AN INVESTIGATION OF THE ESTROGENIC ACTIVITY IN WATER FROM SELECTED DRINKING WATER TREATMENT PROCESSES

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

1. Background and motivation

In recent years there has been increasing evidence that some anthropogenic chemicals as well as naturally occurring estrogens may disrupt the endocrine system by mimicking natural hormones, inhibiting the action of hormones, or altering the normal regulatory function of the immune, nervous and endocrine systems. Endocrine disrupting chemicals (EDCs) can enter the water environment by direct discharge into water, the use of pharmaceuticals and chemicals in households and in agriculture and industry, accidental spills and releases of compounds and indirectly through diffuse sources such as storm water runoff. Natural hormones, including estrogens, can be released into the environment via sewage effluent and from sources such as animal feedlots.

WRC studies have shown that a number of EDCs [e.g. nonyl phenol, chlorinated pesticides, polychlorinated biphenyls (PCBs)] are present in South African surface water and effluent discharges. WRC and other local studies using *in vitro* and *in vivo* biological/biochemical techniques have also demonstrated significant estrogenic activity in surface waters and domestic/industrial effluent discharges. The presence of EDCs in drinking water sources is a matter of great concern and poses the question of how effectively these chemicals are removed by conventional water treatment methods. Due to the wide structural diversity of these chemicals, more than one process may be required for their removal.

2. Objectives

This project was carried out to investigate the estrogenic activity in the water from selected drinking water treatment processes. The specific objectives were:

1) To investigate the occurrence and fate of estrogen mimicking substances in source and treated drinking water using biological/biochemical techniques and chemical analyses;
2) To investigate the removal of estrogen mimicking substances by different drinking water treatment processes; and
3) To make recommendations on the most appropriate combination of tests for the detection of estrogenic activity in drinking water.

This study should be considered as a pilot investigation to assess the presence of estrogenic activity during the various processing steps used by a few selected drinking water treatment facilities. Risk assessment was not part of the scope of the study.

3. Methodology

A battery of *in vivo* and *in vitro* biological/biochemical techniques established by different South African and overseas laboratories were applied. The *in vitro* tests included a recombinant yeast estrogen screen (YES) (Routledge and Sumpter, 1996), an estrogen receptor-mediated chemical activated luciferase gene expression (ER-CALUX) assay (Legler et al., 1999), a *Xenopus laevis* liver slice assay (Hurter

2003; Hurter et al., 2002) and a primary rainbow trout hepatocyte (PRTH) assay (Gagné and Blaise, 2000).

For *in vivo* testing juvenile zebrafish (*Brachyodanio rerio*), 16 to 24 d old, were exposed to water for 14 d where after vitellogenin (VTG) in whole body homogenates was measured directly with an enzyme linked immunosorbant assay (ELISA) (Holbech et al., 2001) and gel electrophoresis (Slabbert et al., 2007a) and indirectly with an alkali-labile phosphate (ALP) assay (Gagné and Blaise, 2000). VTG is synthesised and secreted by the liver of oviparous vertebrates in response to circulating estrogens in mature females. Under normal circumstances no VTG is present in the blood of males. They do, however have a functional VTG gene that can be expressed in the presence of estrogen. The presence of VTG in the blood and liver of males thus indicates the exposure to exogenous estrogens or estrogen mimicking substances.

Chemical analyses were used to establish atrazine and p-nonyl phenol residues in water. In addition, water analysed for the estrogens 17α- and β-estradiol, estrone, 17α-ethinylestradiol and estriol. All the bioassays and chemical analyses were carried out according to published protocols.

Three drinking water treatment plants (A, B and C) in the Gauteng Province were selected for the study. Source water and water from selected treatment processes (e.g. flocculation, sand filtration, chlorination) were tested. Four sampling surveys were carried out during a one year period. For *in vitro* testing, samples were concentrated by means of XAD-7 resin. Ethanol was used to prepare the final extract (8 000-times concentrated). A solid phase column (PPL) was used to concentrate the water for estrogen analysis (10 000-times concentrated) while liquid-liquid extraction [dichloromethane (DCM)] was used for residue analysis (200-times concentrated). Standard protocols were followed.

4. Summary of major results

92% of the samples tested with the YES showed estrogenic activity. On four occasions the estrogenicity in final waters was below the limit of detection (LOD). Estrogenic potencies ranged from 0.036 to 3.189 ng estrogen equivalents (EEQ)/ℓ for source waters to 0.009 to 0.138 ng EEQ/ℓ for final waters. The ER-CALUX assay detected estrogenicity in 56% of the samples. The estrogenic potencies obtained for source water ranged from <LOD to 0.758 ng EEQ/ℓ, while final waters exhibited estrogenic potencies from <LOD to 0.176 ng EEQ/ℓ.

The results obtained with the YES clearly showed a reduction in estrogenicity in the final water. 58% of the samples also showed this reduction with the ER-CALUX assay. With both the YES and ER-CALUX assay there appeared to be an increase in estrogenic activity after the addition of flocculants. With both assays estrogenic effects observed during the 2nd sampling survey were lower than on the other occasions. The results also indicated that the estrogenic activities in the water of Treatment Plant B was generally higher than that of Treatment Plant A followed by that of Treatment Plant C.

Although the YES was able to detect estrogenicity in most of the samples, the ER-CALUX assay was more sensitive. The lowest concentration detected by the YES was 0.009 ng EEQ/ℓ while levels as low as 0.00085 ng EEQ/ℓ were reported for the ER-CALUX assay. The highest estrogenic potencies established with the YES were 3.189 and 4.745 ng EEQ/ℓ. The ER-CALUX assay showed the highest estrogenic potencies for the same samples with 0.758 and 2.620 ng EEQ/ℓ.

The estrogenic potencies obtained with the YES for the source water in this study were similar to those obtained during a previous WRC study on surface water (Slabbert et al., 2007a). In the previous study estrogenic potencies for surface water 0.20 to 2.04 ng EEQ/ℓ while in this study the values ranged from 0.036 to 3.189 ng EEQ/ℓ. The results obtained for source water with the ER-CALUX assay were similar to some of the results reported in a recent WRC study on the water of an urban nature reserve (0.33 to 16.0 ng EEQ/ℓ) (Bornman et al., 2007). No data could be obtained in literature for treated drinking water.

The PRTH assay showed estrogenicity in 64% of the samples. 25% of the water samples showed a reduction in estrogenicity in final water as compared to source water. This reduction in estrogenicity was, however, not noticed for Treatment Plant B. As in the case of the YES and ER-CALUX assay the PRTH assay demonstrated a higher VTG induction by the water of Treatment Plant B.

29% of the samples showed estrogenicity with the zebrafish ALP assay and only 6% with the *Xenopus* liver slice assay. No clear patterns of reduction in estrogenicity during water treatment were noticed for the zebrafish ALP assay and the liver slice assay. The zebrafish ELISA did not detect estrogenicity in any of the samples. Likewise, no VTG band could be detected by means of gel electrophoresis.

In the study by Slabbert et al. (2007a) inductions in VTG of between 20 and 200% were obtained with the zebrafish ALP assay for environmental water. The inductions in the present study by source water is considerably less at between 12 and 68%. As in the present study the zebrafish ELISA showed an absence of estrogenic activity when exposed to sewage effluent samples (Slabbert et al., 2007b). Negative results were also obtained with the liver slice assay when studying environmental waters (Burger, 2007).

The assays used are specific for the detection of estrogens and estrogen mimicking substances. In the PRTH assay and the zebrafish ELISA and ALP assay, VTG concentrations were, in some instances, significantly reduced compared to control VTG. A possible explanation for this could be the presence of anti-estrogens in the water samples. Anti-estrogens block the estrogen receptor, preventing estrogens to bind to the receptor.

Chemical analysis showed that the triazines (simazine, atrazine and terbuthylazine) and p-nonyl phenol were present above the analytical detection limits in some of the water samples. Concentrations ranged from 0.20 (simazine in source water) to 0.70 (atrazine in final water) µg/ℓ. The South African Water Quality Guidelines for domestic use (DWAF, 1996) state a Target Water Quality Range (TWQR) of 0-2.0 µg/ℓ for atrazine to avoid health effects for lifetime exposure. Since atrazine is the most toxic of the triazines, the same TWQR could be applied for simazine and

terbuthylazine. The concentrations detected in the final water samples were on the lower side of the stated TWQR. However, addition or synergism may occur. If this is the case, the concentrations of atrazine and terbuthylazine in the final water of Treatment Plant C (2nd survey) are a concern. No criterium is available for nonyl phenol in drinking water. The concentrations found in the source and final waters were mostly between 0.22 and 0.25 µg/ℓ and in agreement with levels detected from time to time in drinking water (Personal Communication, 2006a).

The triazines do not bind to the estrogen receptor and would, therefore, not have incurred responses in the bioassays used. This could explain why the estrogenic activity in samples collected during the 2nd survey was low, while several samples contained detectable levels of the triazines. Nonyl phenol is an estrogen mimic and could have contributed to the estrogenicity observed in some of the assays.

All the estrogens in the source and final water collected from three drinking water treatment plants during four sampling surveys, were below the limit of detection (<1.0 ng/ℓ).

5. Conclusions

The bioassays clearly showed estrogenic activity in source and treated drinking water. Chemical analysis also indicated that triazines and nonyl phenol were occasionally detected in water samples. The estrogenic compounds 17α- and β-estradiol, estrone, 17α-ethinylestradiol and estriol in all the water samples were below the detection limit of 1.0 ng/ℓ.

The results obtained with the YES and ER-CALUX assay showed a reduction in/removal of estrogenicity by the water treatment processes. The most significant reductions were observed in the final waters, after chlorination.

In general, both assays indicated some increase in estrogenic activity after the addition of flocculants. A reduction of triazines and p-nonyl phenol concentration in final waters was also noticed during 50 to 75% of the sampling occasions.

The data obtained with the YES and ER-CALUX and PRTH assays indicated higher estrogenic activity in the water (source and treated water) of Treatment Plant B. The YES and ER-CALUX assay also indicated that the estrogenic activity in the water of Treatment Plant C was generally the lowest. The PRTH assay, on the other hand, showed the lowest estrogenic activity in the water of Treatment Plant A.

Factors such as heavy rain, high algal loads and changes in flocculant did not appear to have impacted on the water quality. What appeared to be an important factor in the efficiency of treatment plants to reduce/remove estrogenicity was the source water quality.

Water quality is a complex issue. With the few organic chemical analysis carried out it is not possible to link estrogenic effects in bioassays to specific chemicals. Since nonyl phenol is an estrogen mimicking compound, it is expected that this chemical contributed to some of the effects observed. Some of the endocrine disrupting

metals, cadmium, lead and mercury were present above the detection limits in water from Treatment Plants A and B and could have contributed to estrogenicity.

The YES and ER-CALUX and PRTH assays proved to be the most suitable for the detection of estrogenicity in drinking waters. Another candidate for the battery of tests for drinking water testing is the liver slice assay. However, the test will have to be adapted for concentrated samples.

The *in vivo* tests did not perform well in the study. The zebrafish ELISA did not show estrogenic activity in any of the water samples. Although the zebrafish ALP assay detected estrogenicity in some samples, results were erratic and several samples reduced VTG. VTG levels were generally very low in the fish homogenates as a result of dilution. Appropriate responses with the positive control 17β-estradiol were also not obtained. Gel electrophoresis as applied in this study could also not detect VTG in any of the fish exposed to water samples. *In vivo* tests are time consuming and there are many variables that can impose errors in the assays. These tests are, therefore, not recommended for drinking water testing.

6. Recommendations

In vivo tests have an important role to play in environmental water monitoring. It is, therefore, felt that the zebrafish ELISA should be further explored. The exposure protocols should be standardized (fish handling, fish age, etc) and procedures to process fish for shipment should also be investigated (transporting fish in anti-protease buffer as opposed to placing dry fish in containers). It is also recommended that the VTG concentration should be normalized with a total protein determination as the variation in total protein in individual fish may mask significant differences among groups. Inter-laboratory studies should also be carried out with estrogens to validate the test. A study is also required to compare the sensitivities of the different zebrafish VTG antibodies. At the same time the cost of commercially available antibodies versus sensitivity should be assessed.

Efforts should be made to establish the PRTH assay locally. This test has been proven to be a valuable part of the battery of tests performed by Environment Canada for the detection of toxicity and estrogenicity.

The ER-CALUX assay is not available in South Africa. An alternative mammalian cell test is urgently required as part of the battery of bioassays used for drinking water testing. It is expected that the T47D-Kbluc assay, currently being introduced into the University of Pretoria laboratories, will be a suitable alternative to the ER-CALUX assay.

Detection limits and limits of quantification for the whole YES method are not well established. The role of the concentration factor used in the test in determining limits and levels has also not been well defined. It recommended that data calculation is revisited to eliminate unclarities and to ensure standardisation.

The sample extracts for the *in vitro* tests were prepared by means of solid phase extraction using XAD-7. A comparison between a limited number of extracts of spiked water samples and environmental samples using XAD-7 and C18 columns for

extraction showed the best recovery of chemicals was obtained with XAD-7 concentration (Slabbert et al., 2007a). C18 columns are, however, more generally used to prepare sample extracts for *in vitro* tests. It is recommended that a more extensive study is carried out on XAD-7 and C18 extracts to allow firm recommendations.

This study has shown the presence of estrogenic activity in drinking water. In order to establish the cause of these effects, extensive chemical monitoring programmes are recommended. Risk assessment will also be required to provide answers on the risk involved in drinking water exhibiting estrogenicity.

7. Archiving of data generated during the project

Raw data were generated in many different laboratories. Some of the raw data were captured in the form of spectrophotometric print-outs (not connected to an electronic system) and some were recorded on analyses sheets. Because of the difficulty and time-consuming exercise to capture these raw data electronically, it is recommended that the data are kept in storage at the different laboratories.

8. Publications

No publications have been submitted as yet.

9. Capacity building

A number of previously disadvantaged persons received training in methodologies used for the study. Ms A Motsepe, a permanent member of staff at CSIR, was trained to do the water sample concentration. Ms M Moletsane, a National Diploma student doing her in-service training at CSIR, and Ms L Fakude, a contract worker, received training in fish maintenance and breeding. Ms Moletsane was also trained to do the fish exposure studies, protein analysis, ALP analysis and gel electrophoresis. Training was also received in quality assurance aspects related to the techniques. All these persons, as well as another in-service trainee, Ms K Ntsoane, assisted with sample collection. This provided the opportunity to gain insight into the operation of drinking water treatment plants and the different treatment processes.

Although none of the above persons who received training used the project work to obtain further qualifications, the training received constituted the development of scarce skills. M Moletsane is currently enrolled for a B Tech in Biotechnology. She plans to use the zebrafish exposure data for the practical study.

Unfortunately, two of the above persons who received training during the execution of the project, Ms A Motsepe and Ms K Ntsoane, have left the services of the CSIR. Although these resignations might be seen as limitations in capacity building, they are currently employed by institutions in South Africa and therefore the country is still benefiting from the experience gained by them.

At the University of Stellenbosch, Ms ME Esterhuyse, a PhD student, was trained to do the zebrafish VTG ELISA, *X laevis* VTG ELISA, the UniVTG ELISA, as well as the

Xenopus liver culture assay. She now has the competency to do these ELISAs and bioassay independently. Ms F Gordon, a Technical Officer also received training in the *Xenopus* liver bioassay. This was not part of their research activities or duties but represent new capacity that was built.

Because of the delays in sampling caused by problems with fish breeding, it was not possible to do sample concentration on site. No training was, therefore, provided to staff at the water works during the sampling surveys. Staff from the three drinking water treatment plants were invited to a one-day course at CSIR on 18 October 2006. Due to other commitments by one of the plants, the course had to be postponed.

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Abs	Absorbance
ALP	Akali-labile phosphate
ANOVA	Analysis of Variance
AR	Androgen receptor
ATP	Adenosine 5'-triphosphate
CALUX	Chemical activated luciferase gene expression
CPRG	Chlorophenol red-ß-D-galactopyranoside
CV	Coefficient of variance
DCM	Dichloromethane
DDD	1,1-dichloro-2.2-bis(*p*-chlorophenyl) ethane
DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DWAF	Department of Water Affairs
EC_{50}	Concentration giving 50% of the maximum response
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EEQ	Estradiol equivalent
EDC	Endocrine disrupting compound
ELISA	Enzyme linked immunosorbent assay
ER	Estrogen receptor
ERE	Estrogen receptor element
ERG	Estrogen receptor gene
FDA	Fluorescein diacetate
GC	Gas chromatography
GWRC	Gobal Water Research Coalition
hER	Human estrogen receptor
Ig	Immunoglobulin
ITD	Ion trap detector
ITS	Ion trap sensor
LOD	Limit of detection
LOQ	Limit of quantification
mRNA	Messenger ribonucleic acid
MS	Mass spectrophotometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
NRE	Natural Resources and the Environment
OD	Optical density
PBS	Phosphate buffered saline
PCB	Pentachlorophenol
PMSF	Phenyl methyl sulphonyl fluoride
PRTH	Primary rainbow trout hepatocyte
PTFE	Polytetrafluoroethylene
rpm	Revolutions per minute
SABS	South African Bureau of Standards
SD	Standard deviation

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TWQR	Target Water Quality Range
UK	United Kingdom
USA	United States of America
USEPA	United States Environmental Protection Agency
VTG	Vitellogenin
v/v	Volume per volume
WRC	Water Research Commission
w/v	Weight per volume
YES	Recombinant yeast screen

1. INTRODUCTION

The issue of endocrine disrupting chemicals (EDCs) in the environment has received widespread attention during the past decade, not only from the scientific community, but also from the popular media. EDCs are chemicals that interfere with the structure or function of hormone-receptor complexes. These chemicals are thought to mimic natural hormones, inhibit the action of hormones, or alter the normal regulatory function of the immune, nervous and endocrine systems. The USEPA (1997) defines an EDC as 'an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour'. A large number of natural and synthetic chemical compounds have been identified to elicit estrogenic responses including pharmaceuticals, pesticides, industrial chemicals and heavy metals (Ntuli and Slabbert, 1996). Effects in humans for which links with exposure to EDCs have been suggested include breast cancer and endometriosis in women, testicular and prostate cancer in men, abnormalities with regard to sexual development, male infertility, pituitary and thyroid disfunction, immune suppression and changes in neurobehaviour (Aneck-Hahn, 2003).

EDCs can enter water bodies via direct discharge into water; the use of pharmaceuticals and chemicals in households, agriculture and industry; accidental spills and releases of compounds; and indirectly through diffuse sources such as storm water runoff. WRC studies carried out by Meintjies et al. (2000), Bornman et al. (2007) and Burger (2007) have indicated that a number of estrogen related substances are present in South African waters. Many of the substances were found in the Pretoria, Witwatersrand and Vereeniging area. The study by Meintjies et al. (2000) showed that nonyl phenol was present in the Vaal River; lindane, 1,1-dichloro-2.2-bis(*p*-chlorophenyl) ethane (DDD), 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE) and pentachlorophenyls (PCBs) in industrial effluents; and atrazine in dam and river water. The surveys by Bornman et al. (2007) and Burger (2007) confirmed the presence of the above chemicals in environmental waters. The heavy metal endocrine disruptors arsenic, cadmium, lead, and mercury were also found to be present at relatively high levels. WRC (Bornman et al., 2007; Slabbert et al., 2007a,b) and other local studies (Slabbert et al., 2000; 2003) using *in vitro* and *in vivo* biological/biochemical techniques have also demonstrated significant estrogenic activity in surface waters and domestic/industrial effluent discharges.

The presence EDCs in drinking water sources is a matter of great concern and poses the question of how effectively these chemicals are removed by conventional water treatment methods. Due to the wide structural diversity of these chemicals, more than one process may be required for their removal. The aim of this study was, therefore, to investigate the estrogenic activity in the water from selected drinking water treatment processes. The specific objectives were:

1) To investigate the occurrence and fate of estrogen mimicking substances in source and treated drinking water using biological/biochemical techniques and chemical analyses;
2) To investigate the removal of estrogen mimicking substances by different drinking water treatment processes; and
3) To make recommendations on the most appropriate combination of tests for the detection of estrogenic activity in drinking water.

This study should be considered as a pilot investigation to assess the presence of estrogenic activity during the various processing steps used by a few selected drinking water treatment facilities. Risk assessment was not part of the scope of the study.

A battery of *in vitro* and *in vivo* biological/biochemical techniques established by different South African laboratories during the WRC project of Burger (2007) were selected for the study. The *in vitro* tests included a recombinant yeast estrogen screen (YES) (Routledge and Sumpter, 1996), the E-screen (proliferation of MFC-7 mammalian cells) (Soto et al., 1995) and a *Xenopus laevis* liver slice assay (Hurter 2003; Hurter et al., 2002). Because of problems experienced with the suggested mammalian cell test it was decided to replace the E-screen with the T47D-kBluc reporter gene assay (Wilson et al., 2004). When further problems were experienced, i.e. delays in delivery of the cell line, contamination and a faulty luminometer, it was decided to send samples to Bio Detection Systems in Amsterdam for analysis. This laboratory used the estrogen receptor-mediated chemical activated luciferase gene expression (ER-CALUX) assay (Legler et al., 1999). A primary rainbow trout hepatocyte (PRTH) assay (Gagné and Blaise, 2000) was included into the battery of *in vitro* tests when Dr C Blaise of Environment Canada kindly volunteered to use this test on the water samples. For *in vitro* testing a zebrafish test was used. Vitellogenin (VTG) was established directly by means of an enzyme linked immunosorbant assay (ELISA) (Holbech et al., 2001) and gel electrophoresis (Slabbert et al., 2007a), and indirectly by an alkali-labile phosphate (ALP) assay (Gagné and Blaise, 2000).

