bottled water from France using a HepG2 transcriptional activation bioassay,[143,205] Italy using a HELN-ERα luciferase reporter bioassay[108] or the US using the E-screen.[109] A comparison of the estrogenic activity and levels of selected target chemicals (BPA, NP, DEHA, DBP, DEHP, DINP, E_2, E_1 and EE_2) in bottled water from various countries are given in Table 6.3 and Table 6.4 respectively. Only the bottled water in PET was summarised in the table, unless otherwise specified.

© University of Pretoria

Table 6.3: Comparison of the estrogenic activity in bottled water from various countries

Origin of water	Year of publication	Extraction method	Estrogenic bioassay	EEq (ng/L)	Additional information	Reference
South Africa (10 brands)		SPE	YES T47D-KBluc	<dl 0.001 - 0.004		This study
Finland (10 brands)	2015	SPE	Yeast bio-reporter assay	<dl		238
France (2 brands)	2013	SPE	HepG2 transcriptional activation assay	<dl	Carbonated and non-carbonated water exposed to different temperatures	205
France (2 brands)	2014	SPE	HepG2 transcriptional activation assay	<dl	Carbonated and non-carbonated water exposed to sunlight	143
Germany (9 brands)	2009	No extraction	YES	2.65-75.2		133
Italy (9 brands)	2009	SPE	YES	0.027-23.1		132
Italy (5 brands)	2013	SPE	HELN-ERα luciferase reporter assay	<dl		108
US (5 samples)	2010	SPE	E-Screen	<dl	Bottle material not specified	109
Various: France; Germany and Italy (12 brands total)	2011	SPE	E-Screen	0.00096-0.0122		145
Various: England (5 brands); Wales (1 brand); Scotland (1 brand); Italy (3 brands); Ireland (2 brands) and France (2 brands)	2013	SPE	Reporter gene assay using MCF-7 cells	1-34	Samples included 4 glass, one carbonated and 3 flavoured samples	144
Various: Spain (17 brands); France (1 brand) and Portugal (1 brand)	2015	SPE	E-Screen	0.011-0.078		141

<dl Below detection limit

© University of Pretoria

Table 6.4: Comparison of target chemicals in bottled water from various countries

Origin of water	Year of publication	Extraction method	Analysis method#	BPA (ng/L)	NP (ng/L)	DEHA (ng/L)	DBP (ng/L)	DEHP (ng/L)	Additional information	Reference
South Africa (10 brands)		SPE	UPLC-MS	0.55-47.36	<dl	0.80-205.26	99.84-5481	35.65-1431.74		This study
27 countries (77 bottled water samples)	2014	SBSE	GC-MS/MS	-	54	<dl	58-220	<dl		146
Canada (Halifax)	2008	SPME	GC-MS	-	-	<dl	75-317	52-338		148
China (21 brands)	2010	SPE	GC-MS	17.6-324	108-298	-	-	-	Bottle material not specified	135
Egypt	2008	SPE	GC-MS	-	-	<dl	440-1040	<dl		154
France (2 brands)	2013	SPE	GC-MS	-	<dl	<dl	<dl	<dl	Carbonated and non-carbonated water exposed to different temperatures	205
France (2 brands)	2014	SPE	GC-MS	-	<dl	<dl	<dl	<dl	Carbonated and non-carbonated water exposed to sunlight	143
France (25 brands)	2014	On-line SPE	LC-MS/MS	<dl	<dl	-	-	-		112
Greece (8 brands)	2008	LLE	GC-MS	-	-	-	-	150-6 800	Stored up to 3 months and 30 degrees C	153
Greece (6 brands)	2011	LLE	GC-MS	4.6*	7.9*	-	44*	350*	Included 1 brand in polycarbonate container	147
Hungary (3 brands)	2013	LLE	GC-MS	-	-	-	<dl-800	<dl-1.7	Carbonated and non-carbonatedl water, stored up to 48 hours and 60 degrees C	152
India (12 brands)	2016	SPE	GC-MS	-	-	-	30*	200*		156
Iran (6 brands)	2015	MSPE	GC-MS	-	-	-	135 ± 78	217 ± 92	Obtained from factories immediately after production	149
Iran (6 brands)	2015	MSPE	GC-MS	-	-	-	303 ± 172	917 ± 342	Stored up to 45 days at 40 degrees C	149
Italy (71 brands)	2008	SPME	GC-MS	-	-	-	210**	20**	Included PET and glass bottles	155
Italy (6 brands)	2010	SPE	GC-MS	-	-	-	<dl	<dl	Stored at 40 degrees C, 10 days	127
Italy (5 brands)	2013	SPE	LC-ESI-MS/MS	0.83-1.13	<ql	-	-	-		108
Japan (9 brands)	2000	LLE	HPLC-ECD	3-10	19-78	-	-	-		134

© University of Pretoria

Origin of water	Year of publication	Extraction method	Analysis method#	BPA (ng/L)	NP (ng/L)	DEHA (ng/L)	DBP (ng/L)	DEHP (ng/L)	Additional information	Reference
Portugal (6 brands)	2014	SPME	IL-GC-FID(MS)	-	-	-	60-2 940	70-180		121
Spain (5 brands)	2003	SPE	GC-MS	7	<dl	-	59	<ql		111
Spain (5 brands)	3003	SPE	GC-MS	3-11	30-31	-	20-70	39-188	Stored 10 weeks outdoors, up to 30 degrees C	111
Spain (4 brands)	2006	SPME	HPLC	-	-	-	-	<dl		212
Spain (224 bottles)	2014	SPE	GC-MS	37-819	58-2 030	185-6 230	<dl	1 020-13 000	Fresh and stored for 1 year	151
US (6 brands)	2004	SPE	LC-MS/MS	-	<dl	-	-	-		175
US (California) and Afghanistan	2013	Not specified	GC-MS	-	-	<dl	150-380	470-600	Military packaged water filled in California and Afghanistan; stored up to 120 days and 60 degrees C	150

\# GC-MS: gas chromatography-mass spectrophotometry; GC-MS/MS: gas chromatography-tandem mass spectrophotometry; HPLC: high performance liquid chromatography; IL-GC-FID(MS): ionic liquid gas chromatography associated with flame ionization detection or mass spectrometry; LC-ESI-MS/MS: liquid chromatography-electrospray ionization tandem mass spectrometry; LC-MS/MS: liquid chromatography-tandem mass spectrometry; UPLC-MS: ultra-performance liquid chromatography-mass spectrophotometry

* Median concentration ** Average concentration *** Maximum concentration - Not analysed

<dl Below detection limit <ql Below quantification limit

Note: DINP, E_2, E_1 and EE_2 were not included in the table as none of the other studies tested bottled water for DINP. Only one of the studies analysed bottled water for E_2, E_1 and EE_2, but these estrogens were below the detection limit of the analytical method.[108]

© University of Pretoria

BPA was detected in bottled water samples in this study, with concentrations above the ql ranging from 0.55 to 47.36 ng/L. All the bottles were PET bottles, but PET is believed to be PBA-free.[141] However, Fan et al.[208] extracted BPA from 16 brands of PET drinking water bottles in China, with BPA concentrations ranging from 37.7 to 64.1 µg/kg. BPA was also detected in bottled water from China,[135] Greece,[147] Italy,[108] Japan[134] and Spain,[111,151] with concentrations ranging from 0.83 to 819 ng/L. BPA was not detected in bottled water from France.[112] The presence of BPA in bottled water might be explained by BPA contamination of the source water or during the bottling process (from pipelines or disinfectants used), migration from the bottle caps and/or the use of recycled PET.[138,141]

NP was not detected in bottled water in this study. Similarly, NP was not detected in bottled water from France,[112,143,205] Italy[108] and military packaged water filled in California and Afghanistan.[150] In a study that included 77 PET bottled water samples from 27 countries, NP was only detected in one sample[146] and was below the quantification limit in bottled water from Italy.[108] NP was detected in bottled water from China,[135] Greece[147] and Japan.[134] NP was detected in 2% PET bottled water from Spain after filling (62-538 ng/L) and in 7% after one year of storage (58-2 030 ng/L) and the authors suggested that the migration of the NP might be from the HDPE caps.[151] Loyo-Rosales et al.[175] demonstrated that NP migrate from HDPE and PVC, but not from PET.

DEHA was detected in South African bottled water in this study, with concentrations ranging from 1.03 to 205 ng/L in samples that could be quantified. DEHA (283-1 470 ng/L) was also detected in 2% of PET bottled water from Spain after filling and in 6% after one year of storage (185-6 230 ng/L).[151] However, Guart et al.[146] did not find DEHA in bottled water from 27 countries. DEHA was also below the dl in bottled water from Canada,[148] Egypt,[154] France[143,205] and military bottled water filled in California and Afghanistan.[150]

In this study, DBP concentrations above the ql ranged from 99.84 ng/L to 5 481 ng/L (median 201.23 ng/L). DBP was detected in 5% PET bottled water samples from 27 countries at concentrations ranging from 58 to 220 ng/L (median 100 ng/L).[146] DBP was also detected in bottled water from Canada,[148] Egypt,[154] Greece,[147] Hungary,[152]

© University of Pretoria

India,[156] Iran,[149] Italy,[155] Portugal,[121] Spain[111] and military packaged water filled in California and Afghanistan,[150] with concentrations ranging from 20 to 2 940 ng/L. DBP was not detected in PET bottled water from France.[143,205]

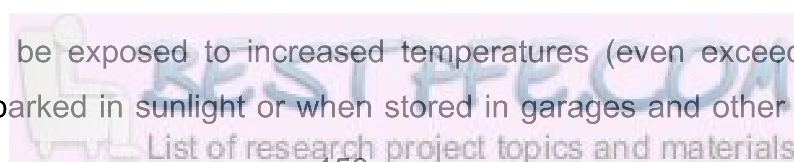
In this study, DEHP concentrations above the ql ranged from 40.95 ng/L to 1431.74 ng/L (median 63.04 ng/L). DEHP was also detected in bottled water from Canada,[148] Greece,[147,153] Hungary,[152] India,[156] Iran,[149] Italy,[155] Portugal,[121] Spain[111,151] and military packaged water filled in California and Afghanistan,[150] with concentrations ranging from 1.7 to 13 000 ng/L. DEHP was not detected in PET bottled water from Egypt[154] and France.[143,205] DEHP (1 520 ng/L) was detected in 0.4% of PET bottled water from Spain after filling and in 5% after one year of storage (1 020-13 000 ng/L).[151] PET does not contain and is not blended with DEHP and therefore DEHP in bottled water was probably present as a contaminant in the water prior to bottling or it was introduced by a processing step before bottling or from the cap liner used.[187]

DINP was detected in bottled water from this study and concentrations above the ql ranged from 5.49 to 15 991 ng/L. No other studies could be found that measured DINP in bottled water.

Phthalates and adipates are not used in the production of PET.[138] Possible sources of phthalates in bottled water include the type of polymer production technology, the quality of raw materials and recycled PET used, infiltration of polymer degradation products into the water source, cross-contamination during bottling and quality of the caps or cap sealing resins.[137,138]

E_2 was not detected in bottled water in this study, but E_1 (0.09-0.98 ng/L) and EE_2 (0.02-0.13 ng/L) was detected. No E_1, E_2 or EE_2 were detected in bottled water from Italy.[108] This was the only study that could be found that analysed bottled water for these estrogens. The occurrence of natural and synthetic estrogens in bottled water is most likely due to their presence in the source water before bottling.

Bottled water can be exposed to increased temperatures (even exceeding 65°C) when left in cars parked in sunlight or when stored in garages and other places not


BEST PFE.COM
List of research project topics and materials

© University of Pretoria

equipped with air-conditioning systems.[137] For sample BTW07 and BTW08, the DBP, DEHA and DINP concentrations were much higher in samples stored at 40°C, indicating the leaching of these chemicals from the plastic bottles into the water content. Similarly, Casajuana and Lacorte[111] reported increased concentrations of NP, DBP, BPA and DEHP in bottled water from Spain after storage for 10 weeks in outdoor conditions reaching temperatures up to 30°C. Average DBP concentrations increased from 135 ng/L to 303 ng/L and DEHP from 217 ng/L to 917 ng/L in bottled water from Iran that was stored for up to 45 days at 40°C.[149] In contrast, Greifenstein et al.[150] reported that DBP and DEHA concentrations were not a function of exposure temperature in military bottled water.