The bioassays selected for the study are no more than tools to identify the presence of estrogenic activity. They are not used to identify reproductive or developmental effects, but are screening tests as recommended for Tier 1 testing (EDSTAC, 1998). Of these the YES and ER-CALUX are well established and internationally used. These two assays are also included in the Global Water Research Coalition (GWRC) EDC toolbox. The *in vivo* tests as well as the liver slice and PRTH assays are not that frequently used, and although set procedures were followed they are still considered to be in the development/optimisation phase.

Chemical analyses were selected on the basis of their occurrence in environmental water during previous studies (Burger, 2007) and the availability of established analytical services. On the recommendation of Ms A Burger and Mr C Schoeman it was decided to conduct chemical analysis on the triazine and p-nonyl phenol residues as well as the estrogens 17α- and β-estradiol, estrone, 17α-ethinylestradiol and estriol.

Three drinking water treatment plants in the Gauteng Province were selected for the study. It was decided to conduct four surveys. The particular plants were selected because 1) a variety of pollution sources in the different catchments could impact on the source waters, e.g. sewage effluent, industrial discharges, and non-point sources (storm water, agricultural activities); 2) previous studies have shown the presence of EDCs and endocrine disrupting activity in the source waters; and 3) considerable information exists with regard to other adverse activities (toxicity, mutagenicity, tumour promotion, carcinogenicity) (Kfir, 1981; Slabbert et al., 1998).

Five local organisations participated in the study. CSIR, Pretoria collected and prepared the samples, conducted the fish exposure study as well as VTG determination by means of gel electrophoresis and ALP, and carried out the YES. The University of Stellenbosch conducted the *Xenopus* liver slice assay and the zebrafish VTG ELISA. The University of Pretoria was responsible for the reporter gene assay. SABS and

Ampath, conducted the residue and estrogen analysis, respectively. Staff at the drinking water treatment plants assisted with information on the treatment processes and sampling.

The test methodologies are described in Chapter 2. This chapter also outlines the sampling and sample preparation procedures and gives a short description of each drinking water treatment plant. The findings of each bioassay/test are presented in Chapter 3. Chapter 4 provides a general discussion comparing the results of the different tests and referring to published data. The conclusions and recommendations are outlined in Chapter 5 and the references are listed in Chapter 6.

The physical-chemical data supplied by the studied drinking water treatment plants are attached in APPENDIX A.

2. MATERIALS AND METHODS

2.1 Sampling

Water samples from three drinking water treatment plants in the Gauteng Province were studied. Sampling was carried out by CSIR staff with the assistance of participating members at treatment plants. Samples were collected on four occasions during the period January 2005 to January 2006.

2.1.1 Drinking water treatment plant information

2.1.1.1 Treatment Plant A

Treatment Plant A produces 60 Mℓ drinking water/d. Source water is abstracted via a 5 km pipeline from a river. The plant uses the following treatment processes: flocculation/dissolved air flotation, sedimentation, sand filtration (no pre-chlorination) and chlorination. Sand filters are back-washed randomly when required (by observation). The supernatant of the back-wash water is returned to the source water while the sludge is disposed of in the sewerage system.

The 1st sampling survey was preceded by a very wet period which resulted in source water dilution. Rain is acidic, causing low pHs throughout the plant with possible effects on the final water quality. Because of the heavy rain the sluice-gate of the dam upstream of the river was opened, resulting in heavy algal loads in the river water. Two flocculants were used at the time, namely polyalum (Ultrafloc 3400, a cationic aluminium chlorohydrate coagulant blend) for clarification and pulverised activated carbon (PAC) to control odour. The sand filter where the sample was taken was back-washed the day before sampling. Less rain fell before the 2nd survey, and algal loads were lower. The condition of the dam water was stable, and as a result only polyalum was used as flocculant. No sand filter back-wash occurred during the 24 h preceding sampling. The 3rd survey was carried out during a relatively dry period and no algal problems were experienced. Polyalum was used for flocculation. One of the flocculation/dissolved air flotation ponds was clogged, and divers and staff were busy trying to solve the problem. No sand filter back-wash was reported for the 24 h before sampling. The 4th survey was carried out a few days before another period of heavy rainfalls. Source water contained considerable algal loads. Polyalum, a flocculant aid (Praestol) and PAC were in use as flocculants. While sampling, municipal workers were busy to drain some of the dissolved air flotation ponds (directly onto the surrounding premises). No sand filter back-wash occurred during the 24 h before sampling.

2.1.1.2 Treatment Plant B

This plant has a treatment capacity of 40 Mℓ/d. Treatment of source water to drinking water takes only 6 h. The source water is abstracted from a dam via an inlet tower. The plant uses the following treatment processes: flocculation, dissolved air flotation and sand filtration (no pre-chlorination), activated carbon filtration and chlorination. Untreated fountain water is added to the activated carbon treated water in a storage reservoir where chlorination is carried out. Sand filter back-wash is automated and each filter is back-washed every 11th h. The back-wash water flows into settling tanks. The supernatant of the settling tanks gravitates back into the river downstream of the dam while the settled sludge is pumped into the sewerage system.

As a result of the heavy rains that fell before the 1st sampling survey some of the fountains in the area were flooded with surface water. This affected the quality of the fountain water that was added to the treated water in the storage reservoir. No problems were highlighted during the 2nd survey. Ferric chloride was used for flocculation during the 1st and 2nd surveys. During the 3rd and 4th surveys problems were experienced with heavy algal loads and resulting tastes and odours. Both ferric chloride and alum/polymer were used for flocculation.

2.1.1.3 Treatment Plant C

Treatment Plant C abstracts water from a dam that receives water via various river systems. During the period of investigation the amount of water purified by the plant was approximately 650 Mℓ/d. The plant uses the following treatment processes: coagulation/flocculation (pH 11), sedimentation, stabilisation, sand filtration (no pre-chlorination), chlorination and chloramination. Lime and activated silica are used for coagulation/flocculation. During the stabilisation step carbon dioxide (CO_2) is applied to correct the pH (7.8-8.4). Ferric chloride is also dosed to aid filtration. Sand filter back-wash is automated and it is, therefore, not possible to assess how long before sampling back-wash occur. The supernatant of the back-wash is returned to the source water while the sludge is disposed of at a sludge disposal site.

No treatment problems were reported during the four sampling surveys. During the 3rd survey heavy algal loads in surface water caused tastes and odours in various Gauteng water supplies. However, the source water to this treatment plant was not affected.

2.1.2 Sample information

The sampling dates and points are summarised in Table 2.1. 22.5 and 4.5 ℓ water was taken for *in vitro* assays and chemical analyses, respectively. 10 ℓ source and chlorinated water was taken for *in vivo* tests with fish. Samples were collected in chemically clean glass containers following standard sampling procedures (Slabbert, 2004).

TABLE 2.1: Sample information

Sampling survey	Sampling date	Treatment Plant	Sampling point	Sample designation
1	25/01/05	A	Source	A-R
2	01/03/05		Sedimentation	A-S
3	12/10/05		Sand filtration	A-SF
4	05/01/06		Chlorination	A-C
1	25/01/05	B	Source	B-R
2	01/03/05		Dissolved air flotation and sand filtration[1]	B-DAFF
3	12/10/05		Activated carbon	B-AC
4	05/01/06		Chlorination	B-C
1	26/01/05	C	Source	C-R
2	28/02/05		Flocculation[2]	C-F
3	13/10/05		Sand filtration	C-SF
4	06/01/06		Chlorination	C-C

[1] Combined sample representing nine operating sand filters
[2] After pH correction (from 11 to 7.8)

Table 2.2 presents the pH, oxygen- and free chlorine concentrations of the water samples upon arrival at the CSIR laboratory. Analytical data supplied by the drinking water treatment plants are included in APPENDIX A for information purposes.

TABLE 2.2: pH, oxygen- and free chlorine concentrations of water samples

Sample	pH				Oxygen concentration (mg/ℓ)				Free chlorine (mg/ℓ)			
	Sampling survey											
	1	2	3	4	1	2	3	4	1	2	3	4
A-R	6.9	6.9	7.4	7.7	5.9	6.2	7.2	5.9	na			
A-S	6.9	7.0	7.5	7.0	6.2	6.4	8.8	6.0				
A-SF	7.0	7.0	7.1	7.8	5.7	6.6	8.4	5.8				
A-C	7.1	6.9	6.8	8.1	5.4	6.8	8.8	6.0	0.31	0.22	0	0
B-R	7.3	6.8	7.0	8.4	5.1	6.1	7.6	6.0	na			
B-DAFF	7.4	6.9	6.6	8.5	5.9	6.7	8.5	6.4				
B-AC	7.4	7.0	6.6	8.2	5.7	6.5	8.1	5.8				
B-C	7.4	6.9	6.7	8.2	5.6	6.3	8.8	6.4	0.14	0.05	0	0
C-R	7.5	7.0	8.0	8.4	6.0	6.3	8.2	7.4	na			
C-F	7.6	7.0	8.4	8.0	5.5	6.6	7.8	8.0				
C-SF	7.5	7.1	8.3	8.1	5.2	6.8	8.8	8.6				
B-C	7.0	7.1	8.2	8.2	5.4	6.5	8.5	8.2	0.03	0.05	0.02	0.01

[1] Concentration after aeration: <0.01 mg/ℓ
na Not analysed

2.1.3 Sample preparation

2.1.3.1 *In vitro* assays

Seven of the 12 water samples collected during a survey were concentrated immediately for the *in vitro* assays, while the other samples were kept at 4ºC until the aspirator bottles in use were available. The samples (20 ℓ) were concentrated by means of XAD-7 resin packed in glass columns (Slabbert, 2004; Slabbert et al., 2007a,b). After extraction the columns were rinsed with Milli-Q® water, air dried, and eluted with reagent grade acetone. The acetone extracts were evaporated to dryness on a Buchi Rotavapour, and the contents re-dissolved in 2.5 mℓ reagent grade ethanol (8 000-times concentration). Each extract was dispensed into four sterile Vacutainers (for assaying by CSIR, University of Stellenbosch, University of Pretoria and Environment Canada) and stored in an upright position at -20ºC until required. The extracts were evaporated to dryness before being shipped to participating laboratories.

2.1.3.2 *In vivo* testing

The chlorinated samples were vigorously aerated for 18 to 24 h before being used in fish tests. The final free chlorine concentrations were <0.01 mg/ℓ.

2.1.3.3 Estrogen analysis

The pH of 1 ℓ water was adjusted to 7. 100 µℓ of a 1 mg/ℓ internal standard (Chrysene d 12) was added to the water [Technologie Zentrum Wasser (TZW), 2002] which was then concentrated by means of a solid phase column (Bond-elute 0.2 g PPL column) (rate: 3 mℓ/min). After extraction, the column was dried with nitrogen gas and eluted with

two volumes of 2 mℓ acetone. The acetone extract was evaporated to dryness using nitrogen gas. The residue was then dissolved in 70 µℓ ethyl acetate/hexane (3:2 v/v) and derivatised with 30 µℓ N-methyl-N-trimethylsilyltrifluoracetamid (MSTFA) and 2% N,O-Bis(trimethylsilyl)acetamid:trimethylchlorsilian:N-trimethylsilylimidazol 3:2:3 (Sylon BTZ). The mixture was left at room temperature for 20 min to react before analysis.

2.1.3.4 Triazine and p-nonyl phenol residue analysis

Two volumes of 1 ℓ water (no pH adjustment and unfiltered) were extracted with the organic solvent dichloromethane (DCM). DCM was added to the samples in separatory funnels. The funnels were shaken vigorously by hand, with periodic venting to release excess pressure. After separation of the liquid phases, the extracts were dried with anhydrous sodium sulphate. The extracts were then evaporated to dryness on a rotavapour. The final extracts (5 mℓ) for triazine and nonyl phenol analysis were prepared with methanol and hexane, respectively, and stored in PTFE Teflon sealed screw-cap vials at 4ºC.

2.2 Test methodologies

2.2.1 *In vitro* assays

2.2.1.1 Recombinant yeast estrogen screen (YES)

The test is based on the metabolism of yellow chlorophenol red-ß-D-galactopyranoside (CPRG) to a red product, which can be assessed spectrophotometrically. The colour reaction from yellow to red is due to the action of ß-galactosidase, which is encoded by the reporter gene *lac-z,* carried by expression plasmids in a transfected yeast cell. A stable deoxyribonucleic acid (DNA) sequence which encodes the human estrogen receptor (hER), is also inserted into the yeast cell. Binding of a suitable substrate to these receptors, thus induces expression of the *lac-z* gene, which leads to the activation of ß-galactosidase through interaction with nuclear transcription factors (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998).

The assay was carried out under sterile conditions in duplicate flat-bottomed 96-well microplates according to the method described by Slabbert et al. (2007a). Each microplate contained a 17ß-estradiol positive control (stock solution in ethanol: 5.448×10^4 ng/ℓ), a negative control (100% ethanol) and the ethanol sample extracts. Samples, as well as the positive control, were serially diluted in 100% ethanol. 10 µℓ of the test sample and controls were placed in the microplate wells. An overnight culture was diluted with fresh growth medium to an absorbance of approximately 1.0 (620 nm). 0.4 mℓ of this preparation and 0.5 mℓ CPRG, were added to 50 mℓ growth medium (assay medium). After evaporation of the ethanol from microplate wells, 200 µℓ of the assay medium were introduced into each well. Plates were sealed with Parafilm, shaken on a microplate shaker for 2 min, and incubated at 32±1°C for 3 d, followed by a further incubation period of 7 d at room temperature (20±2ºC). Plates were shaken for 5 min and allowed to stand for 1 h before absorbance measurements. Absorbance was measured with a microplate reader at 550 and 620 nm, to account for the CPRG colour change and turbidity (yeast growth), respectively.

Absorbance values were imported into a calculation template in Microsoft Excel. To correct for turbidity (each well), the following equation was applied to the data:

$$\text{Corrected absorbance} = Abs_{550\ nm} - [Abs_{620\ nm} - Abs_{negative\ control,\ 620\ nm}]$$

The final corrected absorbance represents the data from two plates.

Non-linear regression analysis (sigmoidal dose-response) was performed on the positive control data to calculate the concentration giving 50% of the maximum response (EC_{50}). Extract absorbance values, as well as the 17ß-estradiol limit of detection [LOD: mean negative control+3SDs (standard deviations)] and limit of quantification (LOQ: mean negative control+10SDs) absorbance values, were interpolated with the positive control curve. Only those extract absorbance values between the 17β-estradiol LOQ and EC_{50} were used for the calculation of estrogenic potency (most linear part of curve). The original sample concentration (8 000-times) was taken into account in the calculation of estrogenic potency and the whole method LOD and LOQ. Three test absorbance values above the 17ß-estradiol LOD indicated significant estrogenicity. The estrogenic potency of water samples and whole method LODs and LOQs are expressed as ng EEQs (estradiol equivalents)/ℓ.

2.2.1.2 Estrogen receptor-mediated chemical activated luciferase gene expression (ER-CALUX) assay

The ER-CALUX assay (Legler et al., 1999) uses T47-D human breast adenocarcinoma cells expressing endogenous ER α and β, which are stably transfected with an estrogen responsive luciferase reporter gene. Exposure to xenoestrogens results in binding to endogenous ER, activation of the receptor, and consequently, binding of the ligand-receptor complex to the ER elements (EREs) present in the promoter region of the stably integrated luciferase gene. This leads to the induction of the luciferase gene, which is easily assayed by lysing cells, adding the substrate luciferin and measuring light output (Legler et al., 2002). The amount of luciferase produced is proportional to the extent of receptor binding and it is quantitated by the reaction with luciferin to produce a light signal.

The assay was performed at 37ºC in 96-well microplates (Nunc). $1x10^4$ cells were seeded per well in assay medium [Dulbecco's Modified Eagles Medium (DMEM)/F12 with stripped serum]. The plates were incubated for 24 h after which the assay medium was replaced with fresh assay medium. After a further incubation period of 24 h, the medium was replaced with fresh medium containing various dilutions of the sample extracts [dry extracts (original volume: 1 mℓ) re-dissolved in 50 µℓ dimethylsulphoxide (DMSO)]. Each dilution was tested in triplicate. After 24 h exposure the cells were lysed and the light output measured with a luminometer (CALUX®). A standard reference curve was constructed on each microplate by exposing cells to serial dilutions of 17ß-estradiol (0.0133 to 27.24 ng/ℓ).

Results of the extracts were interpolated in the standard reference curve. 17ß-estradiol LODs (blank+3SDs), LOQs (blank+10SDs) and EC_{50}s were calculated. Only test data between the 17ß-estradiol LOQ and EC_{50} were quantifiable. The original sample concentration (8 000-times) (paragraph 2.1.3.1) and the volume of solvent used to re-dissolve the dry extract were taken into account in the calculation of estrogenic potency and whole method LOD and LOQ (ng EEQ/ℓ).

The androgen receptor-mediated chemical activated luciferase gene expression (AR-CALUX) assay was applied to samples showing cytotoxicity in the ER-CALUX assay to confirm cytotoxicity. This assay comprises a genetically modified osteosarcoma cell line

(U2OS) in which the firefly luciferase gene coupled to androgen responsive elements as a reporter gene were incorporated to detect androgenic compounds. Two concentrations of the positive control, dihydrotestosterone, were used, namely the EC_{50} (2.9x10 ng/ℓ or $1x10^{-10}$ M) and 100-times EC_{50} ($2.9x10^{3}$ ng/ℓ or $1x10^{-8}$ M).

2.2.1.3 *Xenopus laevis* liver slice assay

In this assay, small pieces of *X. laevis* (African Clawed frog) liver are exposed to culture medium containing test samples in 96-well microplates (Hurter, 2003, Hurter et al., 2002). The vitellogenin (VTG) produced during a 6 d exposure period is measured by means of an in-house Universal Vertebrate VTG (UniVTG) enzyme linked immunosorbant assay (ELISA).

2.2.1.3.1 Animal maintenance

X. laevis breeding stock was obtained from a commercial dealer (Klapmuts, Western Cape). Frogs were maintained in a climate-controlled facility (room temperature: 22±2°C; 14:10 day-night cycle) in glass aquaria (Hurter, 2003). Frogs were fed every week *ad libitum* with commercial Tilapia pellets [AquaNutro (Pty) Ltd, South Africa].

2.2.1.3.2 Liver tissue preparation

A male *X. laevis* frog, unexposed to estrogen prior to the experiment, was decapitated, pithed, and rinsed in 70% ethanol for decontamination of the skin. Liver tissue was removed and transferred into liver culture medium (Hurter, 2003; Hurter et al., 2002). The culture medium consisted of 70% (v/v) RPMI 1640 medium (with L-glutamine, Cambrex, USA) in sterile deionised water (Millipore, USA). A mixture of antibiotics consisting of streptomycin, fungizone, penicillin and gentamycin (Highveld Biological) was added to the medium in accordance with the manufacturer's instructions. Subsequent procedures were carried out in a laminar flow cabinet using aseptic techniques.

2.2.1.3.3 Exposure of liver slices

Cubes of approximately 1 mm^3 were cut from the frog liver. Cubes were transferred to 96-well microplates (NalgeNunc, Denmark) (Hurter, 2003; Hurter et al., 2002). Dry extracts of the water samples were reconstituted with ethanol and added to liver culture medium in order to obtain the original water concentration. The ethanol constituted <0.5% of the final volume. Each exposure was carried out in quadruplicate. After 72 h incubation at 27ºC, the medium in the wells was replaced with fresh medium containing the reconstituted water samples. Following a second 72 h incubation period, the culture media in the wells were collected and VTG determined.

2.2.1.3.4 Xenopus VTG ELISA

VTG transcribed during the 6 d exposure period described above, was quantified using the UniVTG anti-VTG antibodies in the ELISA described by Hurter (2003) and Hurter et al. (2002). In summary, culture medium samples were assayed on a 96-well microplate (Maxisorp, Nunc, Denmark). A 0.9% (w/v) saline (NaCl) solution was used as the ELISA negative (-ve) control. The ELISA positive (+ve) control constituted species specific estrogen induced proteins (including VTG) at a concentration of 2.24 in 0.9% NaCl. Microplate wells were coated with 10% (v/v) sample (medium) in 0.9% phosphate

buffered saline (PBS) and incubated for 2 h. The coating solution was then discarded and the plate rinsed with 0.9% NaCl. Any unbound sites in the wells were then blocked with 5% human serum albumin in 0.9% NaCl for 30 min at room temperature, shaking at 150 rpm. After washing the plate 3-times in 0.9% NaCl, bound peptides were exposed to primary antibody (UniVTG) diluted in 5% human serum albumin solution, incubated at room temperature for 1 h, shaking at 150 rpm. The plate was washed 3-times with 0.9% NaCl followed by exposure to secondary antibody [anti-mouse immunoglobulin (Ig) horseradish peroxidase linked whole antibody from sheep (Amersham Pharmacia Biotech, UK)] diluted 1/5 000 in 0.5% human serum albumin. The plate was incubated for 30 min at room temperature and then washed 3-times with 0.9% NaCl. Soluble tetramethylbenzidine (Roche Diagnostics GmbH, Mannheim, Germany) was used as substrate for peroxidase, and was incubated at room temperature in the dark for 10 min. The chromogenic reaction was stopped by the addition of 167 mM H_2SO_4 per well. The optical density (OD) for each well was measured at 450 nm on a plate reader. The mean OD values were used as indirect evidence of VTG production when compared with OD values of liver slices exposed to control water samples.

Results were subjected to normality and equal variance tests. Failing any of these tests, data were analysed by means of Kruskal-Wallis Analysis of Variance (ANOVA) on ranks followed by Multiple Comparison Procedures (Dunn's Method) for testing the significance ($P<0.05$) between exposure groups and the negative water control. Significance was set to $P<0.05$. Passing both tests, data were analysed using parametric One Way ANOVA followed by Multiple Comparison Procedures (Dunnet's Method) for testing the significance ($P<0.05$) between exposure groups and the negative water control.