In this study, DEHA and DINP concentrations were higher in samples stored in dark conditions compared to samples stored in light conditions. The photolytic degradation of phthalates could be a possible explanation.[137,156] Sunlight did not have an effect on the migration of phthalates and NP from PET bottled water from France.[143] Different brands of PET show variations in leaching behaviour and although temperature can enhance leaching of phthalates from PET bottles, phthalates are also degraded after extended temperature exposure (36 hours at 42 °C).[156] Jeddi et al.[149] reported that phthalate concentrations increased in bottled water with prolonged storage time, regardless of the storage condition. The chemical quality of the raw material and differences in the manufacturing technologies used to manufacture PET bottles could account for the differences in migration observed from PET bottles.[143] It must also be considered that the bottled water samples for this study was purchased from retail stores and that previous poor storage conditions before purchase cannot be excluded.

Although various studies, including this one, indicated the possible migration of chemicals from packaging material into the water content, finding safer alternative packaging material proved to be challenging. Substitute products may be of inferior quality and pose unknown risks.[195] In some applications, BPA has been replaced with bisphenol S (BPS) and bisphenol F (BPF), however, studies indicate that these replacement chemicals also have estrogenic effects comparable to BPA.[45,172] Academic and industrial laboratories are conducting research to develop methods to reduce plasticizer leaching and migration and to use plasticizers or alternatives with

© University of Pretoria

lower toxicity and environmental impact.[186] Another challenge in creating safer plastic alternatives is the production cost, as no one is going to buy the products if it is too expensive. Although many packaging manufacturers are eager to switch to safer alternative packaging material, a lot of development still needs to be done.[172]

6.4. Comparison between distribution point and bottled water

None of the distribution point or bottled water samples in this study was able to reach the maximum activity obtained with E_2 in the bioassays. In a study by Real et al.,[141] bottled water samples were also unable to reach maximum activity using the E-screen. They explained the findings by the presence of compounds with antagonist activity that competes with agonists for binding at the same site on the ER.

In general, estrogenic activity and concentrations of E_1 and E_2 were higher in distribution point water compared to bottled water, but BPA, DBP, DEHA, DINP and EE_2 concentrations were higher in bottled water.

If water treatment plants are not efficient in removing natural and synthetic hormones, it could land up in drinking water. If the source water is contaminated it could even be detected in bottled water. In this study, EE_2 was expected to be found in some of the drinking and bottled water samples (as was found in the PTA08 distribution point samples), but was not expected to be present in all the bottled water samples and at higher concentrations at increased storage temperatures. The median concentration of EE_2 was 0.03 ng/L for bottled water stored at 20°C and 0.09 ng/L for samples stored at 40°C, indicating possible leaching of EE_2 from bottles into the water content. However, EE_2 is a synthetic estrogen and not a component of plastics and therefore not expected to leach from plastic bottles. One possible explanation could be accidental contamination of the samples with EE_2, although all precautionary measures were taken to prevent and limit possible contamination of the samples. The same incubator was used for the 20°C and 40°C exposures, with 40°C exposures being carried out first. The solvents used for the extraction process was ruled out as the source of contamination, as EE_2 was not detected in other samples using the same bottles of extraction solvents. Samples were not prepared and analysed in a specific order for UPLC-MS analysis, and therefore contamination of samples during UPLC-MS analysis was unlikely, as

© University of Pretoria

samples that were prepared and analysed in between the bottled water samples were below the dl for EE_2. This finding should be investigated further in follow-up studies.

It was expected that BPA, DBP, DEHA, DINP concentrations would be higher in bottled water because of the plastic packaging. Although DINP was not one of the initially selected target chemicals for this project, it was included after it was detected in many of the samples. DINP is a commonly used plasticizer and is mostly used in PVC applications to replace DEHP.[60] In Germany and the US, biomonitoring data showed a decrease in DEHP metabolites and an increase in DINP metabolites in recent years, likely due to the increased use of DINP instead of the more heavily regulated DEHP.[193] DINP is also used in polymer related uses (e.g. rubbers) and in inks and pigments, adhesives, sealants, paints and lacquers and lubricants.[184] The DINP found in drinking water is possibly due to the leaching of the chemical from the pipes that transport the water. In bottled water, DINP might be explained by leaching from the packaging material or contamination during the production process. The use of PVC tubing in the production process, for example, can lead to the contamination of food with DINP.[184] Very few studies analysed drinking and bottled water for DINP. However, the results from this study indicate that DINP is present in drinking and bottled water and should be monitored in other drinking water sources.

6.5. Health risk assessment

When analysing water samples using bioassays, trigger values are useful to judge whether further investigation is needed. Exceeding the trigger value does not necessarily mean that a health effect is expected, but further investigation is needed to identify substances responsible for the activity and could ultimately lead to a full risk assessment.[105] Brand et al.[105] derived a trigger value of 3.8 ng/L EEq for estrogenic activity of drinking water samples in the ERα CALUX bioassay. Using this trigger value, the researchers concluded that no human health risks are expected from hormonal activity in Dutch drinking water. The trigger value derived by Brand et al.[105] is higher than the 0.7 ng/L trigger value proposed by Genthe et al.[98] Both used similar methods to determine the trigger value, but different input values (i.e. different safety factors were applied). The more conservative trigger value by Genthe et al.[98] was used for this project as it is more protective.

© University of Pretoria

The greatest health risk was posed by E_1 in distribution point water from Pretoria, with a HQ of 1.56, indicating an unacceptable health risk for a lifetime exposure. This HQ was calculated using the more conservative trigger value suggested by Genthe et al.[98] and under the conservative scenario (with 95th percentile risk). However, E_1 was only detected in four samples and in three different sampling points, indicating that consumers are not continuously exposed to E_1 in their drinking water. It is therefore a more realistic scenario to calculate the HQ using the average concentration. The recalculated HQ was 0.21 and uncertainty analysis revealed that the HQ is expected to fall between 0.011 and 0.629 with 90% certainty, indicating acceptable health risks associated with E_1 in Pretoria distribution point water. A monitoring strategy is however advised, in order to get a more accurate assessment of the frequency of E_1 detections in drinking water, in order to do a more accurate risk assessment.

The highest risk of developing cancer was posed by the levels of DEHP in distribution point water from Cape Town, with a risk of 8.292×10^{-6}. However, this is lower than the acceptable risk level of 1 in 100 000 set by the USEPA, indicating acceptable exposure levels of DEHP in drinking water. DEHP was also the biggest contributor to the total exposure risk of phthalates in drinking water from China.[117]

Overall, this study indicated acceptable human health and carcinogenic risks associated with the consumption of distribution point water. Other countries also reported that the levels of EDCs in drinking water were below the levels expected to have adverse health effects. Tap water from the region of Madrid in Central Spain revealed acceptable health risks based on the HQ values of detected phthalate concentrations in the water samples.[113] Phthalates in drinking water from China did not pose carcinogenic and non-carcinogenic risks, even under the conservative scenario (with 95th percentile risk) (HQ of 4.03×10^{-4} for DBP and 3.66×10^{-3} for DEHP).[117] Santana et al.[121] also reported no expected health risks associated with the levels of phthalates in tap water from Portugal. Caldwell et al.[244] used the Pharmaceutical Assessment and Transport Evaluation model to predict the concentrations of estrogens potentially present in drinking water in the US and compared that to dietary intakes and ADIs. The study found that exposure to estrogens in drinking water was at least 82 times lower than exposure to estrogens

© University of Pretoria

in the diet and 28 times less than ADIs. They concluded that estrogens in drinking water in the US are not causing adverse effects in the residents. Humans produce 50-600 µg/L estrogens daily.[100] Although estrogens can cause deleterious effects in aquatic organisms at very low concentrations (0.1-1 ng/L E_2), the daily intake of steroid hormones in drinking water is only a minute fraction of the normal endogenous secretion of steroids by humans and should therefore have negligible effects on human health.[77,100]

The HQs for BPA, DEHA, DINP and EE_2 were higher in bottled water samples compared to distribution point samples, and the carcinogenic risk associated with DEHA was higher in bottled water samples. In bottled water, the highest potential health risk was posed by EE_2 (HQ = 0.1998). However, the levels of all the target chemicals in bottled water showed acceptable human health and carcinogenic risks. Health risk assessments on bottled water from other countries also showed acceptable health risks. DEHP revealed the greatest health (HQ = 0.012) and carcinogenic (6.5×10^{-7}) risk in bottled water from Iran, when stored at 40°C.[149] Montuori et al.[155] reported that phthalate levels in bottled water from Italy were well below the USEPA reference dose, and therefore do not present a risk for human health. Phthalates in bottled water from India also presented acceptable human health risks based on the tolerable daily intake and reference dose.[156] Using exposure scenarios of preschool children and infants as vulnerable populations, phthalate concentrations in bottled water from Iran showed acceptable health risks and a negligible carcinogenic risk.[149] The daily human intake of BPA is estimated at below 1 µg/kg body weight per day.[93] Bonefeld-Jørgensen et al.[167] reported that the concentrations of BPA-related compounds and alkylphenols found in human fluids were a thousand times lower than the effective concentrations *in vitro*. Santana et al.[121] also reported no health risks associated with the levels of phthalates in bottled water from Portugal.

A challenge in chemical risk assessment is that the number, exposure levels and potencies of chemicals contributing to an adverse outcome is not known.[50] People are exposed to a mixture of chemicals that can act additively and individual EDCs can act via more than one mechanism, resulting in an enhanced effect on the intact organism.[167] Although this study indicated an acceptable human health and

© University of Pretoria

carcinogenic risk associated with the consumption of the distribution point and bottled water, it should be kept in mind that this study only focussed on selected target chemicals. Other hazardous chemicals, not tested for in this study, might also be present in the water samples and would add to the potential health risk. Furthermore, this study only focussed on estrogenic activity and selected target chemicals and did not account for other EDC activities, e.g. androgenic activity, anti-androgenic activity, thyroid activity, etc.

This study only focussed on the consumption of drinking water as an exposure route, but people can also be exposed to the chemicals in drinking water from dermal contact and inhalation of water vapour during bathing or showering (although the oral ingestion exposure is expected to account for more than 90% of the risk).[117] Additional exposure burden may be expected from sources including packed food, air, cosmetics, medical devices, etc.[156] Compared to other dietary sources, drinking water is unlikely to be a significant route of exposure.[105] The consumption of fish from contaminated water sources might result in a daily exposure that is 2.25 times higher than the exposure from drinking water.[244] A study by Stanford et al.[109] showed that EDC exposure through municipal drinking water represents only a small fraction of total exposure. They found that exposure to estrogenic activity was 4 to 21 000 times greater through the consumption of food and beverages than municipal drinking water. Exposure to DEHP from food is generally much higher than from water (as much as 1000 times).[187] Similarly, Kuch and Ballschmiter[115] also concluded that BPA intake from drinking water is negligible compared to the intake by foodstuff that has been in contact with polycarbonate packaging. However, it should also be considered that even though health risk assessment can show no risk, EDCs act at very low doses and their effects may only appear in the long term.[114]

Biomonitoring studies can give a better indication of the total exposure level of an individual or populations. Biomonitoring data can identify novel hazards and high-risk populations and help identify exposure levels that pose health hazards.[19] However, biomonitoring studies are still lacking in South Africa, but could give valuable information on the exposure levels of phthalates and BPA in South African populations.