2.2.1.4 Primary rainbow trout hepatocyte (PRTH) assay

Estrogenic compounds present in test samples will bind intracellularly with the estrogen receptor in PRTH cells and trigger VTG production. VTG will accumulate in the extracellular culture medium and can be measured indirectly via its alkali-labile phosphate (ALP) groups with a microspectrophotometer.

Hepatocytes were collected from 10 to 15 cm long rainbow trout (*Oncorhynchus mykiss*), using a double perfusion method. The livers from three fish were pooled for each study. Cells were distributed in 48-well microplates at a density of $1x10^6$/mℓ in sterile L15 medium (at 320 mOsmol/kg H_2O) supplemented with 2% fetal bovine serum, 1 000 units of penicillin, 10 mg/ℓ streptomycin and 25 µg/ℓ amphotericin B (Gagné et al., 1999). The dry water extracts were re-suspended in 100 µℓ DMSO. Cells were exposed to 0.04, 0.2 and 1% (v/v) of the extracts for 48 h at 15ºC in a humidified incubator. DMSO was used as negative control. For quality control, 17β-estradiol ($1.362x10^6$ ng/ℓ) was used to confirm expected VTG induction by PRTH while potassium chloride (KCl - 8.2 g/ℓ) was used as the cytotoxicity reference toxicant. VTG was established by means of an ALP method (Gagné and Blaise, 2000) and expressed as µg ALP/mℓ PRTH biomass estimated by spectrophotometric reading at 600 nm (see paragraph 2.2.2.4.2).

After medium was removed for ALP analysis, cytoxicity was determined by adding medium containing fluorescein diacetate (FDA) to the microplate wells. FDA interacts with hepatocytes and intracellular esterases then cleave the diacetate groups of the FDA to liberate fluorescein. The latter, a fluorescent and polar molecule, is retained in the cytoplasm of normal cells, but in intoxicated cells the membrane permeability will be impaired and fluorescein will leak out. Hence, after microfluorometry readings,

cytotoxicity will be evidenced by hepatocyte cells that are less fluorescent than normal cells. Cytotoxicity was expressed as % mortality by comparing the intoxicated and normal cell numbers.

All VTG values were corrected for viability [e.g. if a VTG value of 648 (µg ALP/mℓ) and a 10% mortality were obtained, the VTG value is multiplied by 110% which then becomes the theoretical value (712.8) for VTG if 100% of the hepatocytes had been viable]. After correction, estrogenic potential was ascertained by comparing VTG values measured at each exposure concentration with that of the negative control.

2.2.2 *In vivo* methodologies

VTG is synthesised and secreted by the liver of oviparous vertebrates in response to circulating estrogens in mature females. Under normal circumstances no VTG is present in the blood of males. They do, however, have a functional VTG gene that can be expressed in the presence of estrogen. The presence of VTG in the blood and liver of males thus indicates the exposure to exogenous estrogens or estrogen mimicking substances. Zebrafish (*Danio rerio*) is widely used as experimental animal for biological and biotechnological research. This fish has also proved to be very useful to examine VTG expression (Holbech et al., 2001). We have exposed 16 to 24 d old juvenile zebrafish to water samples for 14 d, where after VTG in whole body homogenates was measured directly with an ELISA and gel electrophoresis and indirectly with an ALP procedure.

2.2.2.1 Fish maintenance and breeding

Zebrafish were obtained from the Amatikulu Hatcheries in KwaZulu-Natal. Breeding stock was kept in aerated tap water in 100 ℓ glass tanks (static system) at a water temperature of 28±2ºC according to standard procedures described by Slabbert (2004). The photoperiod in the culture room was regulated with an electronic timer (14:10 h day:night cycle). Each holding tank was equipped with an activated carbon filter to prevent the accumulation of toxic metabolic waste. Fish were fed twice a day with flakes and bloodworms. Fish intended for breeding were fed with excess amounts of bloodworms and crustaceans for a period of 14 d before breeding.

To obtain test fish, 30 to 50 adults were transferred to a breeding basked placed inside a breeding tank in the late afternoon (ISO, 1999; Venter et al*.,* 2005). Breeding tanks (100 ℓ) contained aerated tap water and were equipped with a thermostat (28±2ºC). Spawning is induced by the morning light and is completed after the lights have been switched on for approximately 2 h. The eggs, which are non-adhesive, fall through the mesh in the breeding basket, out of reach of adults. After spawning, the adult fish were returned to their holding tank. After 3 d the eggs started to hatch and the larvae moved to the walls of the tank and later to the water surface. The larvae were fed once a day with oven dried (80°C) boiled egg yolk to optimise the health of the test stock.

During the 14 d after hatching the temperature of the breeding tank was gradually turned down to 23±2ºC (Slabbert, 2004). The water was also reduced to approximately 50 ℓ and gradually replaced with aerated synthetic moderately hard water (Table 2.3).

2.2.2.2 Fish exposure

16 to 24 d old juvenile fish were exposed in ambient light in a temperature controlled

room at a water temperature of 23±2ºC (Slabbert et al*.,* 2004; Venter et al*.,* 2005). The offspring of several females, produced during a 4 d period, were used. Fish were randomly transferred with a fish net from a holding tank into the test containers. Between 80 and 100 fish (to obtain sufficient homogenate for assays) were placed in 250 mℓ sample/control water in a 500 mℓ glass dish (loading: <4 g/ℓ). Moderately hard water (Table 2.3) was used for control testing (negative control). 17ß-estradiol was used as positive control (109 ng/ℓ during the 1st and 2nd sampling surveys, and 1 090 ng/ℓ during the 3rd and 4th sampling surveys). Each test/control was carried out in triplicate. A 14 d exposure period was used (sexual differentiation starts at approximately 38 d). Water was changed twice per week. Fish were fed once a day (oven dried boiled egg yolk) (Venter et al., 2005).

TABLE 2.3: Moderately hard water[1]

KCl	$MgSO_4$	$NaHCO_3$	$CaSO_4.2H_20$	pH[2]	Dissolved oxygen	Hardness	Alkalinity
mg/ℓ					mg/ℓ	As mg/ℓ $CaCO_3$	
4	60	96	60	7.4-7.8	≥6.8	80-100	60-70

1 Prepared with Milli-Q® water
2 Approximate equilibrium pH after aeration

The water temperature was measured at regular intervals. Fish lethality was recorded on a daily basis (Slabbert, 2004). Dead fish were removed to limit bacterial contamination.

2.2.2.3 Preparation of exposed fish for VTG analysis

2.2.2.3.1 ELISA

At the end of the exposure period 10 fish were removed from each test and control container using a Pasteur pipette. The fish were rinsed with buffer [50 mM hydroxymethyl-aminomethane (Tris) base (pH: 7.4), 250 mM sucrose and 0.01% phenyl methyl sulphonyl fluoride (PMSF)] in a Petri dish, dried on filter paper and placed in individual Eppendorf tubes kept on ice. The tubes were stored at -80ºC until dispatch (on dry ice) to the University of Stellenbosch. At the University of Stellenbosch, the fish were initially homogenised individually (1st and 2nd surveys), but subsequently homogenised in pairs (3rd and 4th surveys). Homogenisation was carried out on ice with 500 to 1 000 µℓ buffer (0.01% PMSF in 0.9% NaCl, w/v) and stored at -80ºC if not processed immediately.

2.2.2.3.2 ALP assay and gel electrophoresis

The remaining fish from each test container were pooled by pouring the water through a clean fish net. The fish were rinsed with buffer [50 mM Tris base (pH: 7.4), 250 mM sucrose and 0.01% PMSF], dried on filter paper, and transferred to pre-weighed Eppendorf tubes. After weighing, the fish were homogenised on ice in cold buffer (same as used for rinsing) to a fine homogenous suspension, using 100 mg tissue/mℓ buffer (Venter et al., 2005). The homogenate was centrifuged for 10 min at 4°C in an Eppendorf centrifuge at 11 500 rpm (8 863 g). The supernatant was dispensed in appropriate volumes in separate containers (to avoid thawing and freezing) and stored at -80°C for the ALP assay and gel electrophoresis.

2.2.2.4 VTG analysis

2.2.2.4.1 ELISA

VTG concentrations were determined according to the method outlined by Holbech et al. (2001), using an in-house produced zebrafish monoclonal VTG antibody (Pool and van Wyk, 2007). The wells in a 96-well microplate (Nunc, Roskilde, Denmark) was coated overnight at room temperature with whole body homogenate (~1 µg protein). The coating solution was discarded and the plate rinsed with 0.9% NaCl (w/v). Thereafter any unbound sites in the wells were blocked with 5% human serum albumin in 0.9 % NaCl for 30 min at room temperature, shaking at 150 rpm. After washing the plate 3-times in 0.9% NaCl, wells were exposed to primary antibody (clone B_1G_{12}) diluted in 5% human serum albumin solution at room temperature for 1 h, shaking at 150 rpm. The plate was once again washed 3-times with 0.9% NaCl, followed by exposure to secondary antibody diluted 1/5000 in 0.5% human serum albumin solution. Anti-mouse Ig horseradish peroxidase linked whole-antibody from sheep (Amersham Pharmacia Biotech, UK) was used as secondary antibody. The plate was incubated for 30 min at room temperature and then washed 3-times with 0.9% NaCl. Soluble tetramethylbenzidine (Roche Diagnostics GmbH, Mannheim, Germany) was used as substrate for peroxidase, and was incubated at room temperature in the dark for 10 min. The chromogenic reaction was stopped by the addition 167 mM H_2SO_4 per well. The OD for each well was measured at 450 nm. Dilutions of an in-house VTG standard, normalised against a commercial standard (BioSense Laboratories AS, Norway) was used to construct a VTG standard curve, spanning a range between 4.5 µg/mℓ and 0.15 µg/mℓ VTG. The standard curve generated was used to determine whole body homogenate VTG concentrations from OD values. Relatively little variation in the slopes of the standard curves suggested good inter-assay repeatability (average slope = 9.555±1.564 SD).

Kruskal-Wallis ANOVA on ranks followed by ALL Pairwise Multiple Comparison Procedures (Dunn's Method) was used for testing the significance ($P<0.05$) between exposure groups and the negative water control. Significance was set to $P<0.05$.

2.2.2.4.2 ALP assay

The phosphates in VTG are labile in an alkaline environment. The level of VTG in tissue can thus be determined indirectly in terms of phosphate by first liberating the phosphate under alkaline conditions and then measuring the phosphate with the aid of a colorimetric method. The colour intensity, read at 750 nm, is indicative of the phosphate groups present in VTG, while the reading taken at 450 nm corrects for sample turbidity which may interfere with the blue colour reading taken at 750 nm (Personal Communication, 2003).

To liberate the phosphate, the supernatant (200 µℓ) of the whole body homogenates was precipitated with cold 100% acetone (108 µℓ) for 5 to 10 min (Slabbert et al., 2007a). The precipitate was centrifuged at 10 000 g (4°C) for 20 min. The pellet was then re-suspended in 400 µℓ 1 M sodium hydroxide (NaOH), followed by incubation at 60°C for 30 min with intermittent mixing. The NaOH extract was left to cool down to room temperature before determining the phosphate content.

Phosphate measurements were carried out in 96-well microplates as described by Slabbert et al. (2007a). Each determination was carried out in triplicate. After all the

solutions have been added, the microplate was shaken on a microplate reader for 5 min. Absorbance was recorded at 750 and 450 nm. A standard phosphate curve was constructed to determine the phosphate concentration (mg/mℓ) in fish samples. Dilution factors were applied to the extrapolated phosphate values to correct for the dilution during sample preparation. The results are expressed in terms of total protein (mg ALP/mg protein) (Slabbert et al*.,* 2007a). Total protein was determined by means of a miniaturised Bradford (1976) method.

Data sets were compared by means of Student's t-test (Clarke, 1969) to establish whether or not results differed significantly. P=0.05 (two-tailed test) was used as the level of significance.

2.2.2.4.3 Gel electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for the determination of relative VTG concentrations in the supernatants of whole body homogenates. The method was carried out as described by Slabbert et al*.* (2007a). 50 µg of the sample in terms of protein and 20 µℓ of the molecular weight marker were applied to the gel. After separation of the proteins on the gel, the gel was stained with a Brilliant Blue G-colloidal stain, followed by destaining in an acetic acid/methanol solution. All the stain was removed, except the stain bound to the protein bands. The position of the protein bands were compared with the position of protein molecular weight markers.

2.2.3 Chemical methods

2.2.3.1 Estrogen analysis

Estrogen levels were determined according to the method described by the TZW (2002) using gaschromatography/mass spectrometry (GC/MS). The following instrumentation was used: GC Finnigan ion trap sensor (ITS 40); Split/splitless injector, 250ºC, 2 µL splitless injected for 1.5 min; Column DB 5-MS (30 m x 0.33 mm x 0.25 µm); Helium (ultra pure) carrier gas; and Finnigan ion trap detector (ITD). The temperature programme was set at 50ºC for 2 min, followed by 16ºC/min to 180ºC, then at 5ºC/min to 290ºC, held for 10 min. The ITD and transferline temperature was set at 220ºC. The MS was used at full scan. Estrogens were quantified as a ratio to the internal standard. The following ions were scanned:

	m/z
Estrone	155, 399, 400, 414
17α- and β- estradiol	285, 326, 416, 417
Estriol	311, 386, 414, 504
17α-ethinylestradiol	285, 300, 425, 428

2.2.3.2 Triazine and p-nonyl phenol residue analysis

GC-MS was used to quantify and confirm the chemical compounds, following standard operating procedures. Single determinations were carried out by means of USEPA methodology (1984). Recovery was established by adding known amounts of triazine pesticides and p-nonyl phenol to tap water and analysing these concurrently with the samples.

3. RESULTS AND DISCUSSION

3.1 *In vitro* and *in vivo* assays

3.1.1 Recombinant yeast estrogen screen (YES)

Figure 3.1 shows the typical curves obtained for the positive control 17ß-estradiol after 3, 7 and 10 d incubation using the wavelength combination of 550 and 620 nm. The curves moved up (absorbance values higher) and to the left (lower concentrations) with increased incubation time, rendering the lowest concentrations giving 50% of the maximum response (EC_{50}s) after 10 d (highest sensitivity). The limit of detection (LOD), limit of quantification (LOQ) and EC_{50} values for 17β-estradiol obtained during the study (10 d incubation; 550 and 620 nm) are shown in Table 3.1. LODs ranged from 1.64 to 5.03 ng/ℓ; LOQs from 3.35 to 9.30 ng/ℓ; and LC_{50}s from 10.95 to 20.61 ng/ℓ (Table 3.1).

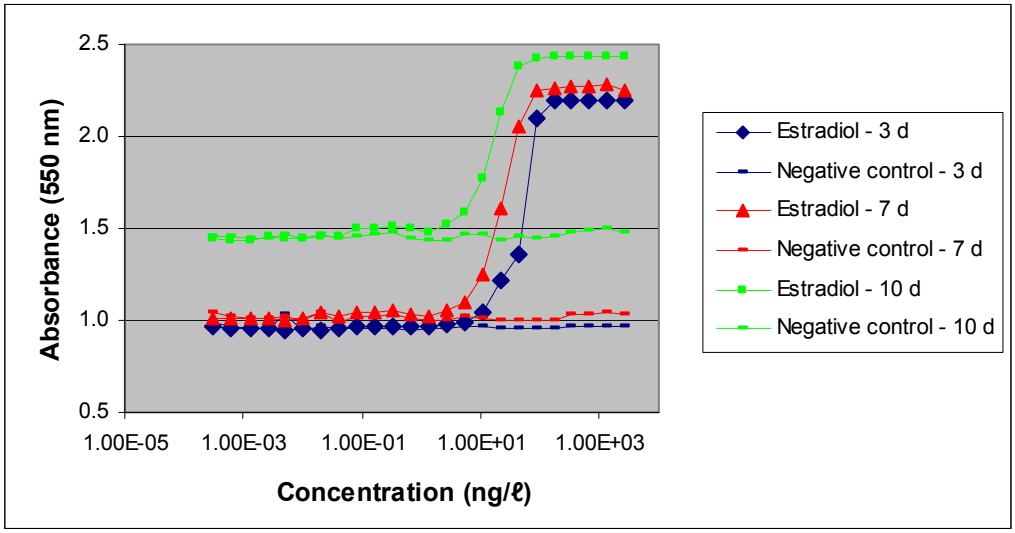

FIGURE 3.1: Typical 17ß-estradiol curves obtained with the YES after 3, 7 and 10 d incubation (550 and 620 nm)

TABLE 3.1: 17ß- estradiol LODs, LOQs and EC_{50}s obtained with the YES

Sampling survey	Sample	LOD (ng/ℓ)	LOQ (ng/ℓ)	EC_{50} (ng/ℓ)
1	A-R, A-S, A-SF, A-C	3.42	6.85	14.61 (13.50-15.82)
	B-R, B-DAFF, B-AC, B-C	3.85	8.13	17.06 (15.27-19.08)
	C-R, C-F, C-SF, C-C	3.49	7.61	16.42 (14.40-18.71)
2	A-R, A-S, A-SF, A-C	3.64	7.14	13.66 (12.85-14.51)
	B-R, B-DAFF, B-AC, B-C	4.34	7.49	14.46 (13.68-15.29)
	C-R, C-F, C-SF, C-C	3.60	6.80	13.44 (12.28-14.70)
3	A-R, A-S, A-SF, A-C	1.64	3.35	10.65 (9.86-11.50)
	B-R, B-DAFF, B-AC, B-C	3.74	6.68	11.77 (11.04-12.55)
	C-R, C-F, C-SF, C-C	1.65	3.50	10.95 (10.09-11.87)
4	A-R, A-S, A-SF, A-C	4.88	9.00	15.78 (14.16-17.58)
	B-R, B-DAFF, B-AC, B-C	3.37	7.97	17.07 (15.29-19.06)
	C-R, C-F, C-SF, C-C	5.03	9.30	20.61 (19.25-22.07)

The estrogenic activities of the tested water samples are shown in Table 3.2. All the samples collected during the 1st and 3rd surveys showed significant estrogenic activity with four to 10 absorbance values >17ß-estradiol LOD. The curves obtained during the 2nd survey were less defined than those obtained during the 1st and 3rd surveys, with between zero and six absorbance values >LOD. Three of the final water samples (A-C, B-C and C-C) did not exhibit significant estrogenic activity (<3 absorbance values >LOD). The curves obtained during the 4th survey were also less defined than those obtained during the 1st and 3rd surveys, with between two and eight absorbance values >LOD. During the 4th survey sample A-C did not exhibit significant estrogenicity (<3 absorbance values >LOD).

The whole method LOD and LOQ, calculated in terms of maximum concentration factors, were 0.009, and between 0.02 and 0.04 ng estradiol equivalents (EEQ)/ℓ, respectively. The estrogenic potency obtained for the source water of Treatment Plant A (A-R) ranged from 0.037 (2nd survey) to 0.761 (1st survey) ng EEQ/ℓ, that obtained for Treatment Plant B (B-R) ranged from 0.115 (2nd survey) to 3.189 (3rd survey) ng EEQ/ℓ, and that obtained for Treatment Plant C (C-R) ranged from 0.036 (2nd survey) to 0.226 (3rd survey) ng EEQ/ℓ (Table 3.2). The final water of Treatment Plant A exhibited an estrogenic potency of between 0.009 (2nd survey) and 0.138 (3rd survey) ng EEQ/ℓ, that of Treatment Plant B an estrogenic potency of between 0.019 (2nd survey) and 0.119 (1st survey) ng EEQ/ℓ and that of Treatment Plant C an estrogenic potency of between 0.017 (2nd survey) and 0.568 (1st survey) ng EEQ/ℓ. Compared to the source waters, all the final waters, except sample C-C collected during the 1st survey, showed reduced estrogenicity. The reduction in estrogenic activity at Treatment Plant A ranged from 66% (3rd survey) to 93% (1st survey), the reduction at Treatment Plant B ranged from >84% (2nd survey) to 99% (3rd survey), and the reduction at Treatment Plant C ranged from >44% (4th survey) to 77% (3rd survey). Sample C-C collected during the 1st survey showed an increase of 462% in estrogenicity. Compared to the source water, most of the samples collected after the initial water treatment processes [sedimentation (A-S), dissolved air flotation and sand filtration (B-DAFF) and flocculation (C-F)] showed an increase in estrogenicity. The estrogenicity of the samples taken before final chlorination [sand filtration (A-SF and C-SF) and activated carbon treatment (B-AC)] was lower than that of the source waters, except for the A-SF samples collected during the 2nd, 3rd and 4th surveys.

3.1.2 Estrogen receptor-mediated chemical activated luciferase gene expression (ER-CALUX) assay

No 17ß-estradiol LODs, LOQs and EC_{50}s were reported by Bio Detection Systems. This is, however, a highly sensitive assay with 17ß-estradiol LODs as low as 0.1 ng/ℓ and EC_{50}s as low as 1.6 ng/ℓ (Murk et al., 2002).

The calculated whole method LOD and LOQ were 0.00085 (0.85×10^{-3}) and 0.0025 (2.55×10^{-3}) ng EEQ/ℓ, respectively. Estrogenic activities exhibited by the water samples are presented in Table 3.3. With the exception of sample B-C, all the estrogenic potencies obtained for samples collected during the 1st survey were quantifiable (>LOQ). A large number of the estrogenic potencies obtained during the other surveys were <LOD or <LOQ. The estrogenic potency of the source water of Treatment Plant A ranged from <LOD (2nd and 4th surveys) to 0.114 (1st survey) ng EEQ/ℓ, that of Treatment Plant B ranged from 0.024 (2nd survey) to 0.758 (3rd survey) ng EEQ/ℓ, and that of Treatment Plant C ranged from <LOD (3rd and 4th surveys) to 0.039 (1st survey) ng EEQ/ℓ. Compared to the source waters, seven of the 12 final waters showed a

TABLE 3.2: Estrogenic activity of water sampled at drinking water treatment plants using the YES

Sampling survey	Sample	Significant estrogenic activity[1] (Y/N)	Estrogenic potency (ng EEQ/ℓ)	Reduction in estrogenicity (compared to R)
1	A-R	Y (9)	0.761	-
	A-S	Y (7)	0.888	+17
	A-SF	Y (7)	0.416	45
	A-C	Y (5)	**0.057**	**93**
	B-R	Y (8)	1.032	-
	B-DAFF	Y (8)	1.024	1
	B-AC	Y (6)	0.322	69
	B-C	Y (5)	**0.119**	**89**
	C-R	Y (6)	0.101	-
	C-F	Y (6)	0.250	+148
	C-SF	Y (6)	**0.098**	**3**
	C-C	Y (5)	0.568	+462
2	A-R	Y (4)	0.037	-
	A-S	Y (5)	0.119	+222
	A-SF	Y (6)	0.076	+105
	A-C	N (0)	**<LOD (0.009)**	**>76**
	B-R	Y (5)	0.115	-
	B-DAFF	Y (6)	0.183	+59
	B-AC	Y (5)	0.093	19
	B-C	N (2)	**<LOQ (0.019)**	**>84**
	C-R	Y (3)	0.036	-
	C-F	Y (3)	0.021	42
	C-SF	Y (4)	0.024	33
	C-C	N (1)	**<LOQ (0.017)**	**>53**
3	A-R	Y (9)	0.405	-
	A-S	Y (10)	0.481	+19
	A-SF	Y (9)	0.442	+9
	A-C	Y (7)	**0.138**	**66**
	B-R	Y (10)	3.189	-
	B-DAFF	Y (10)	4.745	+49
	B-AC	Y (7)	0.413	87
	B-C	Y (4)	**0.040**	**99**
	C-R	Y (8)	0.226	-
	C-F	Y (7)	0.290	+28
	C-SF	Y (8)	0.223	1
	C-C	Y (7)	**0.053**	**77**
4	A-R	Y (6)	0.226	-
	A-S	Y (6)	0.271	+20
	A-SF	Y 6)	0.293	+30
	A-C	N (2)	**<LOQ (0.023)**	**>90**
	B-R	Y (8)	1.126	-
	B-DAFF	Y (7)	1.936	+72
	B-AC	Y (5)	**<LOQ (0.040)**	**>97**
	B-C	Y (3)	0.042	96
	C-R	Y (4)	0.041	-
	C-F	Y (3)	0.069	+68
	C-SF	Y (3)	0.024	42
	C-C	Y (3)	**<LOQ (0.023)**	**>44**

[1] Numbers in brackets after Y/N: Absorbance values >17ß-estradiol limit of detection (LOD)

\+ Increased estrogenicity compared to source water, R

Whole method LOD: 0.009 ng EEQ/ℓ

Whole method limit of quantification (LOQ): 0.02-0.04 ng EEQ/ℓ Values <LOD and <LOQ given as estimates (in brackets) Results in **bold**: Lowest estrogenic potential and highest removal of estrogenicity

TABLE 3.3: Estrogenic activity of water sampled at drinking water treatment plants using the ER-CALUX assay

Sampling survey	Sample	Estrogenic potency (ng EEQ/ℓ)	Reduction in estrogenicity (compared to R)
1	A-R	0.114[1]	-
	A-S	0.320[1]	+181
	A-SF	0.077	33
	A-C	**0.024**	**79**
	B-R	0.081	-
	B-DAFF	0.304	+275
	B-AC	0.035	57
	B-C	**<LOD**	**99**
	C-R	0.039	-
	C-F	0.090	+131
	C-SF	**0.012**	**69**
	C-C	0.176	+351
2	A-R	<LOD	-
	A-S	<LOD	0
	A-SF	<LOD	0
	A-C	<LOD	0
	B-R	0.024	-
	B-DAFF	**<LOD**	**97**
	B-AC	<LOD	97
	B-C	<LOD	97
	C-R	0.007	-
	C-F	<LOQ (0.002)	71
	C-SF	**<LOQ (0.001)**	**86**
	C-C	<LOQ (0.002)	71
3	A-R	0.081	-
	A-S	0.076	6
	A-SF	**<LOD**	**99**
	A-C	0.028	65
	B-R	0.758	-
	B-DAFF	2.620	+246
	B-AC	**<LOD**	**100**
	B-C	<LOD	100
	C-R	<LOD	-
	C-F	<LOQ (0.001)	+18
	C-SF	<LOD	0
	C-C	<LOD	0
4	A-R	<LOD	-
	A-S	0.027	+3 077
	A-SF	0.023	+2 606
	A-C	<LOD	0
	B-R	0.332	-
	B-DAFF	0.444	+34
	B-AC	**<LOD**	**100**
	B-C	<LOD	100
	C-R	<LOD	-
	C-F	<LOQ (0.002)	+135
	C-SF	<LOD	0
	C-C	<LOD	0

[1] Toxic

\+ Increased estrogenicity compared to source water, R

Whole method limit of detection (LOD): 0.00085 (0.85×10^{-3}) ng EEQ/ℓ

Whole method limit of quantification (LOQ): 0.00255 (2.55×10^{-3}) ng EEQ/ℓ Values <LOQ given as estimates (in brackets) Results in **bold**: Lowest estrogenic potential and highest removal of estrogenicity

reduction in estrogenicity. No reduction (0) in estrogenicity was observed for four of the final water samples as a result of the low estrogenic potential (<LOD) of the source water. The final water of Treatment Plant C collected during the 1st survey showed an increase of 351% in estrogenicity. A number of the A-S, B-DAFF and C-F samples showed increased estrogenicity, while the A-SF, B-AC and C-SF samples showed a reduction in estrogenicity.

Samples A-R and A-S collected during the 1st survey exhibited cytotoxicity, as detected with the AR-CALUX assay.

3.1.3 *Xenopus laevis* liver slice assay

Samples collected during the 1st survey induced varied vitellogenin (VTG) production (Figure 3.2) [Kruskal-Wallis analysis of variance (ANOVA), H=25.02, 13df, P=0.023]. Only sample C-C showed significant increased VTG production compared to the negative control. Except for sample A-R, all other samples induced VTG production higher than the negative control. The positive control, 17ß-estradiol, did not exhibit significant VTG production.

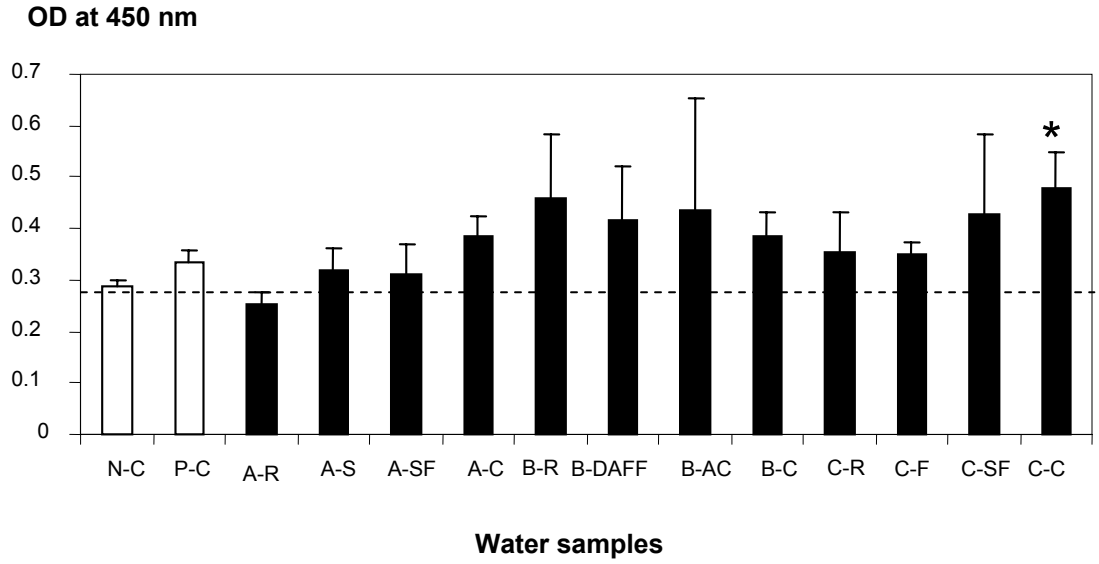

FIGURE 3.2: Average VTG production by *X. laevis* liver slices (quadruple slices for each sample) incubated in extracts of water samples (1st survey) for 6 d. N-C=negative control; P-C=positive control [2.724×10^4 ng/ℓ (10^{-7} M) 17β-estradiol]. The asterisk (*) indicates significant difference (Kruskal-Wallis ANOVA, P<0.05) between the optical densities (ODs) of the water sample and the negative control (VTG production). The horizontal line indicates the mean value for VTG production in the control liver slices

Similar to the exposures carried out during the 1st survey, several samples collected during the 2nd survey showed some degree of VTG induction (Figure 3.3) (Kruskal-Wallis ANOVA, H=29.30, 13df, P=0.006). Only sample C-F induced significant VTG production when compared to the VTG production of the negative control. 17ß-estradiol did not cause significant VTG production.

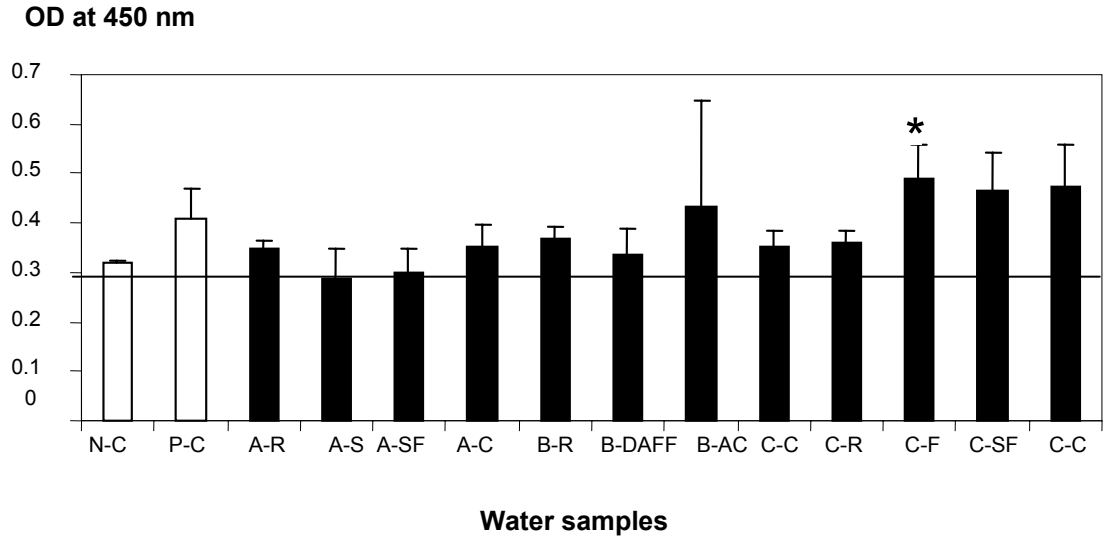

FIGURE 3.3: Average VTG production by *X. laevis* liver slices (quadruple slices for each sample) incubated in extracts of water samples (2nd survey) for 6 d. N-C=negative control; P-C=positive control [2.724×10^4 ng/ℓ (10^{-7} M) 17β-estradiol]. The asterisk (*) indicates significant difference (Kruskal-Wallis ANOVA, $P<0.05$) between the ODs of the water sample and the negative control (VTG production). The horizontal line indicates the mean value for VTG production in the control liver slices

Significant variation in VTG production in liver cultures was found following exposure to water samples collected during the 3rd survey (Figure 3.4) (Kruskall-Wallis ANOVA, $F=2.86$, 13df, $P<0.005$). Although all mean VTG production values are above the negative control mean VTG value, only the C-C sample induced significant VTG production when compared with the VTG induction in the negative control. The positive control, 17ß-estradiol, did not exhibit significant VTG production.

Samples collected during the 4th survey did not induce VTG production in culture when compared to the liver slices exposed in the negative control (Figure 3.5). However, the positive control induced significant VTG production in the liver slices when compared to the liver slices exposed in the negative control (Kruskall-Wallis ANOVA, $H=27.17$, 13df, $P=0.012$).

3.1.4 Primary rainbow trout hepatocyte (PRTH) assay

The positive control, 17ß-estradiol (1.362×10^6 ng/ℓ), confirmed the expected VTG induction by the PRTH assay. Only the water samples of the first three surveys were tested. Most of the samples showed strong toxicity at the 0.2 (96-times concentrated) and 1% (480-times concentrated) test concentrations (30 to 99% mortality). Extract toxicity can either mask the estrogenic potential or will not necessarily reflect the true values of VTG in cytotoxic extracts which have been corrected for viability. In order to reduce the cytotoxicity, VTG values were compared at the lowest test concentration, namely 0.04% (19-times concentrated).

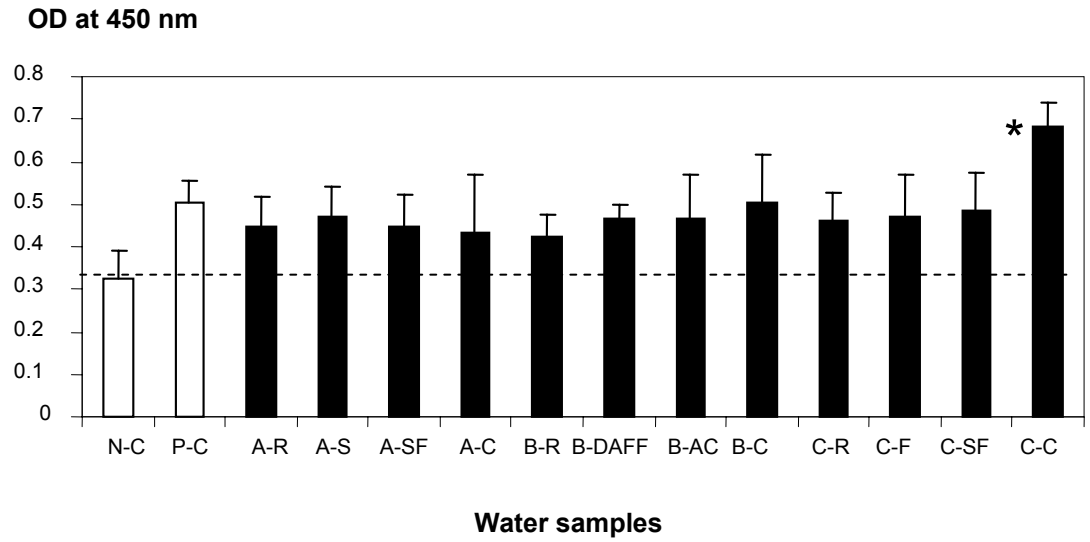

FIGURE 3.4: Average VTG production by *X. laevis liver* slices (quadruple slices for Each sample) incubated in extracts of water samples (3rd survey) for 6 d. N-C=negative control; P-C=positive control [2.724×10^4 ng/ℓ (10^{-7} M) 17β-estradiol]. The asterisk (*) indicates significant difference (Kruskal-Wallis ANOVA, $P<0.05$) between the ODs of the water sample and the negative control (VTG production). The horizontal line indicates the mean value for VTG production in the control liver slices

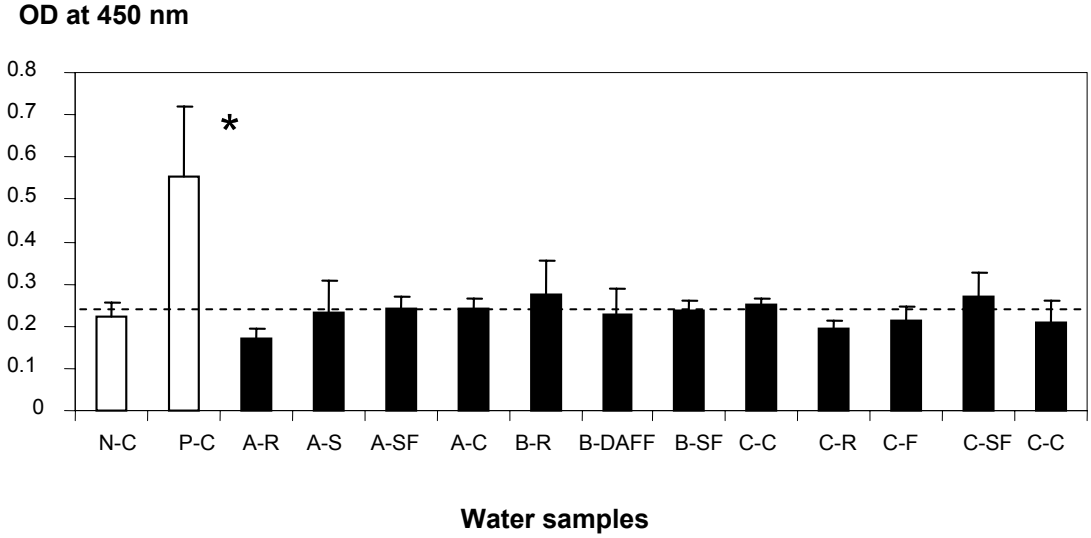

FIGURE 3.5: Average VTG production by *X. laevis liver* slices (quadruple slices for Each sample) incubated in extracts of water samples (4th survey) for 6 d. N-C=negative control; P-C=positive control [2.724×10^4 ng/ℓ (10^{-7} M) 17β-estradiol]. The asterisk (*) indicates significant difference (Kruskal-Wallis ANOVA, $P<0.05$) between the ODs of the positive and negative control (VTG production). The horizontal line indicates the mean value for VTG production in the control liver slices

The results obtained with the PRTH assay are presented in Table 3.4. Some of the samples at the 0.04% concentration still exhibited considerable toxicity. VTG induction by the source water of Treatment Plant A was generally similar to that of the control (VTG induction factor: 0.68 to 1.21), suggesting an absence of estrogenic compounds. The final water of Treatment Plant A collected during the 1st survey exhibited a low VTG induction compared to the source water, while the water collected during the 2nd survey exhibited a high VTG induction compared to the source water. The source water of Treatment Plant B showed relatively high VTG induction values (1.56 to 2.58),

TABLE 3.4: Estrogenic activity of water sampled at drinking water treatment plants using the PRTH assay[1]

Sampling survey	Sample[2]	Mortality (%)	VTG (µg ALP/mℓ PRTH biomass)[3]	VTG induction factor[4]
1	A-R	46	1 741	0.98
	A-S	+2	1 400	0.79
	A-SF	+10	1 050	0.59
	A-C	26	1.152	0.65
	B-R	14	1 838	2.58
	B-DAFF	21	1 334	1.87
	B-AC	39	1 999	2.80
	B-C	+46	2 566	3.60
	C-R	+20	1 518	1.20
	C-F	22	1 220	0.96
	C-SF	13	1 263	1.00
	C-C	14	1 227	**0.97**
2	A-R	57	1 213	0.68
	A-S	+14	1 221	0.69
	A-SF	55	2 388	1.34
	A-C	64	2 684	1.51
	B-R	15	1 111	1.56
	B-DAFF	44	1 612	2.26
	B-AC	3	1 474	2.07
	B-C	8	1 225	1.72
	C-R	31	2 693	2.12
	C-F	49	1 386	1.09
	C-SF	59	1 835	1.45
	C-C	69	1 568	**1.24**
3	A-R	31	2 160	1.21
	A-S	68	958	0.54
	A-SF	5	1 751	0.98
	A-C	66	2 264	1.27
	B-R	17	1 358	1.91
	B-DAFF	+10	1 603	2.25
	B-AC	10	932	1.31
	B-C	31	1 366	1.92
	C-R	69	3 574	2.81
	C-F	68	1 643	1.29
	C-SF	73	1 131	0.89
	C-C	56	896	**0.71**

1 Control: Mortality - 10%; VTG - 713 to 1 778 (µg ALP/mℓ PRTH biomass); Induction factor - 1.0
2 0.04% extract
3 Corrected for mortality
4 Compared to control VTG
\+ Stimulation in viability
Results in **bold:** Final water VTG induction lower as that of the source water, R

suggesting the presence of estrogenic compounds. During all the surveys the final water of Treatment Plant B and some of the water from intermediate treatment processes showed a higher induction in VTG than the source water. The results suggested no removal of estrogenic compounds during water treatment. The VTG induction factor of the source water of Treatment Plant C ranged from 1.20 to 2.81, indicating the presence of endocrine disrupting compounds. In all instances the final water showed lower VTG induction, indicating that treatment reduced/removed estrogenic effects.

Calculating the mean VTG induction factor for the three sampling surveys for each treatment plant and analysing the results by single factor ANOVA (F=15.31; P=0.001), showed that the VTG induction potential of the samples from Treatment Plant B was significantly higher than that of Treatment Plants A and C. Although the mean VTG induction potential of the samples of Treatment Plant A was lower than that of Treatment Plant C, the difference was not significant.

3.1.5 Zebrafish enzyme linked immunosorbant assay (ELISA)

Whole body VTG concentrations varied significantly among the juvenile zebrafish exposed to the samples collected during the 1st survey (Kruskal-Wallis ANOVA, H=17.77, 7df, P=0.013) (Figure 3.6). However, no exposed group showed any increased mean whole body VTG concentration when compared to the negative control exposed fish. When comparing group pairs, no significant differences were found among the fish exposed to source water and final water. Overall, the levels of homogenate VTG concentrations were low with most ODs at the lower-end of the Standard curve. The results indicate that no significant estrogenicity occurred in any of the exposure groups.