© University of Pretoria

6.6. Environmental and Public Health considerations

There is a large debate in the media and in scientific literature about the pros and cons of bottled water vs. tap water.[129] Wilk[130] argue that "...the progressive expansion of water as a commodity is as much the result of a failure of governments to fulfil public obligations, as it is due to the craftiness of the marketers of bottled water." Various campaigns exist that advocate the ban of bottled water. Some of the reasons against bottled water include:

- Bottled water is more expensive than tap water[130]
- Bottled water is generally not safer or purer than tap water[130]
- Chemicals can migrate from bottles into water content[133]
- Bottled water has a much higher carbon footprint than tap water due to the additional processing, transport and refrigeration required for bottled water[130]
- The plastic bottles also contribute to the pollution of the environment.[130,245] Plastic debris are accumulating in terrestrial environments, oceans and shorelines and can take hundreds to thousands of years to decompose by photo-catalysis when exposed to ultraviolet (UV) radiation.[139] The Great Pacific Garbage Patch consists of accumulated trash, including plastic debris, formed by converging ocean currents.[140] When plastic debris start to decompose, micro-plastics are formed that may be ingested by wildlife, with detrimental effects.[139,245] Microplastic particles also bind relatively large amounts of persistent organic pollutants, increasing the exposure of marine organisms when they ingest these particles.[140,245] Plastic debris can also transport non-native and harmful organisms.[139,245]

However, bottled water is a good alternative to tap water when tap water is not available or of poor quality. People are often advised to stockpile bottled water before hurricanes and travellers are warned not to drink from local water supplies in some countries.[130] In South Africa, the Operation Hydrate initiative was started in January 2016, whereby people donated bottled water to be distributed to drought-stricken areas of the country, mainly in the Free State and Eastern Cape.[246,247] Furthermore, bottled water is not always consumed as an alternative to tap water, but rather as a healthier alternative to other beverages. In South Africa, the consumption of soft drinks increased by 68.9% from 1999 to 2012.[128] Soft drink

© University of Pretoria

consumption is estimated at 92.9 L per capita per year, with bottled water contributing only 8.3 L per capita per year. The higher consumption of sugar-sweetened beverages is of concern, as it is associated with obesity and an increase in cardiovascular disease mortality.[128]

The balance between the benefits and potential threats of bottled water presents a challenge to policy makers. It is not feasible to completely ban bottled water, however, the public should be educated to buy, consume and dispose of bottled water in a responsible way. For example, the public should also be advised to always store bottled water according to the instructions, away from sunlight in a cool, dry place and use the water within the expiry date, as increased temperature and extended storage is associated with higher levels of chemical migration. People must also be encouraged to recycle PET bottles. Plastics comprise approximately 10% of municipal waste mass, but 50-80% of the waste on beaches and in the ocean.[139] According to Barnes et al.[139] the major release of plastics to the environment is due to inappropriate waste management and improper human behaviour. Providing incentives for recycling can dramatically increase the fraction of plastics recycled and reduce the plastic input into the environment.[139,140,245] Recycling of PET can introduce contaminants into the material, but advances in recycling technology could address this concern. Wilk[130] suggested that a portion of the profit from bottled water sales should be used to provide clean water systems for people who do not have access to a regular clean water supply.

EE_2, mainly used in oral contraceptives and hormone replacement therapy, was detected in one of the distribution point samples and in all the bottled water samples in this study. The presence of pharmaceuticals in the aquatic environment is a growing concern that should be addressed. Sewage treatment facilities are not equipped to degrade medicinal substances, resulting in the release of these substances into the aquatic environment and potentially drinking water.[82] Efforts should focus on developing cost-effective processes to degrade pharmaceuticals at waste water treatment plants. In conjunction, efforts should be made to mitigate some of the sources of contamination. Strategies to enhance public awareness of the impact of pharmaceuticals in the environment is necessary to encourage people to use and dispose of pharmaceuticals in a more responsible way.[87] For example,

© University of Pretoria

the disposal of unwanted and expired medicine through the toilet, sink or trash is a problem that should be addressed. Kotchen et al.[82] did a survey of 1005 residents in southern California, and revealed that 73.2% of the respondents disposed of pharmaceuticals in the toilet/sink or trash. Only 43% were aware that medical compounds have been found in treated wastewater and in surface waters, but respondents who were aware of the issue were less likely to use these disposal routes.[82] Kotchen et al.[82] recommended the establishment of pharmaceutical disposal programmes with drop-off locations at local pharmacies and to encourage people through awareness campaigns to participate in such programmes. Similar campaigns should also be implemented in South Africa to educate people on the impact of pharmaceuticals in the environment and to establish pharmaceutical disposal programmes in order to reduce the amount of pharmaceuticals that enter wastewater through residential disposal.

According to Chang et al.,[11] greater efforts are required for source reduction of EDCs, limiting exposure of vulnerable populations, treatment and remediation of contaminated sites and the establishment of large-scale monitoring networks. The main source of EDCs in the environment is untreated wastewater and wastewater treatment plant effluents.[12] The effective treatment of wastewater could protect the environment and reduce the contamination of drinking water sources used for tap as well as bottled water. However, the high cost of advanced treatment technologies present a challenge to developing countries.[89] Chlorination is widely used for the disinfection of drinking water due to its low cost, but the formation of potential hazardous by-products is a concern.[101] By combining different treatment processes, drawbacks of individual methods can be overcome and optimal removal efficiencies achieved,[89] for example combining UV photolysis with nanofiltration to decrease the levels of chemicals in the nanofiltration retentate while retaining photolysis by-products by the nanofiltration membrane.[248] The development of a rapid, simple and low-cost procedure for the detection and removal of EDCs and their activity in waste and drinking water is a growing research area[12,101] that is worth investing in.

Test centres able to routinely analyse steroid hormones in water sources are very limited in South Africa and financial capital for analytical equipment for steroid hormone analysis is also a challenge.[249] Bioassays are therefore a feasible option for

© University of Pretoria

the South African context to quantify the biological activity of the total sample.[249] Kunz et al.[73] suggested an EEq-based risk assessment framework for environmental water samples. Samples are screened using bioassays and a risk quotient is calculated by determining the ration between the EEq and the AA-EQS of E_2. A risk quotient above 1 would indicate an intolerable risk and the need for further investigation to determine potential regional sources of the estrogenic contamination and to identify specific chemicals responsible for the activity using chemical analyses. By only doing chemical analyses on areas showing potential risks to the environment, only a fraction of positively tested water samples will require the use of costly high end analytical methods, thereby reducing costs.[73] A similar framework has been suggested by Genthe et al.[98] for estrogenic activity in treated drinking water. By implementing a monitoring strategy in South Africa for source- and drinking water using bioassays, the high costs of analysis for many potential EDCs are reduced. Bioassays can act as an early warning system and areas with potential health risk can be identified and prioritized for further investigation or remedial action. South Africa is a semi-arid country with many communities that do not have access to reliable and adequate quantities of potable water.[250] The reuse of wastewater is becoming an attractive option in regions experiencing water shortages[7,250] but may present health hazards due to high levels of EDCs in wastewater that may not be removed by treatment processes.[7] Applying the monitoring strategy to reused wastewater would therefore be crucial in order to protect people from potential health hazards.

Biomonitoring studies can also play an important role in the health education of the general public. Information on the chemical body burden of a population and the sources and pathways of exposure may prompt individuals to take action to reduce personal and environmental exposures.[19] Another consideration for the South African context is that some poor rural communities make direct use of untreated river water and would therefore have a higher exposure risk.[69] Boiling water was shown to effectively remove phthalates[117] and thyroid receptor antagonistic activity from tap water[110] and can be recommended to communities without access to safe tap water to reduce at least some of the potential risks. Solar disinfection is also recommended for very poor households in areas that don't have access to safe tap water to combat waterborne diseases by exposing water in clear plastic bottles to

BEST PFE.COM
List of research project topics and materials

© University of Pretoria

sunlight for six hours. Schmid et al.[251] reported maximum concentrations of DEHA and DEHP of 46 ng/L and 710 ng/L respectively from the reuse of PET for solar water disinfection and concluded that the chemical micropollutants in the water is a minor problem compared to the risks of microbial contamination.[251] The current study showed the possible migration of DBP, DEHA and DINP from some brands of PET at 40°C. However, this was in bottled water purchased in supermarkets, but the migration potential may differ when bottles are re-used and exposed to higher temperatures and should be investigated for the South African brands of PET and exposure conditions.

Another way to protect the public from EDC exposure is by implementing policies and regulatory practices for the introduction of new chemicals to the market.[45] Based on the precautionary principle, it has been suggested to replace DEHP with less toxic alternatives in order to reduce human exposure.[187] However, according to Kamrin[195] regulations banning the use of phthalates are not likely to provide any public health benefit and may even lead to an overall reduction in public health due to possible inferior quality and unknown risks of substitute products. The development of plastic alternatives should therefore include testing protocols, similar to the TiPED suggested by Schug et al.,[20] to identify potentially hazardous substances as early as possible in the design process. Sweden established a national program to phase-out substances that are persistent, bioaccumulative, carcinogenic, mutagenic and reproductive or endocrine system toxicants and to replace them with safer alternatives.[19] Developing countries, like South Africa can also benefit from programs in countries like Sweden by switching to products that was proven to be safer alternatives in the programs.

People are involuntary and often unknowingly exposed to phthalates and other EDCs via many routes. According to Erythropel et al.[187] it should be the responsibility of the producer to limit exposure and demonstrate the safety of their products. Manufacturers could be encouraged to shift manufacturing processes toward more sustainable products by holding them responsible for the costs of the health effects induced by the use of their products.[45] Although safer products that are being developed to replace harmful chemicals can sometimes be more expensive than the original product, it will lead to a reduction in health related costs.[45] Collaboration

© University of Pretoria

between scientists and manufacturers can be valuable. Bottle caps are often suggested as the source of migration of substances like BPA from PET bottled water that don't contain the chemical. This information is useful for cap and resin distributors to improve and develop products to limit migration of potential harmful substances to water contents.[151]

6.7. Conclusions

People are exposed to EDCs in water, air, soil, food, personal care products and medical devices, making human exposure to environmental EDCs inevitable. Due to their ubiquity in the environment and endocrine disruptive activity, the potential impact of EDCs on public health is a reason for concern. Very limited information is available on estrogenic activity and levels of EDCs in drinking and bottled water from South Africa. This study revealed the presence of BPA, phthalates, an adipate and estrogenic hormones in distribution point water from Pretoria and Cape Town as well as in bottled water available in South Africa. The estrogenic activity and levels of target chemicals were comparable to the levels found in other countries.

The presence of EDCs in drinking and bottled water can be ascribed to the contamination of the source water and ineffective water treatment methodologies or migration from reservoir linings or plastic pipes at water distribution systems or the migration from plastic bottles or caps into bottled water.

Very few studies analysed bottled water for DINP, E_1 and EE_2. However, these chemicals were found in bottled water from this study. The use of DINP has increased in recent years, as it is used to replace the more heavily regulated DEHP. E_1 and EE_2 in bottled water are probably due to the contamination of the source water. The findings from this study indicate the need to include DINP in the monitoring of drinking and bottled water and to include natural and synthetic hormones in the analysis of bottled water.

The hypothesis of this study was that potential human health risks associated with EDCs in water from municipal distribution points would be lower compared to bottled water stored at different storage conditions and that EDCs present in plastic bottles will migrate into the water content at a higher storage temperature, resulting in higher

© University of Pretoria

estrogenic activity and increased human health risk. Although the HQs for BPA, DEHA and DINP were higher in bottled water compared to distribution point water, the greatest non-carcinogenic health risk was posed by E_1 in distribution point water from Pretoria and the highest cancer risk by levels of DEHP in distribution point water from Cape Town. The results from this study indicated the possible migration of DBP, DEHA and DINP from some of the bottled water brands at 40°C. However, health risk assessment revealed acceptable health and carcinogenic risks associated with the consumption of distribution point and bottled water.

Due to the fact that EDCs were frequently detected in Pretoria and Cape Town distribution point and bottled water, a monitoring strategy is recommended that can act as an early warning system. Other municipalities and brands of bottled water should also be included. Strategies to limit source contamination and effectively remove EDCs from source water are also recommended and may include the development of more effective water treatment technologies, the development and use of safer alternatives and public awareness campaigns (e.g. to reduce the use of and recycle plastic products and to dispose of unused pharmaceuticals in a responsible manner).