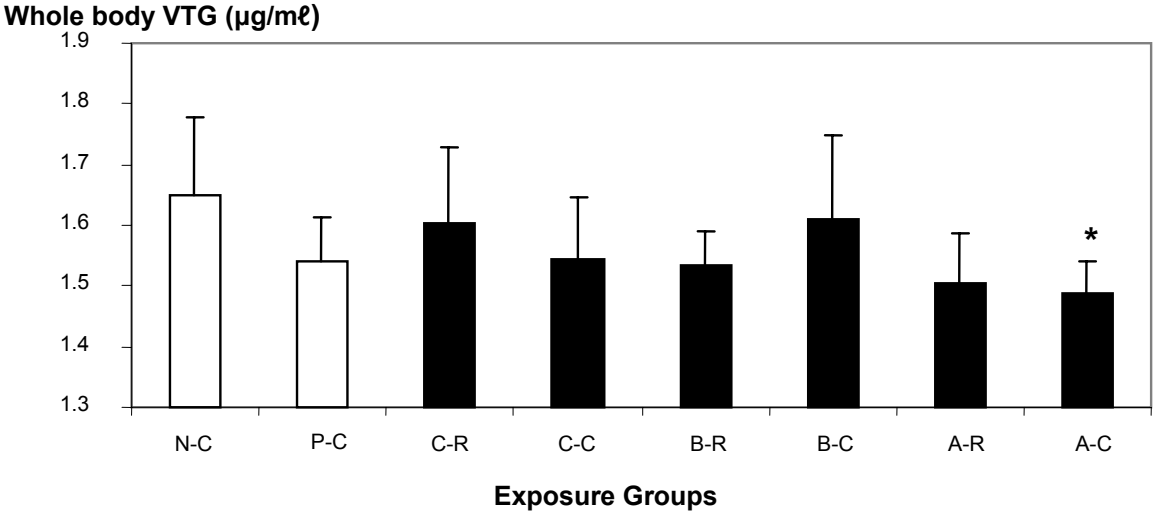

FIGURE 3.6: Average VTG concentrations in whole body homogenates of juvenile zebrafish (N=10/group) exposed to water collected during the 1st survey. N-C=negative control; P-C=positive control (109 ng/ℓ 17β-estradiol). The asterisk (*) indicates significant difference (Kruskal-Wallis ANOVA, P<0.05) of the mean VTG concentration in fish homogenates when compared with fish exposed to the negative control

Similar to the results of the 1st sampling survey, all juvenile zebrafish exposed to source and final waters collected during the 2nd survey showed low homogenate VTG levels with ODs at the lowest end of the standard curve (Figure 3.7). Variation in homogenate VTG levels among fish in the different exposure groups was significant (Kruskal-Wallis ANOVA, H=20.11, 7df. P=0.005). The lowest values were measured in the fish exposed to C-R water, but no significant increases in VTG levels relative to the negative control were observed in any the exposure groups. The results indicated an absence of estrogenicity in this *in vivo* bioassay.

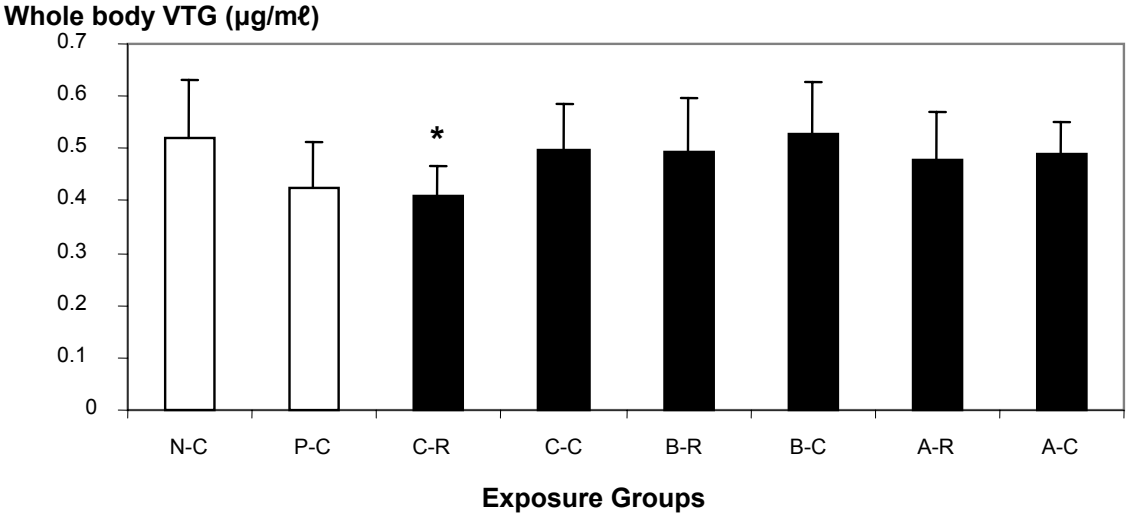

FIGURE 3.7: Average VTG concentrations in whole body homogenates of juvenile zebrafish (N=10/group) exposed to water collected during the 2nd survey. N-C=negative control; P-C=positive control (109 ng/ℓ 17β-estradiol). The asterisk (*) indicates significant difference (Kruskal-Wallis ANOVA, P<0.05) of the mean VTG concentration in fish homogenates when compared with fish exposed to the negative control

The fish exposed to the samples collected during the 3rd survey revealed significant variation among individual fish exposed in the different exposure groups (Kruskal-Wallis ANOVA, H=15.07, 7df, P=0.035) (Figure 3.8). Compared to the negative control no exposure group showed a significant increase in whole body VTG concentration. The mean VTG concentrations were low throughout the exposure groups and it is concluded that no significant estrogenicity was found in any of the exposure groups.

Significant variation in mean whole body VTG levels were also found in fish exposed to the water collected during the 4th survey (Kruskal-Wallis ANOVA, H=14.21, 7df, P=0.048). In spite of this variation the means of the homogenate VTG concentrations did not vary significantly among exposure groups when compared to the negative control (Figure 3.9). The highest mean VTG levels were measured in the fish exposed to A-R and C-R water. As observed with the previous surveys, no significant estrogenicity was detected in any of the samples with this *in vivo* bioassay.

The positive controls included with each survey (Figures 3.6 to 3.9) did not show increased estrogenicity. Fresh test solutions were prepared for each exposure study. It is, therefore, unlikely that preparation errors could have occurred on all occasions. It is

possible that the concentrations selected were too low to induce effects in juvenile fish and, therefore, the absence of activity.

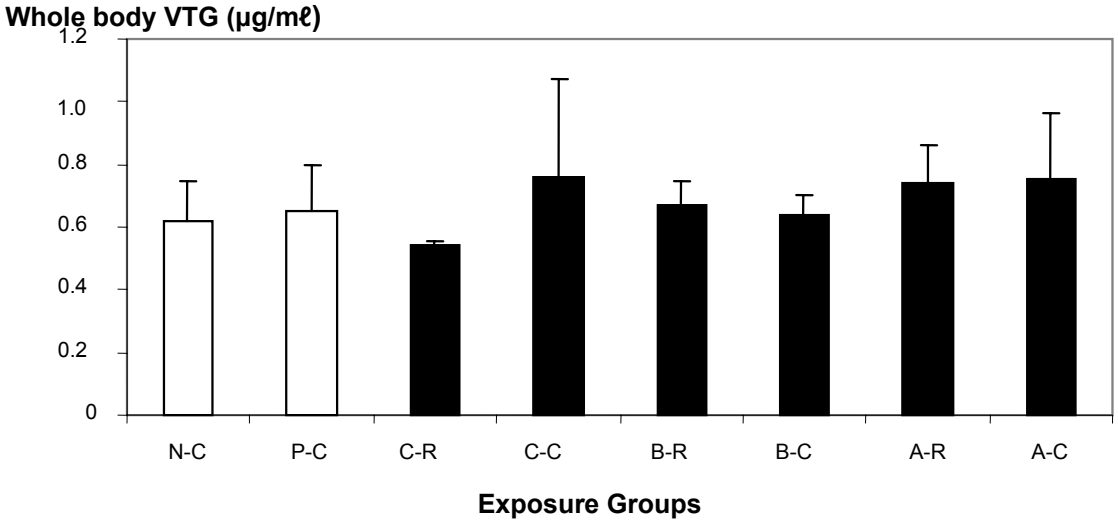

FIGURE 3.8: Average VTG concentrations in whole body homogenates of juvenile zebrafish (N=5/group) exposed to water collected during the 3rd survey. N-C=negative control; P-C=positive control (1 090 ng/ℓ 17β-estradiol). The asterisk (*) indicates significant difference (Kruskal-Wallis ANOVA, P<0.05) of the mean VTG concentration in fish homogenates when compared with fish exposed to the negative control

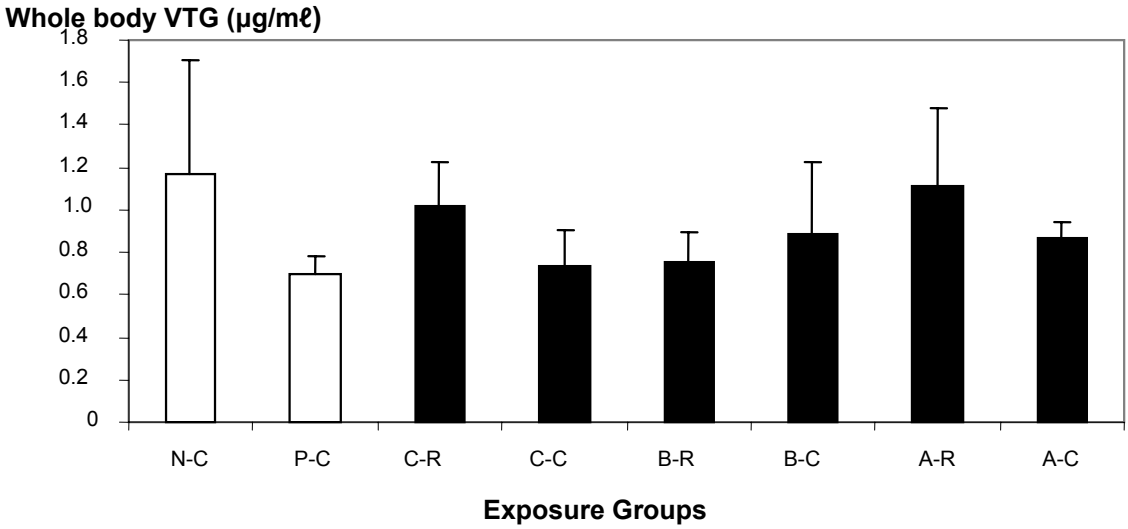

FIGURE 3.9: Average VTG concentrations in whole body homogenates of juvenile zebrafish (N=5/group) exposed to water collected during the 4th survey. N-C=negative control; P-C=positive control (1 090 ng/ℓ 17β-estradiol). The asterisk (*) indicates significant difference (Kruskal-Wallis ANOVA, P<0.05) of the mean VTG concentration in fish homogenates when compared with fish exposed to the negative control

In general, the homogenate VTG concentrations were very low and the fact that VTG was measured at the lower end of the standard curve could have introduced errors in the results. It is doubtful that the low VTG values point to VTG breakdown during preparation and transport (paragraph 3.6). The fish were mostly very small. It is suspected that the low VTG levels were rather due to dilution of the already small fish during homogenation.

3.1.6 Zebrafish alkali-labile phosphate (ALP) assay

VTG levels (mg ALP/mg protein) in juvenile zebrafish kept in moderately hard water (negative control) are shown in Table 3.5. The table presents the means of triplicate exposures (80 to 100 fish per container) and triplicate assays (n=9), as well as the coefficients of variation (CVs). VTG levels ranged from 0.539 to 1.510 mg ALP/mg protein. These values were considerably higher than those obtained during a WRC project on the establishment of *in vivo* techniques for the detection of endocrine disruptors in water systems (Slabbert et al., 2007a). The fluctuation between data sets was also smaller, probably because the protocols improved with time. The CVs ranged from 9 to 25%, which are also much lower than the values obtained previously (Slabbert et al*.,* 2007a). The variation in the protein values, used to express ALP, was generally small (CVs <10%).

TABLE 3.5: VTG levels (mg ALP/mg protein) in juvenile zebrafish kept in moderately hard water

Sample	Mean (n=9)	CV (%)
1	1.341	23
2	1.510	14
3	0.873	14
4	1.359	25
5	0.539	9
6	0.762	10
7	0.991	9

VTG was significantly induced (P=0.05; two-tailed t-test) by the positive control 17ß-estradiol during three of six exposures (Table 3.6). The initial absence in VTG induction could be ascribed to a too low exposure concentration (109 ng/ℓ). A 10-fold increase in the exposure concentration (1 090 ng/ℓ) resulted in an enhanced induction. The absence of induction during the final survey could be due to lower sensitivity of test fish.

TABLE 3.6: Effect of 17ß-estradiol on juvenile zebrafish VTG (mg ALP/mg protein)

17β-estradiol (ng/ℓ)	% Induction (+) or reduction (-)[1]
109	+18
	-19
	+59
1 090	**+73**
	+109
	-6

[1] Compared to fish in moderately hard water
Results in bold indicate significant induction at P=0.05 (two-tailed t-test)

VTG concentrations in whole juvenile zebrafish homogenates exposed to water samples collected from three drinking water treatment plants during four sampling surveys are shown in Figures 3.10 to 3.13. Compared to the negative control, none of the water samples collected during the 1st survey significantly increased VTG concentrations in zebrafish (P=0.05; two-tailed t-test) (Figure 3.10). Instead, significant VTG reduction was exhibited by samples A-R, A-C, C-R and C-C (Figure 3.10). When comparing group pairs, no significant differences were found between fish exposed to source water and final water. VTG was also not significantly induced by the positive control (P=0.05; two-tailed t-test).

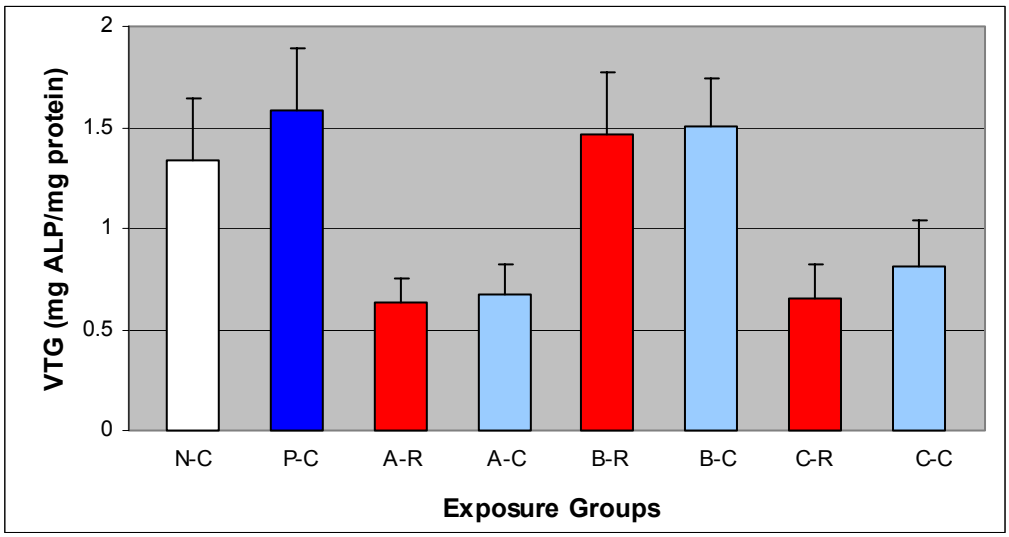

FIGURE 3.10: VTG (mg ALP/mg protein) in whole juvenile zebrafish homogenates (N=9) exposed to water collected during the 1st survey. N-C=negative control; P-C=positive control (109 ng/ℓ 17β-estradiol)

VTG was significantly induced by sample A-R collected during the 2nd sampling survey (P=0.05; two-tailed t-test) (Figure 3.11). Fish exposed to sample B-C showed significant reduction in VTG. Significant differences were found between the source and final waters of Treatment Plants A and B (VTG in fish exposed to final water reduced) (P=0.05; two-tailed t-test). VTG was significantly induced by the positive control (P=0.05; two-tailed t-test).

Compared to the negative control, samples A-R, A-C, B-R and B-C collected during the 3rd survey significantly increased VTG concentrations in zebrafish (P=0.05; two-tailed t-test) (Figure 3.12). When comparing group pairs, significant differences were found between fish exposed to source water and final water of Treatment Plant A (VTG in fish exposed to final water induced) (P=0.05; two-tailed t-test). VTG was significantly induced by the positive control (P=0.05; two-tailed t-test).

Samples B-C and C-C collected during the 4th survey significantly increased VTG concentrations in zebrafish (P=0.05; two-tailed t-test) (Figure 3.13). A-R and B-R caused significant reduction. Significant differences were found between fish exposed to source water and final water of Treatment Plant B (VTG in fish exposed to final water induced) (P=0.05; two-tailed t-test). VTG was not significantly induced by the positive control (P=0.05; two-tailed t-test).

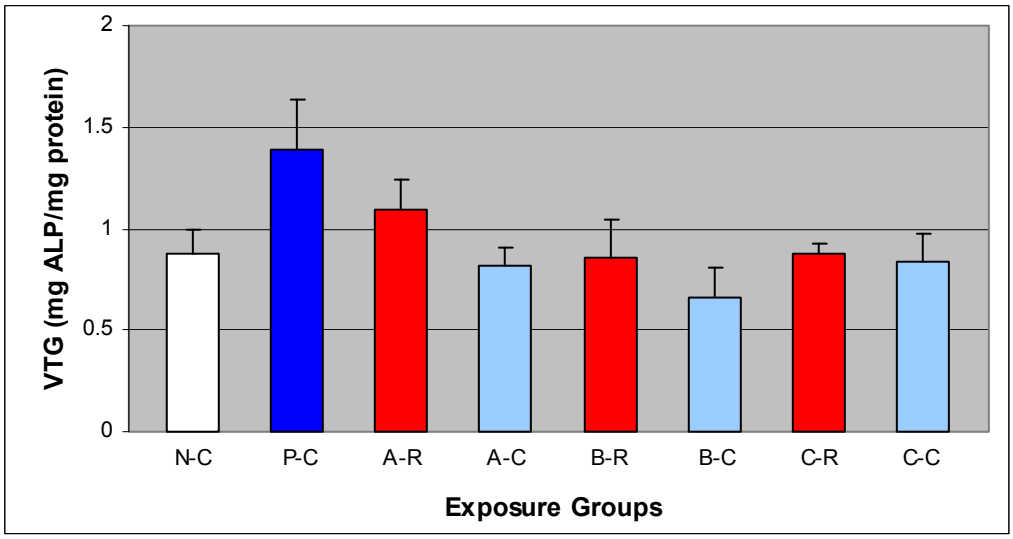

FIGURE 3.11: VTG (mg ALP/mg protein) in whole juvenile zebrafish homogenates (N=9) exposed to water collected during the 2nd survey. N-C=negative control; P-C=positive control (109 ng/ℓ 17β-estradiol)

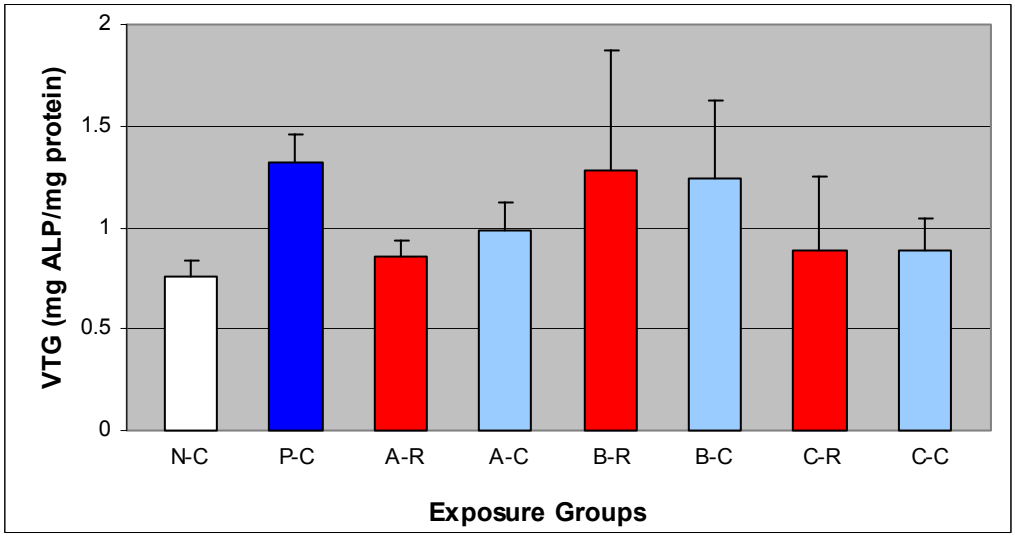

FIGURE 3.12: VTG (mg ALP/mg protein) in whole juvenile zebrafish homogenates (N=9) exposed to water collected during the 2nd survey. N-C=negative control; P-C=positive control (1 090 ng/ℓ 17β-estradiol)

The VTG concentrations of exposure groups were compared to the negative control to derive % effects (induction and reduction). These values are summarised in Table 3.7. The lower VTG concentrations in some of the exposure groups (also shown as a reduction in Table 3.7) could have been due to interferences in spectrophotometric readings. Large precipitates were observed in some instances. Although plates were also read at 450 nm to provide for background readings, interference by the precipitates could not be ruled out.

Exposure groups (water samples and controls) showed large lethalities during the 1st and 4th surveys, particularly during the first few days (Table 3.7). This was possibly due

to a weaker batch of test fish. Another explanation for lethality could be that fish loads in test containers were too high. Unfortunately, the exposure studies could not be repeated because of limited sample volume and unavailability of another batch of test fish. Sample volumes in containers were increased to 500 mℓ as from the 2nd survey to make double sure that fish loads were not too high. All the water samples collected during the 2nd survey, except B-R and the controls, showed some degree of toxicity (13 to 28% lethality). Samples A-C, B-R and B-C collected during the 3rd survey also caused slight lethality (11-18%). Control fish were not affected during the 2nd and 3rd surveys, indicating healthy stock.