6.8. Recommendations

1. Low levels of EDCs were frequently detected in Pretoria and Cape Town distribution point water and a monitoring strategy is therefore recommended for all municipalities. Continued monitoring is vital to alert water suppliers when potential EDCs are introduced into the water system (for example the high DEHP concentrations at the CPT04 distribution point) in order to identify the source and take remedial action as soon as possible.

2. Bioassays can be advantageous in monitoring strategies for source- and drinking water in South Africa. By screening samples in bioassays to identify areas that need further investigation, the high costs of extensive chemical analysis can be reduced. However, in South Africa, bioassays are mainly used by research institutions for specific research projects. There is a great need to establish laboratories that can do these bioassays on a routine basis for monitoring programmes.

© University of Pretoria

3. Grab samples are traditionally used for the analysis of drinking water, as was done for this project. However, this might not be representative of the sample over a period of time. The polar organic compounds integrative sampler (POCIS) has recently been evaluated for its suitability for monitoring contaminants in drinking water.[119] This technology allows for passive sampling over a period of time to provide time-weighted average concentrations of target chemicals in water. The method also allows for the concentration of trace levels of contaminants thereby lowering detection limits.[119] Similar systems should be investigated for the monitoring of EDCs in South African drinking water.

4. For the purposes of this study drinking water samples were taken from distribution points/reservoirs to prevent the possibility of confounding factors in the form of the different types of piping used in private homes (i.e. point of use). As the health risk assessment indicated acceptable risks associated with the consumption of water from distribution points, future research projects should focus on point of use water and the contribution of different types of piping used in homes on the EDC contamination of drinking water.

5. Since this study was conducted, some of the bottled water suppliers changed their bottles and/or water sources. Bottled water should therefore be monitored on a regular basis as the results given in this study might not necessarily be an accurate reflection of the current status of the bottled water. Members of the South African National Bottled Water Association (SANBWA) must adhere to stringent quality standards that involve quality control tests and procedures throughout the bottling process, including the packaging material, source, bottling line and final product.[252] According to Department of Health regulations[253] and the SANBWA standards[252] bottled water must be analysed for several contaminants, including antimony. Antimony is a substance of concern that has been detected in bottled water from other countries and has been suggested as a source of estrogenicity in PET bottled water[138]. It is recommended to add BPA, NP and phthalates to the list of substances that should be regulated in bottled water. Although PET does not contain BPA, NP or phthalates, these substances may be introduced to bottled water through contamination of the source water or during the bottling process (from pipelines

© University of Pretoria

or disinfectants used), migration from the bottle caps and/or the use of recycled PET.

6. Future studies should also include the analysis of degradation products of phthalates in bottled water. Phthalic acid, a degradation product of phthalates was detected at the highest concentrations in bottled water from Italy.[155] Some of DEHPs breakdown products are endocrine disruptors and more toxic than DEHP itself[187] and should be investigated in bottled water from South Africa.

7. Very few studies investigated the presence of natural and synthetic hormones in bottled water. This study reported the presence of E_1 and EE_2 in bottled water from South Africa. Estrogenic potencies of natural and synthetic hormones are several orders of magnitude greater than the potencies of other EDCs, identifying the need to monitor bottled water for the presence and concentrations of E_1, E_2 and EE_2. Furthermore, this study revealed that the concentrations of EE_2 were higher in bottled water stored at 40°C compared to bottled water stored at 20°C, a finding that should be investigated further.

8. The recommended storage conditions for bottled water (as indicated on the label) is in a cool, dry, odourless place, away from direct sunlight. The Department of Health is responsible for regulating and monitoring the bottled water industry, and together with the SANBWA can play an important role to oversee that bottled water is transported and stored correctly by bottled water suppliers.

9. This study focused mainly on the estrogenic activity of distribution point and bottled water, but some of the target chemicals are also known to have androgenic, anti-androgenic and thyroid activity. For example, DBP, DINP, DEHP and DEHA induced thyroid hormone dependent cell proliferation in the T-screen[188] and DBP and DEHP showed androgenic and anti-androgenic activity in MDA-kb2 cells.[189] NP and BPA can also bind to the human progesterone receptor and have antagonistic effects.[181] The interaction of all the different pathways will have an effect on the resultant biological effect on the organism.[188]

© University of Pretoria

Future studies should therefore also include androgenic, anti-androgenic and thyroid activity.

10. There is also a need to standardize bioassay techniques, calculation and reporting methods. A panel of CALUX reporter gene bioassays are available to assess EDC activity in water with high sensitivity and specificity,[90] but it is a commercially available product and therefore not an economical option to use in developing countries. A panel of bioassays suitable for the South African context should therefore be developed and standardized. A WRC funded project developed a South African toolbox of bioassays for estrogenic activity,[222] but this should be expanded to include bioassays for androgen, progesterone, glucocorticoid and thyroid activity as well as bioassays that measure different mechanisms of action.

11. Strategies to limit source contamination are recommended. This may include the development of more effective and economical waste water treatment technologies and public awareness campaigns. The SANBWA is actively involved in facilitating the recycling of PET bottles and also participates in coastal clean-up projects.[252] Similar campaigns can be initiated by the Department of Health to educate people to dispose of unused pharmaceuticals in a responsible manner.

12. There is very little information on bottled and tap water preferences of consumers in peer-reviewed literature.[129] Studies that investigate the factors contributing to the choice of bottled vs tap water would be valuable in understanding consumer's concerns and behaviours.[129] A study investigating the factors affecting South-African consumer's preferences for drinking water is therefore recommended.

13. Biomonitoring studies should be conducted to determine the exposure levels of South African populations to phthalates and BPA. This could give valuable information to identify possible hazards and high-risk populations.

© University of Pretoria

References

1. World Health Organization and United Nations Environment Programme. State of the science of endocrine disrupting chemicals - 2012. Geneva: World Health Organization and United Nations Environment Programme; 2013.

2. Zoeller RT, Brown TR, Doan LL, Gore AC, Skakkebaek NE, Soto AM, et al. Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. Endocrinology. 2012; 153(9):4097-110.

3. Chevalier N, Fenichel P. Endocrine disruptors: new players in the pathophysiology of type 2 diabetes? Diabetes Metab. 2015; 41(2):107-15.

4. Markey CM, Rubin BS, Soto AM, Sonnenschein C. Endocrine disruptors: from Wingspread to environmental developmental biology. J Steroid Biochem Mol Biol. 2002; 83(1-5):235-44.

5. Hamlin HJ, Guillette LJ, Jr. Birth defects in wildlife: the role of environmental contaminants as inducers of reproductive and developmental dysfunction. Syst Biol Reprod Med. 2010; 56:113-21.

6. Woodruff TJ. Bridging epidemiology and model organisms to increase understanding of endocrine disrupting chemicals and human health effects. J Steroid Biochem Mol Biol. 2011; 127(1-2):108-17.

7. Futran Fuhrman V, Tal A, Arnon S. Why endocrine disrupting chemicals (EDCs) challenge traditional risk assessment and how to respond. J Hazard Mater. 2015; 286:589-611.

8. World Health Organization [Internet]. Global assessment of the state-of-the-science of endocrine disruptors 2002. Geneva: World Health Organization; [cited 2012 Feb 29]. Available from: http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/.

9. Burkhardt-Holm P. Endocrine disruptors and water quality: a state-of-the-art review. Int J Water Resour Dev. 2010; 26(3):477-93.

© University of Pretoria

10. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev. 2009; 30(4):293-342.

11. Chang HS, Choo KH, Lee B, Choi SJ. The methods of identification, analysis, and removal of endocrine disrupting compounds (EDCs) in water. J Hazard Mater. 2009; 172(1):1-12.

12. Bolong N, Ismail AF, Salim MR, Matsuura T. A review of the effects of emerging contaminants in wastewater and options for their removal. Desalination. 2009; 239(1-3):229-46.

13. Kiyama R, Wada-Kiyama Y. Estrogenic endocrine disruptors: molecular mechanisms of action. Environ Int. 2015; 83:11-40.

14. Fox MA, Aoki Y. Chapter 2: Environmental contaminants and exposure. In: Woodruff TJ, Janssen SJ, Guillette LJ, Giudice LC, editors. Environmental impacts on reproductive health and fertility. Cambridge: Cambridge University Press; 2010. p. 8-22.

15. Olujimi OO, Fatoki OS, Odendaal JP, Okonkwo JO. Endocrine disrupting chemicals (phenol and phthalates) in the South African environment: a need for more monitoring. Water SA. 2010; 36(5):671-82.

16. Wittassek M, Koch HM, Angerer J, Bruning T. Assessing exposure to phthalates - the human biomonitoring approach. Mol Nutr Food Res. 2011; 55(1):7-31.

17. Connolly L, Ropstad E, Verhaegen S. In vitro bioassays for the study of endocrine-disrupting food additives and contaminants. Trends Analyt Chem. 2011; 30(2):227-38.

18. Jobling S, Reynolds T, White R, Parker MG, Sumpter JP. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ Health Perspect. 1995; 103:582-7.

© University of Pretoria

19. Thornton JW, McCally M, Houlihan J. Biomonitoring of industrial pollutants: health and policy implications of the chemical body burden. Public Health Rep. 2002; 117:315-23.

20. Schug TT, Abagyan R, Blumberg B, Collins TJ, Crews D, DeFur PL, et al. Designing endocrine disruption out of the next generation of chemicals. Green Chem. 2013; 15(1):181-98.

21. Schneider JE, Brozek JM, Keen-Rhinehart E. Our stolen figures: the interface of sexual differentiation, endocrine disruptors, maternal programming, and energy balance. Horm Behav. 2014; 66(1):104-19.

22. Patel S, Zhou C, Rattan S, Flaws JA. Effects of endocrine-disrupting chemicals on the ovary. Biol Reprod. 2015; 93(1):1-9.

23. Uzumcu M, Zama AM, Oruc E. Epigenetic mechanisms in the actions of endocrine-disrupting chemicals: gonadal effects and role in female reproduction. Reprod Domest Anim. 2012; 47:338-47.

24. Schug TT, Janesick A, Blumberg B, Heindel JJ. Endocrine disrupting chemicals and disease susceptibility. J Steroid Biochem Molec Biol. 2011; 127:204-15.

25. Nordkap L, Joensen UN, Blomberg Jensen M, Jorgensen N. Regional differences and temporal trends in male reproductive health disorders: semen quality may be a sensitive marker of environmental exposures. Mol Cell Endocrinol. 2012; 355(2):221-30.

26. Costa EMF, Spritzer PM, Hohl A, Bachega TASS. Effects of endocrine disruptors in the development of the female reproductive tract. Arq Bras Endocrinol Metab. 2014; 58(2):153-61.

27. Frye C, Bo E, Calamandrei G, Calza L, Dessi-Fulgheri F, Fernandez M, et al. Endocrine disrupters: a review of some sources, effects, and mechanisms of actions on behavior and neuroendocrine systems. J Neuroendocrinol. 2012; 24(1):144-59.

© University of Pretoria

28. Cho SC, Bhang SY, Hong YC, Shin MS, Kim BN, Kim JW, et al. Relationship between environmental phthalate exposure and the intelligence of school-age children. Environ Health Perspect. 2010; 118(7):1027-32.

29. Lang IA, Galloway TS, Scarlett A, Henley WE, Depledge M, Wallace RB, et al. Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. JAMA. 2008; 300(11):1303-10.

30. Grandjean P, Bellinger D, Bergman A, Cordier S, Davey-Smith G, Eskenazi B, et al. The Faroes statement: human health effects of developmental exposure to chemicals in our environment. Basic Clin Pharmacol Toxicol. 2007; 102:73-5.

31. Kuo C-H, Yang S-N, Kuo P-L, Hung C-H. Immunomodulatory effects of environmental endocrine disrupting chemicals. Kaohsiung J Med Sci. 2012; 28:S37-42.

32. Rogers JA, Metz L, Yong VW. Review: endocrine disrupting chemicals and immune responses: a focus on bisphenol-A and its potential mechanisms. Mol Immunol. 2013; 53:421-30.

33. Chamorro-Garcia R, Blumberg B. Transgenerational effects of obesogens and the obesity epidemic. Curr Opin Pharmacol. 2014; 19:153-8.

34. Vom Saal FS, Nagel SC, Coe BL, Angle BM, Taylor JA. The estrogenic endocrine disrupting chemical bisphenol A (BPA) and obesity. Mol Cell Endocrinol. 2012; 354(1-2):74-84.

35. Yang O, Kim HL, Weon JI, Seo YR. Endocrine-disrupting chemicals: review of toxicological mechanisms using molecular pathway analysis. J Cancer Prev. 2015; 20(1):12-24.

36. Hu WY, Shi GB, Hu DP, Nelles JL, Prins GS. Actions of estrogens and endocrine disrupting chemicals on human prostate stem/progenitor cells and prostate cancer risk. Mol Cell Endocrinol. 2012; 354(1-2):63-73.

37. Metzler M, Kulling SE, Pfeiffer E, Jacobs E. Genotoxicity of estrogens. Z Lebensm Unters Forsch A. 1998; 206:367-73.

BEST PFE.COM
List of research project topics and materials

© University of Pretoria

38. Dogan S, Simsek T. Possible relationship between endocrine disrupting chemicals and hormone dependent gynecologic cancers. Med Hypotheses. 2016; 92:84-7.

39. Seachrist DD, Bonk KW, Ho S-M, Prins GS, Soto AM, Keri RA. A review of the carcinogenic potential of bisphenol A. Reprod Toxicol. 2016; 59:167-82.

40. Soares A, Guieysse B, Jefferson B, Cartmell E, Lester JN. Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. Environ Int. 2008; 34(7):1033-49.

41. Phillips KP, Foster WG. Key developments in endocrine disrupter research and human health. J Toxicol Environ Health B Crit Rev. 2008; 11(3-4):322-44.

42. Fisher JS. Are all EDC effects mediated via steroid hormone receptors? Toxicology. 2004; 205(1-2):33-41.

43. Cookman CJ, Belcher SM. Classical nuclear hormone receptor activity as a mediator of complex concentration response relationships for endocrine active compounds. Curr Opin Pharmacol. 2014; 19:112-9.

44. Racz L, Goel RK. Fate and removal of estrogens in municipal wastewater. J Environ Monit. 2010; 12(1):58-70.

45. Vandenberg LN, Luthi D, Quinerly DA. Plastic bodies in a plastic world: multi-disciplinary approaches to study endocrine disrupting chemicals. J Clean Prod. 2017; 140:373-85.

46. Vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, et al. Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc Natl Acad Sci USA. 1997; 94:2056-61.

47. Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Jr., Lee DH, et al. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr Rev. 2012; 33(3):378-455.

© University of Pretoria

48. Kortenkamp A. Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. Environ Health Perspect. 2007; 115 Suppl 1:98-105.

49. Svingen T, Vinggaard AM. The risk of chemical cocktail effects and how to deal with the issue. J Epidemiol Community Health. 2016; 70(4):322-3.

50. Kortenkamp A. Low dose mixture effects of endocrine disrupters and their implications for regulatory thresholds in chemical risk assessment. Curr Opin Pharmacol. 2014; 19:105-11.

51. Kortenkamp A. Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology. Int J Androl. 2008; 31(2):233-40.

52. Brian JV, Harris CA, Scholze M, Kortenkamp A, Booy P, Lamoree M, et al. Evidence of estrogenic mixture effects on the reproductive performance of fish. Environ Sci Technol. 2007; 41(1):337-44.

53. Kortenkamp A, Faust M, Scholze M, Backhaus T. Low-level exposure to multiple chemicals: reason for human health concerns? Environ Health Perspect. 2007; 115 Suppl 1:106-14.

54. Muncke J. Exposure to endocrine disrupting compounds via the food chain: is packaging a relevant source? Sci Total Environ. 2009; 407(16):4549-59.

55. Prins GS, Hu W-Y, Shi G-B, Hu D-P, Majumdar S, Li G, et al. Bisphenol A promotes human prostate stem-progenitor cell self-renewal and increases in vivo carcinogenesis in human prostate epithelium. Endocrinology. 2014; 155:805-17.

56. Fagin D. The learning curve. Nature. 2012; 490:462-5.

57. Vaiserman A. Early-life exposure to endocrine disrupting chemicals and later-life health outcomes: an epigenetic bridge? Aging Dis. 2014; 5(6):419-29.

58. Padmanabhan V, Cardoso RC, Puttabyatappa M. Developmental programming, a pathway to disease. Endocrinology. 2016; 157(4):1328-40.

© University of Pretoria

59. Nahar MS, Liao C, Kannan K, Dolinoy DC. Fetal liver bisphenol A concentrations and biotransformation gene expression reveal variable exposure and altered capacity for metabolism in humans. J Biochem Mol Toxicol. 2013; 27(2):116-23.

60. Frederiksen H, Skakkebaek NE, Andersson AM. Metabolism of phthalates in humans. Mol Nutr Food Res. 2007; 51(7):899-911.

61. Hauser R, Meeker JD, Duty S, Silva MJ, Calafat AM. Altered semen quality in relation to urinary concentrations of phthalate monoester and oxidative metabolites. Epidemiology. 2006; 17:682-91.

62. Skinner MK, Manikkam M, Guerrero-Bosagna C. Epigenetic transgenerational actions of endocrine disruptors. Reprod Toxicol. 2011; 31(3):337-43.

63. Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK. Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. PLoS One. 2013; 8(1):e55387. doi:10.1371/journal.pone.0055387.

64. Department of Water Affairs and Forestry [Internet]. Drinking water quality in South Africa. A consumer's guide 2005. Pretoria: Department of Water Affairs and Forestry; [cited 2015 Jul 06]. Available from: http://www.dwaf.gov.za/Documents/Other/DWQM/DWQConsumerPamphletJul05.pdf.

65. Loos R, Wollgast J, Huber T, Hanke G. Polar herbicides, pharmaceutical products, perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and nonylphenol and its carboxylates and ethoxylates in surface and tap waters around Lake Maggiore in Northern Italy. Anal Bioanal Chem. 2007; 387:1469-78.

66. Falconer IR, Chapman HF, Moore MR, Ranmuthugala G. Endocrine-disrupting compounds: a review of their challenge to sustainable and safe water supply and water reuse. Environ Toxicol. 2006; 21(2):181-91.

© University of Pretoria

67. Manickum T, John W. Occurrence, fate and environmental risk assessment of endocrine disrupting compounds at the wastewater treatment works in Pietermaritzburg (South Africa). Sci Total Environ. 2014; 468-469:584-97.

68. Swart JC, Pool EJ, van Wyk JH. The implementation of a battery of in vivo and in vitro bioassays to assess river water for estrogenic endocrine disrupting chemicals. Ecotoxicol Environ Saf. 2011; 74(1):138-43.

69. Genthe B, Le Roux WJ, Schachtschneider K, Oberholster PJ, Aneck-Hahn NH, Chamier J. Health risk implications from simultaneous exposure to multiple environmental contaminants. Ecotoxicol Environ Saf. 2013; 93:171-9.

70. Slabbert JL, Venter EA, Moletsane M, Van Wyk JH, Blaise C, Aneck-Hahn NH. An investigation of the estrogenic activity in water from selected drinking water treatment processes. WRC Report No. 1532/1/08. Pretoria: Water Research Commission of South Africa; 2008.

71. Aneck-Hahn NH, Bornman MS, De Jager C. Oestrogenic activity in drinking waters from a rural area in the Waterberg District, Limpopo Province, South Africa. Water SA. 2009; 35(3):245-51.

72. Bornman MS, Van Vuren JH, Bouwman H, De Jager C, Genthe B, Barnhoorn EJ. The use of sentinal species to determine the endocrine disruptive activity in an urban nature reserve. WRC report number 1505/1/07. Pretoria: Water Research Commission of South Africa; 2007.

73. Kunz PY, Kienle C, Carere M, Homazava N, Kase R. In vitro bioassays to screen for endocrine active pharmaceuticals in surface and waste waters. J Pharm Biomed Anal. 2015; 106:107-15.

74. Martínez-Zapata M, Aristizábal C, Peñuela G. Photodegradation of the endocrine-disrupting chemicals 4n-nonylphenol and triclosan by simulated solar UV irradiation in aqueous solutions with Fe(III) and in the absence/presence of humic acids. J Photochem Photobiol A. 2013; 251:41-9.

© University of Pretoria

75. LaFleur AD, Schug KA. A review of separation methods for the determination of estrogens and plastics-derived estrogen mimics from aqueous systems. Anal Chim Acta. 2011; 696(1-2):6-26.

76. Johnson AC, Belfroid A, Di Corcia A. Estimating steroid oestrogen imputs into activated sludge treatment works and observations on their removal from the effluent. Sci Total Environ. 2000; 256:163-73.

77. Falconer IR. Are endocrine disrupting compounds a health risk in drinking water? Int J Environ Res Public Health. 2006; 3(2):180-4.

78. Filby AL, Neuparth T, Thorpe KL, Owen R, Galloway TS, Tyler CR. Health impacts of estrogens in the environment, considering complex mixture effects. Environ Health Perspect. 2007; 115(12):1704-10.

79. Balaam JL, Grover D, Johnson AC, Jurgens M, Readman J, Smith AJ, et al. The use of modelling to predict levels of estrogens in a river catchment: how does modelled data compare with chemical analysis and in vitro yeast assay results? Sci Total Environ. 2010; 408(20):4826-32.

80. Rodgers-Gray TP, Jobling S, Kelly C, Morris S, Brighty G, Waldock MJ, et al. Exposure of juvenile roach *(Rutilus rutilus)* to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. Environ Sci Technol. 2001; 35(3):462-70.

81. Swartz CH, Reddy S, Benotti MJ, Yin H, Barber LB, Brownawell BJ, et al. Steroid estrogens, nonylphenol ethoxylate metabolites, and other wastewater contaminants in groundwater affected by a residential septic system on Cape Cod, MA. Environ Sci Technol. 2006; 40:4894-902.

82. Kotchen M, Kallaos J, Wheeler K, Wong C, Zahller M. Pharmaceuticals in wastewater: behavior, preferences, and willingness to pay for a disposal program. J Environ Manage. 2009; 90(3):1476-82.

83. Zwiener C. Occurrence and analysis of pharmaceuticals and their transformation products in drinking water treatment. Anal Bioanal Chem. 2007; 387:1159-62.

© University of Pretoria

84. Coetsier C, Lin L, Roig B, Touraud E. Integrated approach to the problem of pharmaceutical products in the environment: an overview. Anal Bioanal Chem. 2007; 387(4):1163-6.

85. Dorne JL, Ragas AM, Frampton GK, Spurgeon DS, Lewis DF. Trends in human risk assessment of pharmaceuticals. Anal Bioanal Chem. 2007; 387(4):1167-72.

86. Nikolaou A, Meric S, Fatta D. Occurrence patterns of pharmaceuticals in water and wastewater environments. Anal Bioanal Chem. 2007; 387(4):1225-34.

87. Touraud E, Roig B, Sumpter JP, Coetsier C. Drug residues and endocrine disruptors in drinking water: risk for humans? Int J Hyg Environ Health. 2011; 214(6):437-41.

88. Daughton CG, Ternes TA. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ Health Perspect. 1999; 107:907-38.

89. Rahman MF, Yanful EK, Jasim SY. Endocrine disrupting compounds (EDCs) and pharmaceuticals and personal care products (PPCPs) in the aquatic environment: implications for the drinking water industry and global environmental health. J Water Health. 2009; 7(2):224-43.

90. Van der Linden SC, Heringa MB, Man H-Y, Sonneveld E, Puijker LM, Brouwer A, et al. Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. Environ Sci Technol. 2008; 42:5814-20.

91. Rudel RA, Melly SJ, Geno PW, Sun G, Brody JG. Identification of alkylphenols and other estrogenic phenolic compounds in wastewater, septage, and goundwater on Cape Cod, Massachusetts. Environ Sci Technol. 1998; 32:861-9.