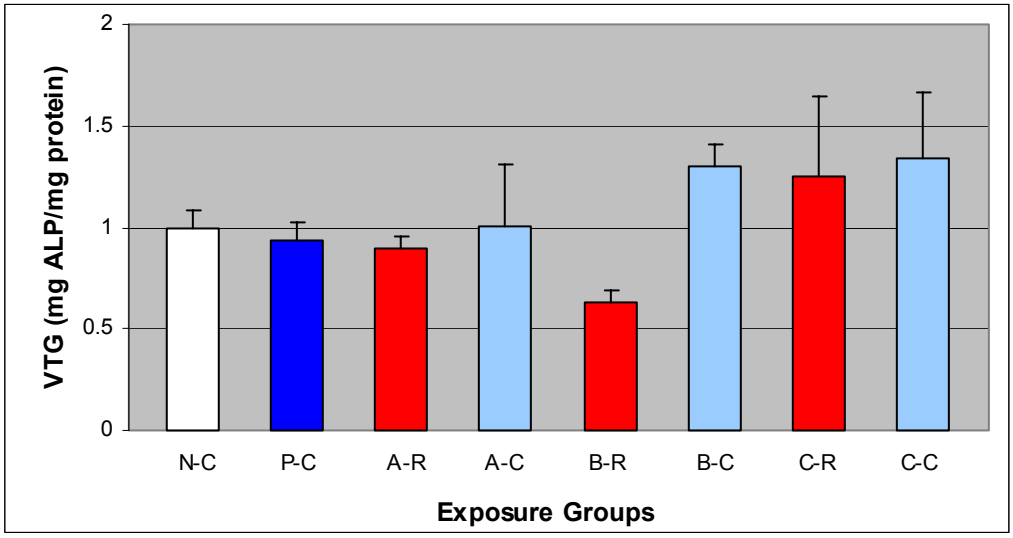

FIGURE 3.13: VTG (mg ALP/mg protein) in whole juvenile zebrafish homogenates (N=9) exposed to water collected during the 4th survey. N-C=negative control; P-C=positive control (1 090 ng/ℓ 17β-estradiol)

A concern that the effect of stress from handling and captivity could have an effect on the VTG levels was raised with the developers of the ALP test (Personal Communication, 2004). According to Environment Canada they are not aware of any reports indicating that stress can have an effect on VTG levels. Adenosine 5'-triphosphate (ATP) levels will be affected by stress, but they proved that the phosphate groups of ATP were not detectable by the ALP method, following ATP injection into test organisms. Phospholipids also do not interfere with the method. It was also proven that phosphate levels are a good indicator of VTG after showing that:

- Phosphate levels were induced in clams and fresh water mussels after 17β-estradiol injections;
- Phosphate levels correlated well with VTG messenger ribonucleic acid (mRNA) levels in rainbow trout hepatocytes; and
- Phosphate levels correlated with the maturation stages of female gonad development in soft-shell clams (*Maia arenaria*) (Personal Communication, 2004).

TABLE 3.7: Effect of water samples on juvenile zebrafish VTG (mg ALP/mg protein)

Survey	Sample	% Lethality	% Induction (+) or reduction (-)[1]
1st	A-R	approximately 50%	**-53**
	A-C		**-50**
	B-R		+9
	B-C		+12
	C-R		**-51**
	C-C		**-40**
2nd	A-R	18	**+25**
	A-C	28	-7
	B-R	10	-2
	B-C	21	**-24**
	C-R	16	+1
	C-C	13	-4
3rd	A-R	6	**+12**
	A-C	14	**+29**
	B-R	11	**+68**
	B-C	18	**+63**
	C-R	0	+16
	C-C	0	+16
4th	A-R	25	**-9**
	A-C	22	+1
	B-R	45	**-36**
	B-C	31	**+31**
	C-R	33	+27
	C-C	47	**+35**

[1] Compared to fish in moderately hard water (negative control)
Results in bold indicate significant induction/reduction at P=0.05 (two-tailed t-test)

3.1.7 Gel electrophoresis

Figure 3.14 shows the bands obtained when juvenile zebrafish were exposed to the controls and water samples. Two very faint bands were noticed between the 100 and 150 kD molecular weight markers. It is possible that these bands correspond to the VTG bands observed in adult female fish (Slabbert et al., 2007a). If this is indeed the case it shows that sexual differentiation starts earlier than the 38 d referred to in literature or that the water used for fish maintenance were contaminated (e.g. aerated tap water, sealant, plastic ware) resulting in low background VTG levels. Exposure to the positive control 17ß-estradiol and water samples did not result in more pronounced bands, suggesting that VTG levels in fish homogenates were actually too low to be detected by the established method. With the way the method is applied (qualitative measurement) it is not expected that differences between VTG levels will be very clear. The results of the 3rd and 4th surveys were similar, namely very faint bands were noticed between the 100 and 150 kD molecular weight markers. Additional gels with fish samples exposed to the positive control did not clarify the observed bands.

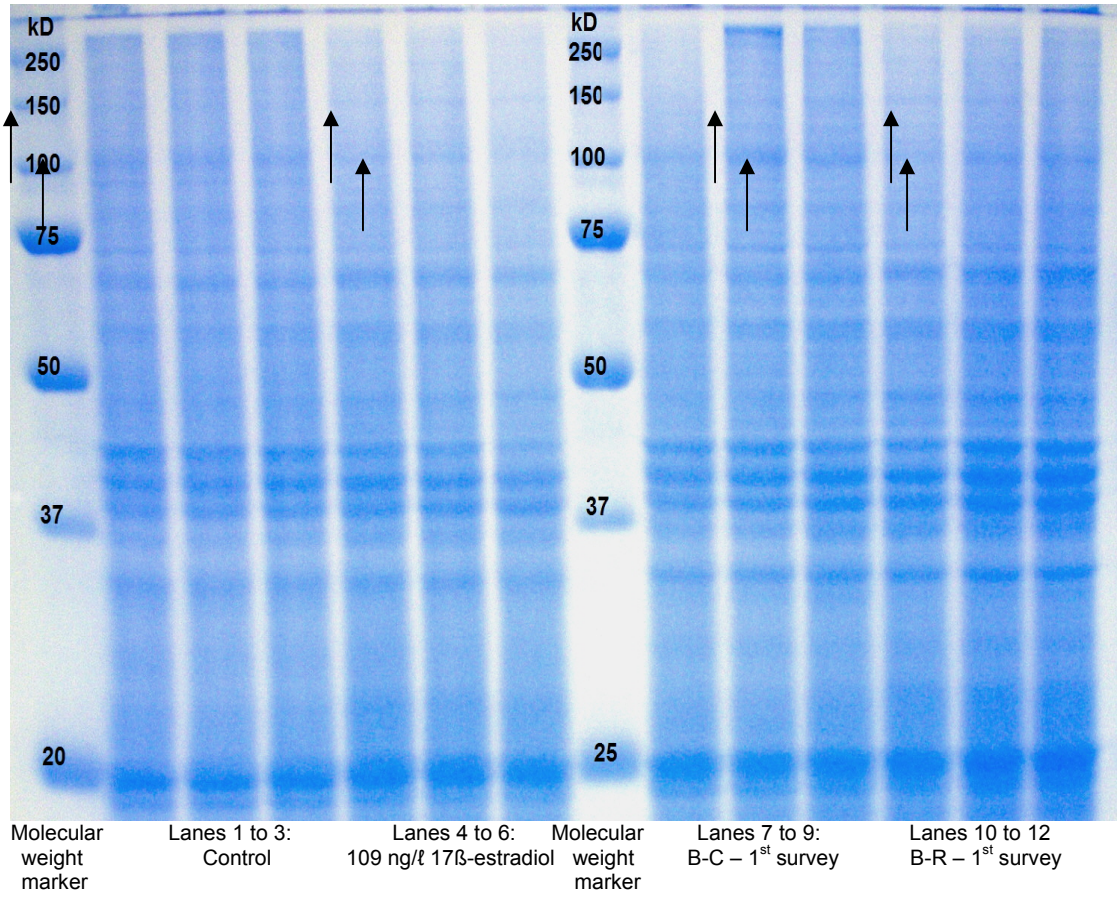

FIGURE 3.14: Band patterns obtained for whole juvenile zebrafish homogenates exposed to controls and water collected from Treatment Plant B

3.2 Chemical analyses

3.2.1 Estrogen analysis

The LOD and LOQ for the analytical procedure are 1.0 and 2.0 ng/ℓ, respectively (taking the concentration factor of 10 000-times into account). All the estrogens analysed for, namely 17α- and β-estradiol, estrone, 17α-ethinylestradiol and estriol, in the source and final water collected from three drinking water treatment plants during four sampling surveys, were <LOD (<1.0 ng/ℓ).

3.2.2 Triazine and p-nonyl phenol residue analysis

The LOD for triazine and p-nonyl phenol residue analysis is 0.15 µg/ℓ (Table 3.8). Results between 0.15 and 0.19 µg/ℓ (LOQ) are not quantified.

The results in Table 3.8 show that five (21%) of the tested water samples (source and final) contained simazine, eight (33%) contained atrazine, three (13%) contained terbuthylazine, and seven (29%) contained p-nonyl phenol at concentrations >LOQ. 10 of the source water samples (83%) and eight of the final water samples (67%) contained one or more of the residues at concentrations >LOQ. In some instances residue concentrations were lower in final water than in source water, but in other instances they were higher.

Simazine was present in the source and final water of Treatment Plant A (A-R and A-C) during the 1st and 2nd surveys. 0.21 µg/ℓ terbuthylazine was also present in sample A-R collected during the 1st survey. The source water collected during the 3rd and 4th surveys contained p-nonyl phenol, while atrazine was present in the final water collected during the 3rd survey.

The source water of Treatment Plant B (B-R) contained simazine and atrazine during the 2nd survey, atrazine and p-nonyl phenol during the 3rd survey and p-nonyl phenol during the 4th survey. p-nonyl phenol was present in the final water during the 1st and 2nd surveys.

Both the source and final water of Treatment Plant C (C-R and C-C) contained some of the residues. p-nonyl phenol was present in the source water of the 1st survey, atrazine and terbuthylazine were present in the source water of the 2nd survey, and atrazine was present in the source water of the 4th survey. The final water contained atrazine and terbuthylazine during the 2nd survey, and atrazine during the 3rd and 4th surveys.

TABLE 3.8: Triazine and p-nonyl phenol concentrations (µg/ℓ) in water samples

Survey	Sample	Simazine	Atrazine	Terbuthyl-azine	p-nonyl phenol
1st	A-R	**0.31**	<LOQ	**0.21**	<LOQ
	A-C	**0.26**	<LOQ	<LOQ	<LOQ
	B-R	<LOD	<LOQ	<LOD	<LOQ
	B-C	<LOQ	<LOQ	<LOD	**0.25**
	C-R	<LOD	<LOQ	<LOD	**0.22**
	C-C	<LOD	<LOQ	<LOD	<LOD
2nd	A-R	**0.28**	<LOD	<LOQ	<LOQ
	A-C	**0.32**	<LOQ	<LOQ	<LOQ
	B-R	**0.20**	**0.24**	<LOQ	<LOD
	B-C	<LOQ	<LOQ	<LOD	**0.24**
	C-R	<LOD	**0.49**	**0.25**	<LOD
	C-C	<LOD	**0.70**	**0.30**	<LOD
3rd	A-R	<LOQ	<LOD	<LOD	**0.24**
	A-C	<LOD	**0.21**	<LOQ	<LOQ
	B-R	<LOQ	**0.24**	<LOQ	**0.24**
	B-C	<LOD	<LOD	<LOD	<LOQ
	C-R	<LOD	<LOQ	<LOD	<LOQ
	C-C	<LOD	**0.27**	<LOQ	<LOD
4th	A-R	<LOD	<LOD	<LOD	**0.39**
	A-C	<LOD	<LOD	<LOD	<LOQ
	B-R	<LOQ	<LOQ	<LOQ	**0.23**
	B-C	<LOD	<LOD	<LOD	<LOD
	C-R	<LOD	**0.29**	<LOQ	<LOQ
	C-C	<LOD	**0.21**	<LOD	<LOD

LOD Limit of detection (0.15 µg/ℓ)
LOQ Limit of quantification (0.19 µg/ℓ)

4. GENERAL DISCUSSION

4.1 *In vitro* and *in vivo* assays

4.1.1 Responses to the positive control 17ß-estradiol

17ß-estradiol is used to verify the sensitivity and performance of *in vitro* and *in vivo* assays. A range of concentrations was used in the yeast estrogen screen (YES) and the estrogen receptor-mediated chemical activated luciferase gene expression (ER-CALUX) assay to establish limits of detection (LODs), limits of quantification (LOQs), and the concentrations giving 50% of the maximum response (EC_{50}s). Single concentrations were applied in the liver slice and primary rainbow trout hepatocyte (PRTH) assays and for zebrafish vitellogenin (VTG) determinations [enzyme linked immunosorbent assay (ELISA), alkali-labile phosphate (ALP) assay and gel electrophoresis].

The data in Table 4.1 shows that the YES and ER-CALUX assay detected 17ß-estradiol at much lower concentrations than the other assays, indicating that these assays are much more sensitive than the other *in vitro* and *in vivo* assays applied. The highest 17ß-estradiol concentrations were used for the liver slice (27 240 ng/ℓ) and the PRTH (1 362 000 ng/ℓ) assays. Higher test concentrations are probably required because these assays are carried out in complex tissue culture medium that could render tissue and cells more protected against chemical contaminants. The 27 240 ng/ℓ 17ß-estradiol concentration exhibited VTG induction in the liver slice assay only during one of four experiments. The estradiol concentration used in the PRTH assay induced the required response during all the experiments. The ELISA could not detect VTG induction in zebrafish exposed to 109 and 1 090 ng/ℓ 17ß-estradiol. Likewise, VTG bands could not be detected when gel electrophoresis was carried out on fish exposed to these concentrations. The ALP assay showed VTG induction during one of three experiments carried out with 109 ng/ℓ 17ß-estradiol, and during two of three experiments carried out with 1 090 ng/ℓ 17ß-estradiol.

TABLE 4.1: Responses of *in vitro* and *in vivo* assays to 17ß-estradiol

Assay	Test concen-tration/s (ng/ℓ)	Response
YES	0.0003-2 724	LOD: 1.64-5.03 ng/ℓ; EC_{50}: 10.95-20.61 ng/ℓ
ER-CALUX	0.0133-27.24	LOD: 0.1 ng/ℓ; EC_{50}: 1.6 ng/ℓ[1]
Liver slice	27 240	1 out of 4 controls positive
PRTH	1 362 000	all of the controls positive
Zebrafish ELISA	109 and 1 090	0 out of 4 controls positive
Zebrafish ALP	109 and 1 090	1 out of 3 (109 ng/ℓ) and 2 out of 3 (1 090 ng/ℓ) controls positive
Zebrafish gel electrophoresis	109 and 1 090	0 out of 4 controls positive

[1] Murk et al. (2002)
LOD: Limit of detection
EC_{50}: Concentration giving 50% of the maximum response

A comparison between the 17ß-estradiol LODs and EC_{50}s showed that the ER-CALUX assay was considerably more sensitive than the YES (Table 4.1). Similar findings were made by Murk et al. (2002), reporting LODs of 0.1 and 2.7 ng/ℓ, and EC_{50}s of 1.6 and 27 ng/ℓ, for the ER-CALUX assay and YES, respectively. A study by Kinnberg (2003),

comparing the detection potential of various *in vitro* assays, showed that the YES 17ß-estradiol LODs ranged from ≤1.0 to 3.0 ng/ℓ and EC_{50}s ranged from 27 to 218 ng/ℓ. Incubation periods of between 2 and 10 d were used. Although no clear correlation was noticed between the estrogenic potencies and incubation times reported in the study of Kinnberg (2003), studies by the UK Environment Agency (1997) and Beresford et al. (2000) showed that the YES can be made more sensitive by using longer incubation periods. This was also found during a previous WRC study when various incubation periods for the YES were evaluated (Slabbert et al., 2007a). Because of the increased sensitivity, an incubation period of 10 d was selected for testing water samples.

In studies by Hurter (2003), Hurter et al. (2002) and Burger (2007) concentrations ranging from 1 000 to 1 000 000 ng/ℓ 17ß-estradiol exhibited significant VTG induction in the liver slice assay. The fact that the same performance was not noticed in the present study indicates that the methodology requires standardization.

The two 17ß-estradiol concentrations used for positive control testing in the zebrafish test were in agreement with the concentrations used in literature to examine estrogenicity in fish. Studies by Panter et al. (1998) showed a significant reduction in the testis mass in adult male fathead minnows exposed to 320 and 1 000 ng/ℓ 17ß-estradiol for 21 d, while no effects were observed at concentrations ≤100 ng/ℓ. VTG induction was noticed at considerably lower 17ß-estradiol concentrations. A 10- to 100-fold increase in plasma VTG occurred in adult male fathead minnows exposed to 27 ng/ℓ 17ß-estradiol for 7 to 21 d (Parks et al., 1999). Newly hatched fathead minnows, exposed for 30 d to 17ß-estradiol concentrations ranging from 25 to 100 ng/ℓ, showed dose-dependent increases in whole body VTG (Tyler et al., 1999). 17ß-estradiol concentrations ranging from 10 to 1 000 ng/ℓ were also found to induce VTG in adult zebrafish and whole body homogenates (BATTELLE, 2002).

The observation that the 109 and 1 090 ng/ℓ concentrations used in the present study induced VTG only during some of the experiments indicates that the exposure and tissue preparation methodology requires optimization. High increases in VTG levels were detected by the ELISA described in paragraph 2.2.2.1.3 in the blood plasma of adult male zebrafish exposed to 17ß-estradiol during previous WRC studies (Slabbert et al., 2007b; Burger, 2007). In the present study the ELISA did not detect VTG induction in fish exposed to 17ß-estradiol, suggesting that the ELISA was not sufficiently sensitive to detect VTG in whole body homogenates, or that the ELISA methodology requires optimization. Measurement of VTG with the ALP assay showed that 17ß-estradiol concentrations ranging from 10.9 to 1 090 ng/ℓ exhibited significant VTG induction in juvenile zebrafish during a previous WRC study (Slabbert et al., 2007a). The fact that the ALP assay did not detect VTG induction on all occasions during the present study indicates that the ALP methodology requires refinement. As in a previous WRC study (Slabbert et al., 2007a), the gel electrophoresis methodology described in paragraph 2.2.2.4.3 was not sufficiently sensitive to detect VTG induction in juvenile zebrafish exposed to 109 and 1 090 ng/ℓ 17ß-estradiol.

4.1.2 Responses to water samples

Table 4.2 summarises the responses obtained with the different assays. The YES detected estrogenicity in 92% of the water samples. This was followed by the PRTH assay with 64% of the samples showing estrogenicity. 56% of the samples tested positive with the ER-CALUX assay and 29% with the zebrafish ALP assay. Only 6% of

the samples showed estrogenicity in the liver slice assay. The zebrafish ELISA did not detect estrogenicity in any of the water samples.

TABLE 4.2: Summary of the responses of *in vitro* and *in vivo* assays to water samples collected during four surveys (Y - estrogenicity detected; N - no estrogenicity detected)

Sampling survey	Sample	YES	ER-CALUX assay	*X. laevis* liver slice assay	PRTH assay	Zebrafish ELISA	Zebrafish ALP assay
1	A-R	Y	Y	N	N	N	N
	A-S	Y	Y	N	N	na	
	A-SF	Y	Y	N	N		
	A-C	Y	Y	N	N	N	N
	B-R	Y	Y	N	Y	N	N
	B-DAFF	Y	Y	N	Y	na	
	B-AC	Y	Y	N	Y		
	B-C	Y	N	N	Y	N	N
	C-R	Y	Y	N	Y	N	N
	C-F	Y	Y	N	N	na	
	C-SF	Y	Y	N	N		
	C-C	Y	Y	Y	N	N	N
2	A-R	Y	N	N	N	N	Y
	A-S	Y	N	N	N	na	
	A-SF	Y	N	N	Y		
	A-C	N	N	N	Y	N	N
	B-R	Y	Y	N	Y	N	N
	B-DAFF	Y	N	N	Y	na	
	B-AC	Y	N	N	Y		
	B-C	N	N	N	Y	N	N
	C-R	Y	Y	N	Y	N	N
	C-F	Y	Y	Y	Y	na	
	C-SF	Y	Y	N	Y		
	C-C	N	Y	N	Y	N	N
3	A-R	Y	Y	N	Y	N	Y
	A-S	Y	Y	N	N	na	
	A-SF	Y	N	N	N		
	A-C	Y	Y	N	Y	N	Y
	B-R	Y	Y	N	Y	N	Y
	B-DAFF	Y	Y	N	Y	na	
	B-AC	Y	N	N	Y		
	B-C	Y	N	N	Y	N	Y
	C-R	Y	N	N	Y	N	N
	C-F	Y	Y	N	Y	na	
	C-SF	Y	N	N	N		
	C-C	Y	N	Y	N	N	N
4	A-R	Y	N	N	na	N	N
	A-S	Y	Y	N		na	
	A-SF	Y	Y	N			
	A-C	N	N	N		N	N
	B-R	Y	Y	N		N	N
	B-DAFF	Y	Y	N		na	
	B-AC	Y	N	N			
	B-C	Y	N	N		N	Y
	C-R	Y	N	N		N	N
	C-F	Y	Y	N		na	
	C-SF	Y	N	N			
	C-C	Y	N	N		N	Y
Total Y		44	27	3	23	0	7
Total N		4	21	45	13	24	17

na Not analysed

The results in Table 4.2 show that the detection potential of tests was enhanced when concentrated samples were tested (compared to direct exposure to water samples). The only exception was the liver slice assay, where samples were diluted to the original sample concentration before testing to simulate real environmental conditions. As a consequence only three water samples exhibited estrogenicity.

The YES and ER-CALUX assay (Table 4.2) showed a 60% agreement in results (Y and N). The ER-CALUX assay only showed a positive response on one occasion (2nd survey, C-C), when the result obtained with the YES was negative. In all other instances the YES detected estrogenicity while the ER-CALUX assay results were negative. The data presented in Figure 4.1 show a linear relationship [correlation (R)=0.9265] between the positive responses (>whole method LOD) obtained with the YES and ER-CALUX assay for water samples. 56% of the PRTH assay results were in agreement with the YES results and 53% were in agreement with the ER-CALUX assay results (Y and N). The PRTH assay showed estrogenicity in three samples (2nd survey, A-C, B-C and C-C) while the YES results were negative. Nine samples tested positive in the PRTH assay while the ER-CALUX assay did not show estrogenicity. All seven positive responses obtained with the zebrafish ALP assay were in agreement with the YES results while only three agreed with the ER-CALUX assay results. The three positive results obtained with the liver slice assay were in agreement with the YES results, while only two were in agreement with the ER-CALUX assay results.

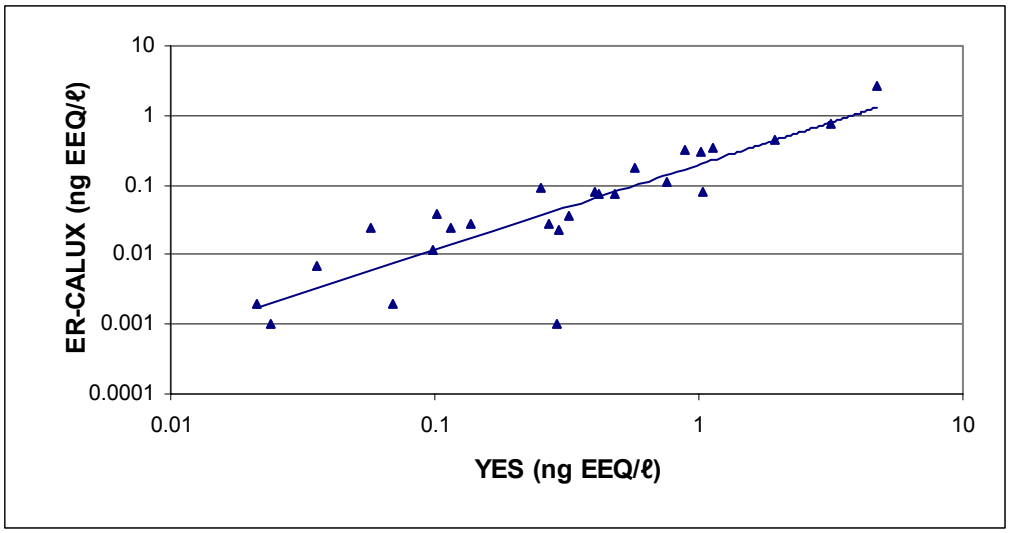

FIGURE 4.1: Comparison between the positive responses (>whole method LOD) obtained with the YES and ER-CALUX assay for water samples

Although the YES detected estrogenicity in the majority of water samples, the ER-CALUX assay was between 2- and 13-times more sensitive than the YES. The whole method LOD of the YES was 0.009 ng EEQ/ℓ (Table 3.2) and that of the ER-CALUX was 0.00085 ng EEQ/ℓ (Table 3.3). The highest estrogenic potencies established with the YES were 3.189 ng EEQ/ℓ (3rd survey, B-R) and 4.745 ng EEQ/ℓ (3rd survey, B-DAFF) (Table 3.2 and Figure 4.1). The ER-CALUX assay showed the highest estrogenic potencies for the same samples with 0.758 ng EEQ/ℓ for B-R and 2.620 ng EEQ/ℓ for B-DAFF (Table 3.3 and Figure 4.1).

Both the YES and ER-CALUX assay showed lower estrogenic activity in the water samples during the 2nd survey (Tables 3.2 and 3.3). This could be due to the dilution of source water as a result of the heavy rains in the Gauteng area during the period preceding sampling. The YES and ER-CALUX assay results also indicated that the estrogenic activities in the water of Treatment Plant B were generally higher than that of Treatment Plant A followed by that of Treatment Plant C. These findings are in agreement with the results of the PRTH assay (Table 3.4) which clearly demonstrated higher VTG induction by the water of Treatment Plant B

The assays used are specific for the detection of estrogens and estrogen mimicking substances. In the PRTH assay and the zebrafish ELISA and ALP assays, VTG concentrations were, in some instances, significantly reduced compared to control VTG (Table 3.4 and Figures 3.6, 3.7, 3.10, 3.11, and 3.13). A possible explanation for this could be the presence of anti-estrogens in the water samples. Anti-estrogens block the estrogen receptor, preventing estrogens to bind to the receptor.

4.1.3 Assay responses to water samples and data reported in literature

Table 4.3 shows that, with the exception of the results of the 2nd survey (which were very low), the YES estrogenic potencies obtained for the source waters of Treatment Plants A and B were similar to the potencies obtained for surface waters collected in the same areas during a previous WRC study (Slabbert et al., 2007a). However, the estrogenic potencies obtained for the source water of Treatment Plant C, which was abstracted from a dam, were considerably lower than the potencies obtained for the water sampled from a river in the area (Slabbert et al., 2007a). The source water of Treatment Plant B and surface water collected in the area of Treatment Plant B showed the highest estrogenic activity (Table 4.3). In an earlier study on 25 surface water samples collected from the Vaal, Jukskei and Hennops River catchments estrogenic potencies obtained with the YES ranged from 0.027 to 23.5 ng EEQ/ℓ (Arijs et al., 2002). The higher estrogenic activities were noticed at points of sewage discharge. In the ER-CALUX assay estrogenic potencies of source waters ranged from <0.00085 to 0.756 ng EEQ/ℓ (Tables 3.3 and 4.4). The results obtained for the source waters of Treatment Plants A and C were considerably lower than the results obtained during a WRC study on surface water in the area of Treatment Plant B (Bornman et al., 2007). The estrogenicity obtained for B-R (Table 4.4) was at the lower side of the values reported by Bornman et al. (2007). The ALP assay showed that some of the source waters of Treatment Plants A and B were estrogenic (12 to 68% VTG induction) while C-R was not estrogenic (Table 3.7). During a previous WRC study (Slabbert et al., 2007a) surface water collected in the vicinity of Treatment Plants A and C caused significant estrogenicity (as much as 200% VTG induction) using the ALP assay while no estrogenicity was noticed in water taken in the vicinity of Treatment Plant B.

TABLE 4.3: YES estrogenic potencies of source and surface water

Present study		Survey carried out during 2003-2004[1]	
Source water	**Estrogenic potency (ng EEQ/ℓ)**	**Surface water**	**Estrogenic potency (ng EEQ/ℓ)**
A-R	0.037-0.761	HD	0.20-0.65
B-R	0.115-3.189	RD	0.83-2.04
C-R	0.036-0.226	VB	0.55-1.24

[1] Slabbert et al. (2007a)

TABLE 4.4: ER-CALUX assay estrogenic potencies of source and surface water

Present study		Survey carried out during 2005-2006[1]	
Source water	**Estrogenic potency (ng EEQ/ℓ)**	**Surface water**	**Estrogenic potency (ng EEQ/ℓ)**
A-R	<0.00085-0.114	HD	nd
B-R	0.024-0.758	RD	0.33-16.0
C-R	<0.00085-0.039	VB	nd

[1] Bornman et al. (2007)
nd Not determined

As in the present study, an absence of estrogenic activity (no significant VTG induction) was reported during previous WRC studies when adult zebrafish were exposed to surface water (Burger, 2007) and sewage effluent samples (Slabbert et al., 2007b). Negative results were also obtained with the liver slice assay (Burger, 2007) and zebrafish gel electrophoresis (Slabbert et al., 2007a) during previous WRC studies on surface water.

Table 4.5 shows that the estrogenic potencies obtained with the YES and ER-CALUX assay for surface waters in other parts of the world are in some instances similar to our findings but in other instances the potencies are much higher.

TABLE 4.5: Estrogenic potencies reported for surface water in other countries

Country	Assay	Estrogenic potency (ng EEQ/ℓ)	Author/s
United Kingdom	YES	<24	Thomas et al., 2001
Belgium		2.75-81	Witters et al., 2001
Japan		10	Tashiro et al., 2003
The Netherlands	ER-CALUX	0.068-0.469	Murk et al., 2002
		0.02-0.04	Vethaak et al., 2002
		0.08-9.4	Bogers et al., 2007

The estrogenic potencies obtained in this study for the final water using the YES were between 0.009 and 0.138 ng EEQ/ℓ. The potencies obtained with the ER-CALUX were considerably lower (<0.00085 to 0.028 ng EEQ/ℓ). In a study by KIWA in the Netherlands on drinking water samples originating from the river Meuse, no response was found (<0.02 ng EEQ/ℓ) in any of the test samples with the ER-CALUX (Bogers et al., 2007).

4.2 Chemical analyses

4.2.1 Estrogen concentrations and data reported in literature

The present study indicated that the estrogens 17α- and β-estradiol, estrone, 17α-ethinylestradiol and estriol in source and final water were below the LOD (<1.0 ng/ℓ). These values are in agreement with findings of Vethaak et al. (2002) who reported the following values for estrogens in surface water in The Netherlands: 17β-estradiol - <0.8 to 1.0 ng/ℓ; 17α-estradiol - <0.3 to 0.4 ng/ℓ; estrone - <0.3 to 7.2 ng/ℓ; and 17α-ethynylestradiol - <0.3 to 0.4 ng/ℓ. 17ß-estradiol was also found to be <LOD in surface waters collected in the vicinity of the three treatment plants during a previous WRC study (Burger, 2007). The concentrations of the other estrogens were however much

higher than those reported in literature for surface water in Europe: estriol - <LOD to 411.13 ng/ℓ; estrone - 22.05 to 151.5 ng/ℓ; 17α-ethynylestradiol - <LOD to 321.0 ng/ℓ (Burger, 2007).

4.2.2 Triazine and p-nonyl phenol residue concentrations and data reported in literature

Chemical analysis (Table 3.8) showed that the triazines (simazine, atrazine and terbuthylazine) and p-nonyl phenol were present above the analytical detection limits in some of the source and final water samples. Concentrations ranged from 0.20 (simazine, 2nd sampling, B-R) to 0.70 (atrazine, 2nd sampling, C-C) µg/ℓ. The concentrations reported for atrazine and terbuthylazine are in agreement with concentrations found in surface water in the vicinity of the treatment plants during a previous WRC study (Burger, 2007). The South African Water Quality Guidelines for Domestic Use (DWAF, 1996) state a Target Water Quality Range (TWQR) of ≤2.0 µg/ℓ for atrazine to avoid health effects for lifetime exposure. Since atrazine is the most toxic of the triazines, the same TWQR could be applied for simazine and terbuthylazine. The concentrations detected in the final water samples (Table 3.8) were on the lower side of the stated TWQR. Even if the chemicals were additive or synergistic it is not expected that the upper concentration of 2.0 µg/ℓ would be exceeded (e.g. final water of Treatment Plant C, 2nd survey).

p-Nonyl phenol concentrations in source water ranged from <LOD to 0.39 µg/ℓ (Table 3.8). These values are in agreement with the results reported by Burger (2007) for surface water in the vicinity of the treatment plants (<LOD to 0.65 µg/ℓ). The results obtained in the present study are on the lower side of the concentrations reported by Vethaak et al. (2002) for surface waters in The Netherlands (0.11 to 4.1 µg/ℓ). Concentrations in the final water ranged from <LOD to 0.25 µg/ℓ (Table 3.8). These concentrations are similar to concentrations detected from time to time in drinking water (Personal Communication, 2006a). In a study on eleven Canadian drinking water treatment plants it was found that nonyl phenol in the final water ranged from <LOD to 6.7 µg/ℓ (Berryman et al., 2004). There is no South African criterium for nonyl-phenol (DWAF, 1996). The recommended acute and chronic criteria for the protection of aquatic life in the USA are 28 and 6.6 µg/ℓ, respectively (USEPA, 2006). Canada has a criterium of 1 µg/ℓ for surface water (Berryman et al., 2004).

Historical data for triazine and nonyl phenol residues were not available for Treatment Plants A and B. Treatment Plant C reported that two surveys were carried out on their final water and both times the residues were below the LODs (simazine and atrazine: 0.1 µg/ℓ; terbuthylazine: <1.0 µg/ℓ; nonyl phenol not detected - no detection limit given) (Personal Communication, 2006b).

4.2.3 Heavy metal and mineral concentrations and data reported in literature

Heavy metals such as arsenic, cadmium, mercury and lead have endocrine disrupting properties. Table 4.6 shows that the heavy metal concentrations in the final water of Treatment Plant A and in the source and final water of Treatment Plant C (APPENDIX A) were below the TWQRs (no health effects expected) specified by DWAF (1996) for domestic use. The source water of Treatment Plant A was not analysed. Arsenic and mercury were present at concentrations >TWQRs in some of the surface water samples collected in the vicinity of Treatment Plants A and C (Table 4.6) during an earlier WRC study (Burger, 2007). Surface water in the vicinity of Treatment Plant A also contained

lead at concentrations >TWQR (Burger, 2007). Cadmium was detected at concentrations >TWQR in a number of source and final waters of Treatment Plant B. In addition, all the source and final waters of Treatment Plant B contained lead at concentrations >TWQR. Cadmium and lead were also present at concentrations >TWQRs in fountain water that is blended with the final water (APPENDIX A). The water of Treatment Plant B was not analysed for arsenic and mercury. Data on surface water sampled in the vicinity of Treatment Plant B (Burger, 2007) showed that these two heavy metals were occasionally present at concentrations >TWQRs (Table 4.6). The cadmium and lead concentrations in surface water (Burger, 2007) were slightly lower than those reported for source water in the present study.

TABLE 4.6: A comparison between heavy metal concentrations (µg/ℓ) in the source and final water of treatment plants and data reported in literature

Heavy metal (µg/ℓ)	Treatment Plant						TWQR[1]
	A		B		C		
	Source water	Final water	Source water	Final water	Source water	Final water	
Arsenic	na (0.41-11.9)	5.0	na (0.32-12.4)	na	<5.0 (0.26-31.3)	<5.0	≤10
Cadmium	na (<LOD-3.0)	3.0	1.9-6.7 (<LOD-3.7)	1.6-9.4	<3.0 (0.30-2.8)	<3.0	≤5.0
Mercury	na (0.92-6.6)	1.0	na (0.58-7.0)	na	<1.0 (1.1-8.9)	<1.0	≤1.0
Lead	na (0.63-22.2)	10	19-30 (1.2-16.7)	20-29	<10 (2.2-7.1)	<10	≤10

[1] DWAF (1996)
LOD Limit of detection
na Not analysed
TWQR Target Water Quality Range – no health effects expected
Results in brackets: Values reported in a previous WRC study on surface water in the vicinity of the treatment plants (Burger, 2007)

The data supplied by the Water Treatment Plants (APPENDIX A) showed that the mineral concentrations in the final water of Treatment Plants A and C were also within the required TWQRs (no health effects expected) DWAF, 1996). Aluminium and iron concentrations in source water of Treatment Plant C were considerably higher than the TWQRs. Some source and treated waters of Treatment Plant B contained high concentrations iron, while manganese was occasionally present at high concentrations in the source water. Calcium and chloride concentrations in some of the source and treated waters of Treatment Plant B were also found to exceed the TWQRs.

4.3 Removal efficiency of treatment processes

4.3.1 *In vitro* and *in vivo* assays

An increase in estrogenic activity was noticed with both the YES and ER-CALUX assay after the initial water treatment processes (A-S, B-DAFF, C-F) (Tables 3.2 and 3.3), suggesting that the flocculants contained estrogenic compounds. However, in most instances there was a large reduction in estrogenicity by the following treatment steps,

namely sand filtration (A-SF and C-F) and activated carbon treatment (B-AC), after the initial increase in estrogenicity. The YES results showed that the highest removal of estrogenicity was achieved after chlorination (Table 3.2). 58% of the samples also showed a reduction in estrogenicity in final water in the ER-CALUX assay (Table 3.3). The results suggest that the chlorination process used by all the treatment plants was successful in reducing estrogenic activity. This is contrary to findings by Lenz et al. (2004). In their study on the removal efficiencies of selected oxidative drinking water treatment processes, they found that ozone and chlorine dioxide reduced estrogenicity in final water. Chlorination removed chemicals such as nonyl phenol and bisphenol below detection limits, but estrogenic activities increased possibly as a result of the formation of daughter products exerting greater estrogenic activity than the parent substances (Lenz et al., 2004). The PRTH assay also demonstrated a reduction in estrogenicity in the final water of Treatment Plant C (Table 3.4). This reduction in estrogenic activity was, however, not noticed for Treatment Plant B where relatively high estrogenicity was observed in the source water.

The estrogenic potency of the source water of Treatment Plant B was much higher than that of the other treatment plants. Since the estrogenicity in the final water of all three plants was similar, the reduction in estrogenicity by Treatment Plant B, therefore, appeared to be much higher than in the case of the other plants.

The YES and ER-CALUX assay showed a large increase in estrogenicity in the final water of Treatment Plant C during the 1st sampling survey. VTG was also induced in the liver slice assay. This could be ascribed to a breakthrough of estrogen mimicking chemicals at the treatment plant, or since all three assays were carried out on the same concentrated sample, to contamination during sample preparation.

4.3.2 Chemical analyses

In general, no clear patterns of removal were observed when the chemical data of source and final water were compared. In some instances chemical concentrations were lower in final water than in source water, but in other instances they were higher.

Table 4.7 shows that during the 1st sampling survey the final water of Treatment Plant A showed a 16% reduction in the simazine concentration, but during the 2nd survey there was a 14% increase. During the 2nd and 3rd surveys atrazine concentrations in the final water of Treatment Plant C were approximately 40% higher than in the source water. However, during the 4th survey a reduction of 28% was noticed. During the first three surveys nonyl phenol concentrations were between 21 and 60% higher in the final water of Treatment Plant B. A reduction of 67% was observed during the 4th survey.

Cadmium, cobalt, iron and lead concentrations in the final water of Treatment Plant B were in some instances lower and in other instances higher than in the source water (Table 4.8). On all sampling occasions chromium, copper and zinc concentrations were higher in the final water. Increases in chromium and zinc were as high as 230 and 340%, respectively. Manganese was reduced in all instances. The removal efficiency of the treatment plant with reference to aluminium, arsenic and mercury could not be established, as data were not supplied by the treatment plant. Increases and reductions in zinc concentrations were observed in the final water of Treatment Plant C (Table 4.8). Copper concentrations were the same in source and final water, or higher in final water. Aluminium, iron and manganese were in all instances reduced by the water treatment

processes. No observations with regard to the treatment efficiency of Treatment Plant A were possible because source water data were not available.

TABLE 4.7: Reduction (%) of triazine and nonyl phenol during water treatment (comparing source and final water quality)

Survey	Treatment Plant	Simazine	Atrazine	Terbuthyl-azine	p-nonyl phenol
1	A	16	<LOD/LOQ	10	<LOD/LOQ
	B	<LOD/LOQ		<LOD/LOQ	+32
	C				32
2	A	+14			<LOD/LOQ
	B	5	21		+60
	C	<LOD/LOQ	+40	+20	<LOD/LOQ
3	A		+15	<LOD/LOQ	+21
	B		21		+21
	C		+42		<LOD/LOQ
4	A		<LOD/LOQ		51
	B				67
	C		28		<LOD/LOQ

\+ Increase in concentration
<LOD/LOQ Both source and final water below limit of detection or limit of quantification

TABLE 4.8: Reduction (%) of heavy metals during water treatment (comparing source and final water quality)[1]

Chemical	Treatment Plant B	Treatment Plant C
Aluminium	na	93-99
Arsenic	na	<LOD
Cadmium	15-+40	
Cobalt	71-+80	
Chromium	+28-+230	
Copper	+5-+168	0-+100
Iron	55-+151	94-98
Mercury	na	<LOD
Manganese	16-96	70
Lead	3-+27	<LOD
Zinc	+27-+340	20-+100

[1] APPENDIX A
na Not analysed
\+ Increase in concentrations
<LOD Both source and final water below limit of detection

4.4 Comparing assay responses and chemical data

One of the advantages of using *in vitro* and *in vivo* assays is that no unknown chemical component in water, including metabolites, or combined effects of chemicals is overlooked. In order to explain assay responses, an extensive list of chemical analyses will be required. In this study only a few endocrine disrupting chemicals (estrogens, atrazines, nonyl phenol) that proved to be a problem during previous studies (Burger, 2007) were included for analysis. Data on general determinants, including heavy metals, were supplied by the treatment plants. These data were mostly sketchy, and

analyses were often not carried out on the same dates when samples were collected by the CSIR.

Chemical analyses indicated that the estrogen concentrations in source and final water were <LOD (1.0 ng/ℓ). However, the YES and ER-CALUX assay are highly sensitive to estrogens. It is, therefore, possible that estrogens contributed to the responses observed with the two assays, especially in the case of the source water.

Triazines are endocrine disruptors, but do not bind to the estrogen receptor and would, therefore, not have incurred responses in the assays used in this study. This could explain why the estrogenic activity in samples collected during the 2nd survey was low, while several samples contained detectable levels of the triazines. Nonyl phenol is an estrogen mimic. An EC_{10} (concentration giving 10% of the maximum response) of 88.82 µg/ℓ was reported for nonyl phenol using the YES (van den Belt et al., 2004). This concentration will be about 10-times lower with the ER-CALUX assay. At the 400-times concentration of water used in the present study it is expected that nonyl-phenol played a role in the effects observed in some of the *in vitro* assays (nonyl phenol concentration in final water: 0.24 µg/ℓ x 400 = 96 µg/ℓ).