92. Geens T, Goeyens L, Covaci A. Are potential sources for human exposure to bisphenol-A overlooked? Int J Hyg Environ Health. 2011; 214(5):339-47.

93. Kang JH, Kondo F, Katayama Y. Human exposure to bisphenol A. Toxicology. 2006; 226(2-3):79-89.

© University of Pretoria

94. Campbell CG, Borglin SE, Green FB, Grayson A, Wozei E, Stringfellow WT. Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine disrupting compounds in water: a review. Chemosphere. 2006; 65(8):1265-80.

95. Matthiessen P, Arnold D, Johnson AC, Pepper TJ, Pottinger TG, Pulman KG. Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. Sci Total Environ. 2006; 367(2-3):616-30.

96. Chopra AK, Sharma MK, Chamoli S. Bioaccumulation of organochlorine pesticides in aquatic system - an overview. Environ Monit Assess. 2011; 173(1-4):905-16.

97. Dabrowski JM. Development of pesticide use maps for South Africa. S Afr J Sci. 2015; 111(1/2):52-8.

98. Genthe B, Steyn M, Aneck-Hahn NH, Van Zijl C, De Jager C. The feasibility of a health risk assessment framework to derive guidelines for oestrogen activity in treated drinking water. WRC Report No. 1749/1/09. Pretoria: Water Research Commission of South Africa; 2010.

99. Fatoki OS, Bornman M, Ravandhalala L, L Chimuka L, Genthe B, Adeniyi A. Phthalate ester plasticizers in freshwater systems of Venda, South Africa and potential health effects. Water SA. 2010; 36(1):117-26.

100. Pereira RO, Postigo C, de Alda ML, Daniel LA, Barcelo D. Removal of estrogens through water disinfection processes and formation of by-products. Chemosphere. 2011; 82(6):789-99.

101. Silva CP, Otero M, Esteves V. Processes for the elimination of estrogenic steroid hormones from water: a review. Environ Pollut. 2012; 165:38-58.

102. Padhye LP, Yao H, Kung'u FT, Huang C-H. Year-long evaluation on the occurrence and fate of pharmaceuticals, personal care products, and endocrine disrupting chemicals in an urban drinking water treatment plant. Water Res. 2014; 51:266-76.

© University of Pretoria

103. Brouckaert C, Mhlanga F, Mashava A. Investigation into methods for the development of a protocol for quantitative assessment of industrial effluents for permitting of discharge to sewer. An eThekwini case study. WRC Report No 1734/1/13. Pretoria: Water Research Commission of South Africa; 2013.

104. Berryman D, Houde F, DeBlois C, O'Shea M. Nonylphenolic compounds in drinking and surface waters downstream of treated textile and pulp and paper effluents: a survey and preliminary assessment of their potential effects on public health and aquatic life. Chemosphere. 2004; 56:247-55.

105. Brand W, de Jongh CM, van der Linden SC, Mennes W, Puijker LM, van Leeuwen CJ, et al. Trigger values for investigation of hormonal activity in drinking water and its sources using CALUX bioassays. Environ Int. 2013; 55:109-18.

106. Gou Y-Y, Lin S, Que DE, Tayo LL, Lin D-Y, Chen K-C, et al. Estrogenic effects in the influents and effluents of the drinking water treatment plants. Environ Sci Pollut Res. 2016; 23(9):8518-28.

107. Lv X, Xiao S, Zhang G, Jiang P, Tang F. Occurrence and removal of phenolic endocrine disrupting chemicals in the water treatment processes. Sci Rep. 2016; 6:22860.

108. Maggioni S, Balaguer P, Chiozzotto C, Benfenati E. Screening of endocrine-disrupting phenols, herbicides, steroid estrogens, and estrogenicity in drinking water from the waterworks of 35 Italian cities and from PET-bottled mineral water. Environ Sci Pollut Res Int. 2013; 20(3):1649-60.

109. Stanford BD, Snyder SA, Trenholm RA, Holady JC, Vanderford BJ. Estrogenic activity of US drinking waters: a relative exposure comparison. J Am Water Works Assoc. 2010; 102(12):55-65.

110. Shi W, Hu X, Zhang F, Hu G, Hao Y, Zhang X, et al. Occurrence of thyroid hormone activities in drinking water from eastern China: contributions of phthalate esters. Environ Sci Technol. 2012; 46(3):1811-8.

© University of Pretoria

111. Casajuana N, Lacorte S. Presence and release of phthalic esters and other endocrine disrupting compounds in drinking water. Chromatographia. 2003; 57:649-55.

112. Colin A, Bach C, Rosin C, Munoz JF, Dauchy X. Is drinking water a major route of human exposure to alkylphenol and bisphenol contaminants in France? Arch Environ Contam Toxicol. 2014; 66(1):86-99.

113. Dominguez-Morueco N, Gonzalez-Alonso S, Valcarcel Y. Phthalate occurrence in rivers and tap water from central Spain. Sci Total Environ. 2014; 500-501:139-46.

114. Esteban S, Gorga M, González-Alonso S, Petrovic M, Barceló D, Valcárcel Y. Monitoring endocrine disrupting compounds and estrogenic activity in tap water from central Spain. Environ Sci Pollut Res. 2014; 21(15):9297-310.

115. Kuch HM, Ballschmiter K. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. Environ Sci Technol. 2001; 35:3201-6.

116. Li L, Wang J, Zhou S, Zhao M. Development and characterization of an immunoaffinity monolith for selective on-line extraction of bisphenol A from environmental water samples. Anal Chim Acta. 2008; 620:1-7.

117. Liu X, Shi J, Bo T, Li H, Crittenden JC. Occurrence and risk assessment of selected phthalates in drinking water from waterworks in China. Environ Sci Pollut Res. 2015; 22(14):10690-8.

118. Luks-Betlej K, Popp P, Janoszka B, Paschke H. Solid-phase microextraction of phthalates from water. J Chromatogr A. 2001; 938:93-101.

119. Metcalfe C, Hoque ME, Sultana T, Murray C, Helm P, Kleywegt S. Monitoring for contaminants of emerging concern in drinking water using POCIS passive samplers. Environ Sci Process Impacts. 2014; 16(3):473-81.

© University of Pretoria

120. Sodré FF, Locatelli MAF, Jardim WF. Occurrence of emerging contaminants in Brazilian drinking waters: a sewage-to-tap issue. Water Air Soil Pollut. 2010; 206(1-4):57-67.

121. Santana J, Giraudi C, Marengo E, Robotti E, Pires S, Nunes I, et al. Preliminary toxicological assessment of phthalate esters from drinking water consumed in Portugal. Environ Sci Pollut Res Int. 2014; 21(2):1380-90.

122. Serodio P, Nogueira JM. Considerations on ultra-trace analysis of phthalates in drinking water. Water Res. 2006; 40(13):2572-82.

123. Shao B, Hu J, Yang M, An W, Tao S. Nonylphenol and nonylphenol ethoxylates in river water, drinking water, and fish tissues in the area of Chongqing, China. Arch Environ Contam Toxicol. 2005; 48:467-73.

124. Stackelberg PE, Furlong ET, Meyer MT, Zaugg SD, Henderson AK, Reissman DB. Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. Sci Total Environ. 2004; 329:99-113.

125. Renner R. Exposure on tap drinking water as on overlooked source of lead. Environ Health Perspect. 2010; 118(2):A69-A74.

126. Romero J, Ventura F, Gomez M. Characterization of paint samples used in drinking water reservoirs: identification of endocrine disruptor compounds. J Chromatogr Sci. 2002; 40:191-7.

127. Ceretti E, Zani C, Zerbini I, Guzzella L, Scaglia M, Berna V, et al. Comparative assessment of genotoxicity of mineral water packed in polyethylene terephthalate (PET) and glass bottles. Water Res. 2010; 44(5):1462-70.

128. Ronquest-Ross L-C, Vink N, Sigge GO. Food consumption changes in South Africa since 1994. S Afr J Sci. 2015; 111(9-10):1-12.

129. Doria MF. Bottled water versus tap water: understanding consumers' preferences. J Water Health. 2006; 04(2):271-6.

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

130. Wilk R. Bottled Water: the pure commodity in the age of branding. J Consum Cult. 2006; 6(3):303-25.

131. Rodwan JG, Jr. [Internet]. 2012 Market report findings. Alexandria, VA: International Bottled Water Association; [cited 2016 July 13]. Available from: http://www.bottledwater.org/economics/industry-statistics.

132. Pinto B, Reali D. Screening of estrogen-like activity of mineral water stored in PET bottles. Int J Hyg Environ Health. 2009; 212(2):228-32.

133. Wagner M, Oehlmann J. Endocrine disruptors in bottled mineral water: total estrogenic burden and migration from plastic bottles. Environ Sci Pollut Res Int. 2009; 16(3):278-86.

134. Toyo'oka T, Oshige Y. Determination of alkylphenols in mineral water contained in PET bottles by liquid chromatography with coulometric detection. Anal Sci. 2000; 16:1071-6.

135. Li X, Ying GG, Su HC, Yang XB, Wang L. Simultaneous determination and assessment of 4-nonylphenol, bisphenol A and triclosan in tap water, bottled water and baby bottles. Environ Int. 2010; 36(6):557-62.

136. Sax L. Polyethylene terephthalate may yield endocrine disruptors. Environ Health Perspect. 2010; 118(4):445-8.

137. Mihucz VG, Záray G. Occurrence of antimony and phthalate esters in polyethylene terephthalate bottled drinking water. Appl Spectrosc Rev. 2016; 51(3):183-209.

138. Bach C, Dauchy X, Chagnon MC, Etienne S. Chemical compounds and toxicological assessments of drinking water stored in polyethylene terephthalate (PET) bottles: a source of controversy reviewed. Water Res. 2012; 46(3):571-83.

139. Barnes DK, Galgani F, Thompson RC, Barlaz M. Accumulation and fragmentation of plastic debris in global environments. Philos Trans R Soc Lond B Biol Sci. 2009; 364(1526):1985-98.

© University of Pretoria

140. Claudio L. Our food: packaging & public health. Environ Health Perspect. 2012; 120(6):A233-A7.

141. Real M, Molina-Molina JM, Jimenez-Diaz I, Arrebola JP, Saenz JM, Fernandez MF, et al. Screening of hormone-like activities in bottled waters available in southern Spain using receptor-specific bioassays. Environ Int. 2015; 74:125-35.

142. Muncke J, Myers JP, Scheringer M, Porta M. Food packaging and migration of food contact materials: will epidemiologists rise to the neotoxic challenge? J Epidemiol Community Health. 2014; 68(7):592-4.

143. Bach C, Dauchy X, Severin I, Munoz JF, Etienne S, Chagnon MC. Effect of sunlight exposure on the release of intentionally and/or non-intentionally added substances from polyethylene terephthalate (PET) bottles into water: chemical analysis and in vitro toxicity. Food Chem. 2014; 162:63-71.

144. Plotan M, Frizzell C, Robinson V, Elliott CT, Connolly L. Endocrine disruptor activity in bottled mineral and flavoured water. Food Chem. 2013; 136(3-4):1590-6.

145. Wagner M, Oehlmann J. Endocrine disruptors in bottled mineral water: estrogenic activity in the E-Screen. J Steroid Biochem Mol Biol. 2011; 127(1-2):128-35.

146. Guart A, Calabuig I, Lacorte S, Borrell A. Continental bottled water assessment by stir bar sorptive extraction followed by gas chromatography-tandem mass spectrometry (SBSE-GC-MS/MS). Environ Sci Pollut Res Int. 2014; 21(4):2846-55.

147. Amiridou D, Voutsa D. Alkylphenols and phthalates in bottled waters. J Hazard Mater. 2011; 185(1):281-6.

148. Cao XL. Determination of phthalates and adipate in bottled water by headspace solid-phase microextraction and gas chromatography/mass spectrometry. J Chromatogr A. 2008; 1178(1-2):231-8.