The endocrine disrupting metals, cadmium and lead, were present >TWQRs (no health effects expected) stated for domestic use (DWAF, 1996) in the source and final water of Treatment Plant B and could have contributed to the responses observed with some of the assays. No information was available on arsenic and mercury.

Organochlorine pesticides and phthalates were occasionally found to be present at concentrations >LOD in the vicinity of the source water of Treatment Plant B (Bornman et al., 2007) (e.g. lindane: 14.04 µg/ℓ; p,p'-DDT: 2.27 µg/ℓ; p,p'-DDD: 1.10 µg/ℓ; DEP: 3.77 mg/ℓ). It is possible that these chemicals contributed to the estrogenic activity observed.

5. CONCLUSIONS AND RECOMMENDATIONS

- The objectives of this study, namely 1) to investigate the occurrence and fate of estrogen mimicking substances in source and treated drinking water using biological/biochemical techniques and chemical analyses; 2) to investigate the removal of estrogen mimicking substances by different drinking water treatment processes; and 3) to make recommendations on the most appropriate combination of tests for the detection of estrogenic activity in drinking water, were met.

- The bioassays clearly showed estrogenic activity in source and treated drinking water. Chemical analysis also indicated that triazine and nonyl phenol residues, and the heavy metal endocrine disruptors, cadmium and lead, were occasionally present above the analytical detection limits (LODs) in some of the source and final waters.

- The results obtained with the yeast estrogen screen (YES) and the estrogen receptor-mediated chemical activated luciferase gene expression (ER-CALUX) assay showed a reduction in/removal of estrogenicity by the water treatment processes. The most significant reductions were observed in the final waters, after chlorination. In general, both assays indicated some increase in estrogenic activity after the addition of flocculants. No clear patterns of removal were observed when chemical data of source and final water were compared. In some instances chemical concentrations were lower in final water than in source water, and in other instances the chemical concentrations were higher.

- The data obtained with the YES and ER-CALUX and primary rainbow trout hepatocyte (PRTH) assays indicated higher estrogenic activity in the water (source and treated water) of Treatment Plant B. The YES and ER-CALUX assay also indicated that the estrogenic activity in the water of Treatment Plant C was generally the lowest. The PRTH assay, on the other hand, showed the lowest estrogenic activity in the water of Treatment Plant A.

- Water quality is a complex issue. As an extensive chemical survey was not carried out on the water samples it is not possible to link estrogenic activity to the presence of specific chemicals. Since nonyl phenol is an estrogen mimicking compound, it is expected that this chemical contributed to some of the estrogenic effects observed. Some of the endocrine disrupting metals present in source and final water, especially in the case of Treatment Plant B, could have played a role in the observed estrogenic effects. Although chemical analyses indicated that estrogens were below the <LOD, the YES and ER-CALUX assay are very sensitive to estrogen. It is, therefore, possible that these chemicals also contributed to the estrogenic effects observed.

- The lower estrogenic potencies of source and treated water during the 2nd sampling survey could have been due to the heavy rains and consequent dilution of estrogens and estrogen mimicking substances during the period preceding sampling. Factors such as high algal loads and the use of different types of flocculants did not appear to have impacted on the water quality.

- The YES and ER-CALUX and PRTH assays proved to be the most suitable for the detection of estrogenicity in drinking waters. Another candidate for the battery of tests for drinking water testing is the liver slice assay. However, the test will have to be adapted to test concentrated samples.

- The *in vivo* tests did not perform well in the study. The zebrafish ELISA did not detect estrogenic activity in any of the water samples. Although the zebrafish ALP assay detected estrogenicity in some samples, results were erratic and several samples reduced VTG. VTG levels were generally very low in the fish homogenates as a result of dilution. Appropriate responses with the positive control 17β-estradiol were also not obtained. Gel electrophoresis, as applied in this study, could also not detect VTG in fish exposed to water samples and the positive control. *In vivo* tests are time consuming and there are many variables that can impose errors in the assays. These tests are, therefore, not recommended for drinking water testing.

- *In vivo* tests, however, have an important role to play in environmental water monitoring. It is, therefore, felt that the zebrafish ELISA should be further explored. The exposure protocols should be standardized (fish handling, fish age, etc) and procedures to process fish for shipment should also be investigated (transporting fish in anti-protease buffer as opposed to placing dry fish in containers). It is also recommended that the VTG concentration should be normalized with a total protein determination as the variation in total protein in individual fish may mask significant differences among groups. Inter-laboratory studies should also be carried out with estrogens to validate the test. A study is also required to compare the sensitivities of the different zebrafish VTG antibodies. At the same time the cost of commercially available antibodies versus sensitivity should be assessed.

- Efforts should be made to establish the PRTH assay locally. This test has been proven to be a valuable part of the battery of tests performed by Environment Canada for the detection of toxicity and estrogenicity.

- The ER-CALUX assay is not available in South Africa. An alternative mammalian cell test is urgently required as part of the battery of bioassays used for drinking water testing. It is expected that the T47D-Kbluc assay, currently being introduced into the University of Pretoria laboratories, will be a suitable alternative to the ER-CALUX assay.

- Detection limits and limits of quantification for the whole YES method are not well established. The role of the concentration factor used in the test in determining limits and levels has also not been well defined. It recommended that data calculation is revisited to eliminate unclarities and to ensure standardisation.

- The sample extracts for the *in vitro* tests were prepared by means of solid phase extraction using XAD-7. A comparison between a limited number of extracts of spiked water samples and environmental samples using XAD-7 and C18 columns for extraction showed the best recovery of chemicals was obtained with XAD-7 concentration (Slabbert et al., 2007a). C18 columns are, however, more generally used to prepared sample extracts for *in vitro* tests. It is recommended

that a more extensive study is carried out on XAD-7 and C18 extracts to allow firm recommendations.

- This study has shown the presence of estrogenic activity in drinking water. In order to establish the cause of these effects, extensive chemical monitoring programmes are recommended. Risk assessment will also be required to provide answers on the risk involved in drinking water exhibiting estrogenicity.

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APPENDIX A

PHYSICAL-CHEMICAL DATA SUPPLIED BY THE STUDIED DRINKING WATER TREATMENT PLANTS

TABLE A.1: Physical-chemical quality of the final (A-C) water of Treatment Plant A during the periods of the 3rd and 4th sampling surveys[1]

Determinand	3rd Sampling survey	4th Sampling survey	
	29/11/05	07/02/06	07/03/06
pH	7.5	8.4	na
Colour*	8.0	1.0	na
Turbidity (NTU)	1.23	0.26	na
Conductivity (mS/m)	61	48	na
Hardness ($CaCO_3$)*	167	na	126
Alkalinity ($CaCO_3$)*	137	50	84
Calcium (Ca)*	35	26	28
Magnesium (Mg)*	18.8	16.3	13.1
Potassium (K)*	15.2	9.2	7.4
Sodium (Na)*	52.3	57.4	40.7
Ammonia (N)*	1.10	0.02	0.04
Nitrite (N)*	0.27	0.10	0.10
Nitrate (N)*	1.18	0.51	1.13
Sulphate (SO_4)*	56	47	37
Orthophosphate (PO_4)*	0.01	0.10	0.10
Chloride (Cl)*	66.0	54.5	55.8
Fluoride (F)*	0.32	0.18	0.35
Aluminium (Al)**	50	na	na
Arsenic (As)**	5.0	na	na
Cadmium (Cd)**	3.0	na	na
Cobalt (Co)**	10	na	na
Total chromium (Cr)**	10	10	10
Copper (Cu)**	10	na	na
Iron (Fe)**	13	10	10
Mercury (Hg)**	1.0	na	na
Manganese (Mn)**	10	10	10
Nickel (Ni)**	10	na	na
Lead (Pb)**	10	na	na
Antimony (Sb)**	5.0	na	na
Selenium (Se)**	5.0	na	na
Vanadium (V)**	50	na	na
Zinc (Zn)**	10	50	10
TOC*	4.52	na	0.45

[1] No data were available for the periods when the 1st and 2nd surveys were carried out. Only the final (A-C) water quality was analysed

* mg/ℓ

** µg/ℓ

na Not analysed

TOC Total organic carbon

TABLE A.2: Physical-chemical quality of the source (B-R) and treated water (B-DAFF, B-AC and B-C) of Treatment Plant B during the periods of the 1st and 2nd sampling surveys

Determinand	1st Sampling survey				2nd Sampling survey			
	B-R	B-DAFF	B-AC	B-C	B-R	B-DAFF	B-AC	B-C
	31/01/05				07/03/05			
pH	na	na	na	7.6	9.0	8.7	8.4	8.2
Colour*	29	17	15	7.0	35	13	11	4.0
Turbidity (NTU)	na	na	na	0.21	4.93	0.31	0.20	0.18
Conductivity (mS/m)	na	na	na	49	52	51	51	48
Hardness ($CaCO_3$)*	na	na	na	146	139	137	138	151
Alkalinity ($CaCO_3$)*	121	108	107	107	132	124	121	117
Calcium (Ca)*	na	na	na	30	34	33	34	38
Magnesium (Mg)*	na	na	na	17.2	12.8	12.9	12.9	13.5
Potassium (K)*	na	na	na	9.5	9.4	9.5	9.4	8.5
Sodium (Na)*	na	na	na	54.6	49.3	49.6	48.8	44.4
Ammonia (N)*	0.55	0.04	0.06	0.04	0.14	0.03	0.04	0.02
Nitrite (N)*	0.06	0.002	0.002	0.001	0.05	0.002	0.01	0.001
Nitrate (N)*	0.33	0.85	0.93	0.84	0.33	0.58	0.74	0.68
Sulphate (SO_4)*	47	46	46	42	49	48	48	43
Phosphate (PO_4)*	0.41	0.05	0.05	0.05	0.22	0.20	0.22	0.19
Chloride (Cl)*	na	na	na	57.1	55.4	56.1	56.2	53.4
Fluoride (F)*	na	na	na	na	0.24	na	na	0.23
Cadmium (Cd)**	na	na	na	2.0	na	na	na	na
Cobalt (Co)**	na	na	na	na	9.1	13	18	14
Total chromium (Cr)**	na	na	na	na	9.4	6.7	1.8	12
Copper (Cu)**	na	na	na	17	4.0	3.0	3.6	4.2
Iron (Fe)**	na	na	na	66	191	74	58	87
Manganese (Mn)**	na	na	na	6.3	222	11	7.7	8.0
Nickel (Ni)**	na	na	na	23	22	20	9.1	12
Lead (Pb)**	na	na	na	20	20	11	16	20
Zinc (Zn)**	na	na	na	47	5.0	1.0	2.0	22
TOC*	na	na	na	6.21	na	na	na	5.05

* mg/ℓ
** µg/ℓ
na Not analysed
TOC Total organic carbon

TABLE A.3: Physical-chemical quality of the source (B-R) and treated water (B-DAFF, B-AC and B-C) of Treatment Plant B during the periods of the 3rd and 4th sampling surveys

Determinand	3rd Sampling survey				4th Sampling survey			
	B-R	B-DAFF	B-AC	B-C	B-R	B-DAFF	B-AC	B-C
	10/10/05				04/01/06			
pH	8.2	7.6	7.5	7.4	9.6	9.5	9.4	9.2
Colour*	23	12	10	6.0	34	12	10	5.0
Turbidity (NTU)	1.97	0.24	0.24	0.27	10.30	0.32	0.27	0.24
Conductivity (mS/m)	60	59	59	55	57	57	56	54
Hardness ($CaCO_3$)*	142	142	139	136	141	138	138	136
Alkalinity ($CaCO_3$)*	143	126	125	129	54	54	54	54
Calcium (Ca)*	35	35	34	34	35	34	34	33
Magnesium (Mg)*	13.5	13.4	13.0	12.7	13.2	13.0	13.1	13.0
Potassium (K)*	12.8	12.2	12.0	10.3	13.0	12.7	13.1	11.5
Sodium (Na)*	56.6	55.2	55.9	37.0	60.1	59.9	59.3	54.9
Ammonia (N)*	1.90	0.33	0.10	0.02	0.28	0.01	0.01	0.01
Nitrite (N)*	0.17	0.21	0.04	0.001	0.15	0.004	0.002	0.002
Nitrate (N)*	1.71	3.54	3.82	3.45	0.43	0.91	0.94	0.89
Sulphate (SO_4)*	64	64	63	55	58	55	55	51
Phosphate (PO_4)*	0.17	0.17	0.18	0.15	0.18	0.17	0.16	0.15
Chloride (Cl)*	70.0	69.5	69.8	66.9	141	138	138	136
Fluoride (F)*	na	na	na	na	0.24	0.27	0.23	0.20
Cadmium (Cd)**	1.9	1.8	1.0	1.6	6.7	8.5	9.2	9.4
Cobalt (Co)**	18	7.3	11	5.3	6.1	9.8	7.3	11
Total chromium (Cr)**	11	13	31	15	9.1	18	6.8	30
Copper (Cu)**	4.1	4.9	8.6	11	11	8.3	13	12
Iron (Fe)**	80	63	44	201	205	85	114	102
Manganese (Mn)**	1.9	1.8	1.0	1.6	74	9.8	8.5	9.0
Nickel (Ni)**	na	na	na	na	na	na	na	na
Lead (Pb)**	30	28	29	29	19	36	23	24
Zinc (Zn)**	15	14	14	39	11	7.0	10	14
TOC*	na	na	na	na	na	na	na	na

* mg/ℓ
** µg/ℓ
na Not analysed
TOC Total organic carbon

TABLE A.4: Physical-chemical quality of fountain water blended with the final (B-C) water of Treatment Plant B during the periods when the four sampling surveys were carried out

Determinand	Sampling survey			
	1st	2nd	3rd	4th
	31/01/05	07/03/05	10/10/05	04/01/06
pH	na	8.2	8.1	8.1
Colour*	1.0	1.0	1.0	2.0
Turbidity (NTU)	na	0.04	0.1	0.03
Conductivity (mS/m)	na	23	23	23
Hardness ($CaCO_3$)*	na	140	111	120
Alkalinity ($CaCO_3$)*	120	124	118	58
Calcium (Ca)*	na	32	23	25
Magnesium (Mg)*	na	15	13	14
Potassium (K)*	na	0.6	0.8	0.7
Sodium (Na)*	na	2.5	2.5	2.7
Ammonia (N)*	na	0.02	0.09	0.03
Nitrite (N)*	0.001	0.001	0.003	0.002
Nitrate (N)*	0.38	0.71	0.51	0.43
Sulphate (SO_4)*	2.0	2.4	11	1.3
Phosphate (PO_4)*	0.01	0	0.02	0.01
Chloride (Cl)*	na	4.3	1.9	120
Fluoride (F)*	na	0.08	na	0.09
Cadmium (Cd)**	na	6.0	1.1	9.6
Cobalt (Co)**	na	13	5.8	4.0
Total chromium (Cr)**	na	7.2	4.3	24
Copper (Cu)**	na	2.4	3.6	9.3
Iron (Fe)**	na	62	34	88
Manganese (Mn)**	na	6.5	1.1	3.6
Nickel (Ni)**	na	2.1	na	na
Lead (Pb)**	na	21	15	23
Zinc (Zn)**	na	1.0	11	10
TOC*	na	na	na	na

* mg/ℓ
** µg/ℓ
na Not analysed
TOC Total organic carbon

TABLE A.5: Physical-chemical quality of the source (C-R) and final (C-C) water of Treatment Plant C during the periods of the 1st and 2nd sampling surveys

Determinand	1st Sampling survey				2nd Sampling survey	
	C-R		C-C		C-R	C-C
	03/01/05	31/01/05	24/01/05	31/01/05	28/02/05	
pH	7.9	7.5	8.1	7.8	7.9	8.3
Colour*	96	37	6.0	<5.0	45	5.0
Turbidity (NTU)	145	130	0.23	0.17	120	0.25
Conductivity (mS/m)	17	17	17	18	18	20
Hardness ($CaCO_3$)*	54	63	62	64	65	74
Alkalinity ($CaCO_3$)*	73	73	61	70	80	70
Calcium (Ca)*	12	14	20	22	14	26
Magnesium (Mg)*	5.7	6.8	3.2	2.4	7.2	2.3
Potassium (K)*	1.6	1.5	1.7	1.4	1.8	1.8
Bromium (Br)*	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Sodium (Na)*	6.1	6.6	7.2	6.9	6.9	7.5
Silica (Si)*	6.7	5.8	5.1	5.1	6.8	5.3
Ammonia (N)*	<0.05	0.30	0.08	0.07	0.06	0.10
Nitrite (N)*	0.13	0.05	<0.03	0.19	0.07	0.09
Nitrate (N)*	<0.10	<0.10	0.20	<0.10	0.21	0.19
Sulphate (SO_4)*	14	15	14	14	20	20
Phosphate (PO_4)*	<0.05	0.05	<0.05	<0.05	<0.05	<0.05
Phosphor (P)*	<0.04	<0.04	0.13	<0.04	0.10	0.07
Chloride (Cl)*	<5.0	5.3	8.0	7.7	6.2	9.0
Fluoride (F)*	0.16	0.23	0.18	0.22	0.27	0.28
Sulphur (S)*	3.4	4.0	3.8	4.0	4.5	4.5
Aluminium (Al)**	850	290	<10	20	840	<10
Arsenic (As)**	na	<5.0	<5.0	<5.0	<5.0	<5.0
Boron (B)**	20	20	10	10	10	<10
Cadmium (Cd)**	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
Cobalt (Co)**	<15	<15	<15	<15	<15	<15
Total chromium (Cr)**	<10	<10	<10	<10	<10	<10
Copper (Cu)**	<10	10	<10	20	<10	<10
Iron (Fe)**	480	320	30	20	570	10
Mercury (Hg)**	na	<1.0	<1.0	<1.0	<1.0	<1.0
Manganese (Mn)**	<3.0	10	<3.0	<3.0	10	<3.0
Molibdenum (Mo)**	<10	<10	<10	<10	<10	<10
Nickel (Ni)**	<15	<15	<15	<15	<15	<15
Lead (Pb)**	<10	<10	<10	<10	<10	<10
Antimony (Sb)**	na	<5.0	<5.0	<5.0	<5.0	<5.0
Selenium (Se)**	na	<5.0	<5.0	<5.0	<5.0	<5.0
Vanadium (V)**	<30	<30	<30	<30	<30	<30
Zinc (Zn)**	10	10	<8.0	20	10	<8.0
Cyanide (CN)*	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
DOC*	na	na	na	na	na	na

* mg/ℓ
** µg/ℓ
na Not analysed
DOC Dissolved organic carbon

TABLE A.6: Physical-chemical quality of the source (C-R) and final (C-C) water of Treatment Plant C during the periods of the 3rd and 4th sampling surveys

Determinand	3st Sampling survey				4th Sampling survey		
	C-R		C-C		C-R	C-C	
	03/10/05	31/10/05	10/10/05	17/10/10	30/01/06	09/01/06	16/01/06
pH	8.2	7.9	8.4	8.3	7.5	7.6	7.4
Colour*	112	13	<5.0	6.0	96	<5.0	<5.0
Turbidity (NTU)	74	70	0.33	0.22	150	0.14	0.27
Conductivity (mS/m)	18	17	24	23	15	21	17
Hardness ($CaCO_3$)*	64	70	87	81	49	70	65
Alkalinity ($CaCO_3$)*	63	61	83	79	47	69	65
Calcium (Ca)*	14	16	28	25	11	24	20
Magnesium (Mg)*	7.2	7.2	4.2	4.3	5.5	2.3	3.5
Potassium (K)*	2.6	2.6	2.3	2.2	2.4	2.2	2.2
Bromium (Br)*	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Sodium (Na)*	8.2	9.4	8.9	7.4	7.4	8.3	8.4
Silica (Si)*	10	8.4	6.2	6.0	5.1	4.7	4.8
Ammonia (N)*	<0.05	0.11	<0.05	<0.05	<0.05	<0.05	<0.05
Nitrite (N)*	0.05	0.11	<0.03	0.12	0.14	0.11	0.05
Nitrate (N)*	0.18	0.13	0.16	0.20	0.66	0.23	0.12
Sulphate (SO_4)*	15	14	13	13	13	13	14
Phosphate (PO_4)*	<0.05	0.19	<0.05	<0.05	<0.05	<0.05	<0.05
Phosphor (P)*	0.29	0.27	0.10	0.13	0.09	0.13	0.04
Chloride (Cl)*	6.2	8.0	9.3	8.8	6.5	9.0	9.6
Fluoride (F)*	0.26	0.20	0.19	0.18	0.21	0.17	0.18
Sulphur (S)*	4.4	4.4	4.1	3.9	4.3	3.9	4.0
Aluminium (Al)**	5 000	1 100	<10	10	190	<10	<10
Arsenic (As)**	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
Boron (B)**	20	20	10	<10	90	10	<10
Cadmium (Cd)**	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
Cobalt (Co)**	<15	<15	<15	<15	<15	<15	<15
Total chromium (Cr)**	<10	<10	<10	<10	<10	<10	<10
Copper (Cu)**	20	10	<10	<10	20	20	10
Iron (Fe)**	2 900	870	20	40	130	30	10
Mercury (Hg)**	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Manganese (Mn)**	10	10	<3.0	<3.0	10	10	10
Molibdenum (Mo)**	<10	<10	<10	<10	<10	<10	<10
Nickel (Ni)**	<15	<15	<15	<15	<15	<15	<15
Lead (Pb)**	<10	<10	<10	<10	<10	<10	<10
Antimony (Sb)**	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
Selenium (Se)**	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
Vanadium (V)**	<30	<30	<30	<30	<30	<30	<30
Zinc (Zn)**	30	10	<8.0	<8.0	<8.0	<8.0	10
Cyanide (CN)*	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
DOC*	na	na	6.8	na	na	4.3	na

* mg/ℓ
** µg/ℓ
na Not analysed
DOC Dissolved organic carbon