© University of Pretoria

149. Jeddi MZ, Rastkari N, Ahmadkhaniha R, Yunesian M. Concentrations of phthalates in bottled water under common storage conditions: do they pose a health risk to children? Food Res Int. 2015; 69:256-65.

150. Greifenstein M, White DW, Stubner A, Hout J, Whelton AJ. Impact of temperature and storage duration on the chemical and odor quality of military packaged water in polyethylene terephthalate bottles. Sci Total Environ. 2013; 456-457:376-83.

151. Guart A, Bono-Blay F, Borrell A, Lacorte S. Effect of bottling and storage on the migration of plastic constituents in Spanish bottled waters. Food Chem. 2014; 156:73-80.

152. Keresztes S, Tatar E, Czegeny Z, Zaray G, Mihucz VG. Study on the leaching of phthalates from polyethylene terephthalate bottles into mineral water. Sci Total Environ. 2013; 458-460:451-8.

153. Leivadara SV, Nikolaou AD, Lekkas TD. Determination of organic compounds in bottled waters. Food Chem. 2008; 108(1):277-86.

154. Mohamed MA, Ammar AS. Quantitative analysis of phthalates plasticizers in traditional Egyptian foods (koushary and foul medams), black tea, instant coffee and bottled waters by solid phase extraction-capillary gas chromatography-mass spectroscopy. Am J Food Technol. 2008; 3(5):341-6.

155. Montuori P, Jover E, Morgantini M, Bayona JM, Triassi M. Assessing human exposure to phthalic acid and phthalate esters from mineral water stored in polyethylene terephthalate and glass bottles. Food Addit Contam Part A. 2008; 25(4):511-8.

156. Selvaraj KK, Mubarakali H, Rathinam M, Harikumar L, Sampath S, Shanmugam G, et al. Cumulative exposure and dietary risk assessment of phthalates in bottled water and bovine milk samples: a preliminary case study in Tamil Nadu, India. Hum Ecol Risk Assess. 2016; 22(5):1166-82.

© University of Pretoria

157. Pfeifer D, Chung YM, Hu MC. Effects of low-dose bisphenol A on DNA damage and proliferation of breast cells: the role of c-Myc. Environ Health Perspect. 2015; 123(12):1271-9.

158. Geens T, Aerts D, Berthot C, Bourguignon JP, Goeyens L, Lecomte P, et al. A review of dietary and non-dietary exposure to bisphenol-A. Food Chem Toxicol. 2012; 50(10):3725-40.

159. Birnbaum LS, Bucher JR, Collman GW, Zeldin DC, Johnson AF, Schug TT, et al. Consortium-based science: the NIEHS's multipronged, collaborative approach to assessing the health effects of bisphenol A. Environ Health Perspect. 2012; 120(12):1640-4.

160. Christensen KL, Lorber M, Koslitz S, Bruning T, Koch HM. The contribution of diet to total bisphenol A body burden in humans: results of a 48 hour fasting study. Environ Int. 2012; 50:7-14.

161. Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. Environ Health Perspect. 2008; 116:39-44.

162. Carwile JL, Luu HT, Bassett LS, Driscoll DA, Yuan C, Chang JY, et al. Polycarbonate bottle use and urinary bisphenol A concentrations. Environ Health Perspect. 2009; 117(9):1368-72.

163. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). Reprod Toxicol. 2007; 24:139-77.

164. Trasande L, Attina TM, Blustein J. Association between urinary bisphenol A concentration and obesity prevalence in children and adolescents. JAMA. 2012; 308(11):1113-21.

165. Erler C, Novak J. Bisphenol a exposure: human risk and health policy. J Pediatr Nurs. 2010; 25(5):400-7.

166. Rochester JR. Bisphenol A and human health: a review of the literature. Reprod Toxicol. 2013; 42:132-55.

© University of Pretoria

167. Bonefeld-Jorgensen EC, Long M, Hofmeister MV, Vinggaard AM. Endocrine-disrupting potential of bisphenol A, bisphenol A dimethacrylate, 4-n-nonylphenol, and 4-n-octylphenol in vitro: new data and a brief review. Environ Health Perspect. 2007; 115 Suppl 1:69-76.

168. Bastos Sales L, Kamstra JH, Cenijn PH, Van Rijt LS, Hamers TL, J. Effects of endocrine disrupting chemicals on in vitro global DNA methylation and adipocyte differentiation. Toxicol In Vitro. 2013; 27:1634-43.

169. Carwile JL, Michels KB. Urinary bisphenol A and obesity: NHANES 2003-2006. Environ Res. 2011; 111(6):825-30.

170. European Food Safety Authority. Toxicokinetics of bisphenol A: scientific opinion of the panel on food additives, flavourings, processing aids and materials in contact with food (AFC). EFSA J. 2008; 759:1-10.

171. Ginsberg G, Rice DC. Does rapid metabolism ensure negligible risk from bisphenol A? Environ Health Perspect. 2009; 117(11):1639-43.

172. Seltenrich N. A hard nut to crack: reducing chemical migration in food-contact materials. Environ Health Perspect. 2015; 123(7):A174-9.

173. Inoue K, Kondo S, Yoshie Y, Kato K, Yoshimura Y, Horie M, et al. Migration of 4-nonylphenol from polyvinyl chloride food packaging films into food simulants and foods. Food Addit Contam. 2001; 18(2):157-64.

174. Chang CH, Tsai MS, Lin CL, Hou JW, Wang TH, Tsai YA, et al. The association between nonylphenols and sexual hormones levels among pregnant women: a cohort study in Taiwan. PLoS One. 2014; 9(8):e104245. doi: 10.1371/journal.pone.0104245.

175. Loyo-Rosales JE, Rosales-Rivera GC, Lynch AM, Price CP, Torrents A. Migration of nonylphenol from plastic containers to water and a milk surrogate. J Agric Food Chem. 2004; 52(7):2016-20.

© University of Pretoria

176. Forte M, Di Lorenzo M, Carrizzo A, Valiante S, Vecchione C, Laforgia V, et al. Nonylphenol effects on human prostate non tumorigenic cells. Toxicology. 2016; 357-358:21-32.

177. Yadetie F, Arukwe A, Goksøyr A, Male R. Induction of hepatic estrogen receptor in juvenile Atlantic salmon in vivo by the environmental estrogen, 4-nonylphenol. Sci Total Environ. 1999; 233(1-3):201-10.

178. Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology. 1993; 132(6):2279-86.

179. Soto AM, Justica H, Wray JW, Sonnenschein C. P-Nonylphenol: an estrogenic xenobiotic released from'modified' polystyrene. Environ Health Perspect. 1991; 92:167-73.

180. Preuss TG, Gurer-Orhan H, Meerman J, Ratte HT. Some nonylphenol isomers show antiestrogenic potency in the MVLN cell assay. Toxicol In Vitro. 2010; 24(1):129-34.

181. Scippo ML, Argiris C, Van De Weerdt C, Muller M, Willemsen P, Martial J, et al. Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. Anal Bioanal Chem. 2004; 378(3):664-9.

182. Giger W, Brunner PH, Schaffner C. 4-Nonylphenol in sewage-sludge: accumulation of toxic metabolites from nonionic surfactants. Science. 1984; 225:623-5.

183. Latini G. Monitoring phthalate exposure in humans. Clinica Chimica Acta. 2005; 361:20-9.

184. Hays SM, Aylward LL, Kirman CR, Krishnan K, Nong A. Biomonitoring equivalents for di-isononyl phthalate (DINP). Regul Toxicol Pharmacol. 2011; 60(2):181-8.

185. Heudorf U, Mersch-Sundermann V, Angerer J. Phthalates: toxicology and exposure. Int J Hyg Environ Health. 2007; 210(5):623-34.

© University of Pretoria

186. Rahman M, Brazel C. The plasticizer market: an assessment of traditional plasticizers and research trends to meet new challenges. Prog Polym Sci. 2004; 29(12):1223-48.

187. Erythropel HC, Maric M, Nicell JA, Leask RL, Yargeau V. Leaching of the plasticizer di(2-ethylhexyl)phthalate (DEHP) from plastic containers and the question of human exposure. Appl Microbiol Biotechnol. 2014; 98(24):9967-81.

188. Ghisari M, Bonefeld-Jorgensen EC. Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol Lett. 2009; 189(1):67-77.

189. Shen O, Du G, Sun H, Wu W, Jiang Y, Song L, et al. Comparison of in vitro hormone activities of selected phthalates using reporter gene assays. Toxicol Lett. 2009; 191(1):9-14.

190. Okubo T, Suzuki T, Yokoyama Y, Kano K, Kano I. Estimation of estrogenic and anti-estrogenic activities of some phthalate diesters and monoesters by MCF-7 cell proliferation assay in vitro. Biol Pharm Bull. 2003; 26(8):1219-24.

191. Takeuchi S, Iida M, Kobayashi S, Jin K, Matsuda T, Kojima H. Differential effects of phthalate esters on transcriptional activities via human estrogen receptors alpha and beta, and androgen receptor. Toxicology. 2005; 210(2-3):223-33.

192. Kruger T, Long M, Bonefeld-Jorgensen EC. Plastic components affect the activation of the aryl hydrocarbon and the androgen receptor. Toxicology. 2008; 246(2-3):112-23.

193. Johns LE, Cooper GS, Galizia A, Meeker JD. Exposure assessment issues in epidemiology studies of phthalates. Environ Int. 2015; 85:27-39.

194. Swan SH. Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environ Res. 2008; 108(2):177-84.

195. Kamrin MA. Phthalate risks, phthalate regulation, and public health: a review. J Toxicol Environ Health B Crit Rev. 2009; 12(2):157-74.

© University of Pretoria

196. Colón I, Caro D, Bourdony CJ, Rosario O. Identification of phthalate esters in the serum of young Puerto Rican girls with premature breast development. Environ Health Perspect. 2000; 108:895-900.

197. Tranfo G, Caporossi L, Paci E, Aragona C, Romanzi D, De Carolis C, et al. Urinary phthalate monoesters concentration in couples with infertility problems. Toxicol Lett. 2012; 213:15-20.

198. Gray LE, Jr., Ostby J, Furr J, Price M, Veeramachaneni DNR, Parks L. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol Sci. 2000; 58:350-65.

199. Noriega NC, Howdeshell KL, Furr J, Lambright CR, Wilson VS, Gray LE, Jr. Pubertal administration of DEHP delays puberty, suppresses testosterone production, and inhibits reproductive tract development in male Sprague-Dawley and Long-Evans rats. Toxicol Sci. 2009; 111(1):163-78.

200. Swan SH, Liu F, Hines M, Kruse RL, Wang C, Redmon JB, et al. Prenatal phthalate exposure and reduced masculine play in boys. Int J Androl. 2010; 33(2):259-69.

201. Xie C, Zhao Y, Gao L, Chen J, Cai D, Zhang Y. Elevated phthalates' exposure in children with constitutional delay of growth and puberty. Mol Cell Endocrinol. 2015; 407:67-73.

202. Axelsson J, Rylander L, Rignell-Hydbom A, Lindh CH, Jonsson BA, Giwercman A. Prenatal phthalate exposure and reproductive function in young men. Environ Res. 2015; 138:264-70.

203. Axelsson J, Rylander L, Rignell-Hydbom A, Jonsson BA, Lindh CH, Giwercman A. Phthalate exposure and reproductive parameters in young men from the general Swedish population. Environ Int. 2015; 85:54-60.

204. Duty SM, Singh NP, Silva MJ, Barr DB, Brock JW, Ryan L, et al. The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay. Environ Health Perspect. 2003; 111:1164-69.

© University of Pretoria

205. Bach C, Dauchy X, Severin I, Munoz JF, Etienne S, Chagnon MC. Effect of temperature on the release of intentionally and non-intentionally added substances from polyethylene terephthalate (PET) bottles into water: chemical analysis and potential toxicity. Food Chem. 2013; 139(1-4):672-80.

206. Zhou SN, Moody RP, Aikawa B, Yip A, Wang B, Zhu J. In vitro dermal absorption of di(2-ethylhexyl) adipate (DEHA) in a roll-on deodorant using human skin. J Toxicol Environ Health A. 2013; 76(3):157-66.

207. Cao XL, Zhao W, Churchill R, Dabeka R. Di-(2-ethylhexyl) adipate in selected total diet food composite samples. J Food Prot. 2013; 76(11):1985-8.

208. Fan YY, Zheng JL, Ren JH, Luo J, Cui XY, Ma LQ. Effects of storage temperature and duration on release of antimony and bisphenol A from polyethylene terephthalate drinking water bottles of China. Environ Pollut. 2014; 192:113-20.

209. Filer D, Patisaul HB, Schug T, Reif D, Thayer K. Test driving ToxCast: endocrine profiling for 1858 chemicals included in phase II. Curr Opin Pharmacol. 2014; 19:145-52.

210. Leusch FDL, De Jager C, Levi Y, Lim R, Puijker L, Sacher F, et al. Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters. Environ Sci Technol. 2010; 44(10):3853-60.

211. Sanfilippo K, Pinto B, Colombini MP, Bartolucci U, Reali D. Determination of trace endocrine disruptors in ultrapure water for laboratory use by the yeast estrogen screen (YES) and chemical analysis (GC/MS). J Chromatogr B Analyt Technol Biomed Life Sci. 2010; 878(15-16):1190-4.

212. Kayali N, Tamayo FG, Polo-Diez LM. Determination of diethylhexyl phtalate in water by solid phase microextraction coupled to high performance liquid chromatography. Talanta. 2006; 69(5):1095-9.

213. Aneck-Hahn NH, De Jager C, Bornman MS, Du Toit D. Oestrogenic activity using a recombinant yeast screen assay (RCBA) in South African laboratory water sources. Water SA. 2005; 31(2):253-6.

© University of Pretoria

214. Arditsoglou A, Voutsa D. Determination of phenolic and steroid endocrine disrupting compounds in environmental matrices. Environ Sci Pollut Res. 2008; 15(3):228-36.

215. Liu R, Zhou JL, Wilding A. Simultaneous determination of endocrine disrupting phenolic compounds and steroids in water by solid-phase extraction–gas chromatography–mass spectrometry. J Chromatogr A. 2004; 1022(1-2):179-89.

216. Beronius A, Hanberg A, Zilliacus J, Ruden C. Bridging the gap between academic research and regulatory health risk assessment of endocrine disrupting chemicals. Curr Opin Pharmacol. 2014; 19:99-104.

217. Mueller SO. Xenoestrogens: mechanisms of action and detection methods. Anal Bioanal Chem. 2004; 378(3):582-7.

218. Genthe B, Steyn M. Health risk assessment protocol for endocrine disrupting chemicals. WRC Project No KV 206/08. Pretoria: Water Research Commission of South Africa; 2008.

219. Zoeller RT, Vandenberg LN. Assessing dose-response relationships for endocrine disrupting chemicals (EDCs): a focus on non-monotonicity. Environ Health. 2015; 14:42.

220. USEPA. Exposure Factors Handbook: 2011 edition. Washington, DC: National Centre for Environmental Assessement; 2011.

221. Waters [Internet]. Oasis glass cartridges care and use manual. Milford, USA: Waters Corporation; [cited 24 Dec 2014]. Available from: http://www.waters.com/waters/support.htm?lid=10103462&type=USRM.

222. De Jager C, Aneck-Hahn NH, Barnhoorn IEJ, Bornman MS, Pieters R, Van Wyk JH, et al. The compilation of a toolbox of bio-assays for detection of estrogenic activity in water. WRC report no 1816/1/10. Pretoria: Water Research Commission of South Africa; 2011.

BESTPFE.COM
List of research project topics and materials

© University of Pretoria

223. Routledge EJ, Sumpter JP. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem. 1996; 15(3):241-8.

224. Wilson VS, Bobseine K, Gray LE, Jr. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. Toxicol Sci. 2004; 81(1):69-77.

225. Dhooge W, Arijs K, D'Haese I, Stuyvaert S, Versonnen B, Janssen C, et al. Experimental parameters affecting sensitivity and specificity of a yeast assay for estrogenic compounds: results of an interlaboratory validation exercise. Anal Bioanal Chem. 2006; 386(5):1419-28.

226. USEPA. Risk assessment guidelines of 1986. USA: United States Environmental Protection Agency; 1987.

227. USEPA. Guidelines for exposure assessment. USA: United States Environmental Protection Agency; 1992.

228. WHO. Human health risk assessment toolkit. Geneva: World Health Organization; 2010.

229. Genthe B, Steyn M. Chapter 11: Health risk assessment. In: Bornman MS, Van Vuren JHJ, Bouwman H, De Jager C, Genthe B, Barnhoorn IEJ, editors. Endocrine disruptive activity and the potential health risk in an urban nature reserve WRC report number 1505/1/07. Pretoria: Water Research Commission of South Africa; 2007. p. 178-203.

230. World Health Organization. The World Health Report 2004. Changing History. Geneva: World Health Organization; 2004.

231. Risk*Assistant™ [computer program]. Hampshire Research Institute. Alexandria, VA: Thisle Publishers; 1995.

© University of Pretoria

232. Beresford N, Routledge EJ, Harris CA, Sumpter JP. Issues arising when interpreting results from an in vitro assay for estrogenic activity. Toxicol Appl Pharmacol. 2000; 162(1):22-33.

233. Jugan ML, Oziol L, Bimbot M, Huteau V, Tamisier-Karolak S, Blondeau JP, et al. In vitro assessment of thyroid and estrogenic endocrine disruptors in wastewater treatment plants, rivers and drinking water supplies in the greater Paris area (France). Sci Total Environ. 2009; 407(11):3579-87.

234. Leusch FD, van den Heuvel MR, Chapman HF, Gooneratne SR, Eriksson AM, Tremblay LA. Development of methods for extraction and in vitro quantification of estrogenic and androgenic activity of wastewater samples. Comp Biochem Physiol C. 2006; 143(1):117-26.

235. Avbersek M, Zegura B, Filipic M, Uranjek-Zevart N, Heath E. Determination of estrogenic potential in waste water without sample extraction. J Hazard Mater. 2013; 260:527-33.

236. Conley JM, Evans N, Mash H, Rosenblum L, Schenck K, Glassmeyer S, et al. Comparison of in vitro estrogenic activity and estrogen concentrations in source and treated waters from 25 U.S. drinking water treatment plants. Sci Total Environ. 2016; S0048-9697(16):30303-5.

237. Bergamasco AM, Eldridge M, Sanseverino J, Sodre FF, Montagner CC, Pescara IC, et al. Bioluminescent yeast estrogen assay (BLYES) as a sensitive tool to monitor surface and drinking water for estrogenicity. J Environ Monit. 2011; 13(11):3288-93.

238. Omoruyi IM, Pohjanvirta R. Estrogenic activity of wastewater, bottled waters and tap water in Finland as assessed by a yeast bio-reporter assay. Scand J Public Health. 2015; 43(7):770-5.

239. Cargouet M, Perdiz D, Levi Y. Evaluation of the estrogenic potential of river and treated waters in the Paris area (France) using in vivo and in vitro assays. Ecotoxicol Environ Saf. 2007; 67(1):149-56.

© University of Pretoria

240. Torres NH, Aguiar MM, Ferreira LFR, Américo JHP, Machado ÂM, Cavalcanti EB, et al. Detection of hormones in surface and drinking water in Brazil by LC-ESI-MS/MS and ecotoxicological assessment with Daphnia magna. Environ Monit Assess. 2015; 187(6):379. doi: 10.1007/s10661-015-4626-z.

241. Carvalho ARM, Cardoso VV, Rodrigues A, Ferreira E, Benoliel MJ, Duarte EA. Occurrence and analysis of endocrine-disrupting compounds in a water supply system. Environ Monit Assess. 2015; 187(3):139. doi: 10.1007/s10661-015-4374-0.

242. Benotti MJ, Trenholm RA, Vanderford BJ, Holady JC, Stanford BD, Snyder SA. Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. Environ Sci Technol. 2009; 43:597-603.

243. United States Environmental Protection Agency. 2012 Edition of the drinking water standards and health advisories. Washington, DC: Office of Water US Environmental Protection Agency; 2012.

244. Caldwell DJ, Mastrocco F, Nowak E, Johnston J, Yekel H, Pfeiffer D, et al. An assessment of potential exposure and risk from estrogens in drinking water. Environ Health Perspect. 2010; 118(3):338-44.

245. Thompson RC, Moore CJ, vom Saal FS, Swan SH. Plastics, the environment and human health: current consensus and future trends. Phil Trans R Soc B. 2009; 364(1526):2153-66.

246. Pretorius W [Internet]. Operation Hydrate delivers 4 million litres of water, and still counting. News24; [updated 2016 Feb 03; cited 2016 Jun 6]. Available from: http://www.news24.com/SouthAfrica/News/operation-hydrate-delivers-4-million-litres-of-water-and-still-counting-20160203.

247. Operation Hydrate [Internet]. #OperationHydrate. Johannesburg: Operation Hydrate; [cited 2016 Mar 26]. Available from: http://www.operationhydrate.org.

248. Sanches S, Rodrigues A, Cardoso VV, Benoliel MJ, Crespo JG, Pereira VJ. Comparison of UV photolysis, nanofiltration, and their combination to remove hormones from a drinking water source and reduce endocrine disrupting activity. Environ Sci Pollut Res. 2016; 23(11):11279-88.

© University of Pretoria

249. Manickum T, John W. The current preference for the immuno-analytical ELISA method for quantitation of steroid hormones (endocrine disruptor compounds) in wastewater in South Africa. Anal Bioanal Chem. 2015; 407(17):4949-70.

250. Adewumi JR, Ilemobade AA, Van Zyl JE. Treated wastewater reuse in South Africa: overview, potential and challenges. Resour Conserv Recy. 2010; 55(2):221-31.

251. Schmid P, Kohler M, Meierhofer R, Luzi S, Wegelin M. Does the reuse of PET bottles during solar water disinfection pose a health risk due to the migration of plasticisers and other chemicals into the water? Water Res. 2008; 42(20):5054-60.

252. South African National Bottled Water Association [Internet]. About bottled water. Johannesburg: South African National Bottled Water Association; [cited 2016 Oct 21]. Available from: http://www.sanbwa.org.za/water.asp.

253. Department of Health [Internet]. Regulations relating to all packaged water: GNR. 718 of 28 July 2006. Pretoria: Department of Health; [updated 2010 May 26; cited 2016 Oct 7]. Available from: http://www.health.gov.za/index.php/shortcodes/2015-03-29-10-42-47/2015-04-30-09-10-23/2015-04-30-09-11-35/category/210-regulations-packaged-water-and-other-beverages?download=792:regulations-relating-to-all-packaged-water-r718-2006.

© University of Pretoria

Appendix A: Bottled water sample codes

Sample code	Brand name
BTW01	Valpré
BTW02	Highland
BTW03	Tsitsikamma Crystal
BTW04	Bonaqua
BTW05	Below Zero
BTW06	Bené
BTW07	Aquartz
BTW08	Aquellé
BTW09	Woolworths
BTW10	Nestlé Pure Life

© University of Pretoria

Appendix B: Ethics approval

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

4/03/2013

Approval Notice
New Application

Ethics Reference No.: 48/2013

Title: Estrogenic activity, target endocrine disrupting chemical levels and potential health risks of bottled water and water from selected distribution points in Pretoria and Cape Town.

Dear Catherina van Zijl

The **New Application** for your research received on the 20/02/2013, was approved by the Faculty of Health Sciences Research Ethics Committee on the 27/02/2013

Please note the following about your ethics approval:
- Ethics Approval is valid for 3 years, till the end of February 2016 .
- Please remember to use your protocol number (48/2013) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
Standard Conditions:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

• Tel:012-3541330 • Fax:012-3541367 Fax2Email: 0866515924 • E-Mail: manda@med.up.ac.za
• Web: //www.healthethics-up.co.za • H W Snyman Bld (South) Level 2-34 • Private Bag x 323, Arcadia, Pta, S.A., 0007

© University of Pretoria