WRC PROGRAMME ON ENDOCRINE DISRUPTING COMPOUNDS (EDCs) VOLUME 1 STRATEGIC RESEARCH PLAN FOR ENDOCRINE DISRUPTERS IN SOUTH AFRICAN WATER SYSTEMS

AEC Burger

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Water Research Commission

WRC PROGRAMME ON ENDOCRINE DISRUPTING COMPOUNDS (EDCs)

VOLUME 1

STRATEGIC RESEARCH PLAN FOR ENDOCRINE DISRUPTERS IN SOUTH AFRICAN WATER SYSTEMS

COMPILED FOR THE

WATER RESEARCH COMMISSION

BY

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Report on consultancy K8/447

Status of Endocrine Disrupter Chemical (EDC) information in South Africa and to assess the capacity and capability of laboratories to generate data on chemical and biological analysis of these substances

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FOREWORD

Research into EDC (Endocrine Disrupting Compounds / Chemicals) has become of great importance worldwide. Although on a smaller scale, EDC research in South Africa has increased It was imperative that South Africa become involved in this field, because the possible adverse effect of EDCs in water could impact on wildlife and human populations. The EDC Research Programme of the Water Research Commission was launched in order to coordinate and extend current capacity to conduct EDC research and monitoring currently being undertaken by several groups of researchers in the country.

This was the first effort to conduct an integrated research programme involving Universities, Technikons, the water treatment sector, government departments and parastatal research institutions.

The Research Programme on EDCs will be presented in 4 parts:

Volume 1 : Strategic Planning

The development of a strategic research plan to determine occurrence of EDCs in South African water systems. Report Number KV 143 /05

Volume 2 : Implementation Phase I and Phase II (2002-2005)

Consolidated report of research consultancies and projects which saw the implementation of a limited survey on the occurrence of selected EDCs in environmental water and effluent from water treatment systems

Report Number 1469/1/05 (Publication expected December 2005)

Volume 3: Strategic Planning (2006-2010)

Consultancy to be awarded to extend the strategic plan into a five-year plan for future research actions on EDCs in South Africa including remedial actions to limit the risk to ecosystem health and the human population

Volume 4: Implementation Phase III (2006-2010)

Research Projects to be awarded to implement the management plan for extended research and remedial actions.

EXECUTIVE SUMMARY

Endocrine Disrupting Chemicals (EDCs) are defined as chemicals that interfere with the structure or function of hormone-receptor complexes. They can cause endocrine disruptive effects at exposure levels up to a million times lower than carcinogen exposure levels of concern. Internationally, the negative impact of EDCs on health is evident and no longer an issue of dispute. Examples include the increase of testicular and prostatic cancer, the higher incidence of undescended testis and hypospadia, the decline in male reproductive health and fertility, and the very likely causal effect on cognitive and immunological development of children.

The presence of EDCs in South African water systems may have an impact on the health of the South African population and wild life. Limited research had been done on possible contamination by EDCs in the country. These projects were mainly small studies undertaken at Universities and the CSIR. A need existed for a coordinated program involving all researchers and other role players such as government departments, industry and water suppliers as EDC research requires a multidisciplinary approach involving research in disciplines such as zoology, physiology, toxicology and analytical chemistry.

The main objective was to develop a strategic plan to conduct research on EDCs in the country in order to determine the occurrence of EDCs in water and endeavor to determine the possible risk to the human population and wild life. In order to conduct risk assessment reliable data is needed on occurrence, magnitude and frequency of pollution. Very little data existed in the country on contamination of EDCs in water systems. Most of the existing data was collected with toxicity in mind and could not be used because the detection limits in the studies were too high. EDC effects can occur at levels of million times lower than the toxicity effect. Certain EDCs, such as synthetic hormones and phthalates are not regarded as toxicants and no data existed on the occurrence and levels of these substances in water systems.

During a workshop held in Pretoria in 1999 a need was expressed by the stakeholders for reliable and relevant data on the levels of EDCs in South African water systems. This project was proposed in order to lay the foundation for a programme to investigate the occurrence, magnitude and effect of Endocrine Disrupter Chemicals (EDCs) in South African water systems so that it may be determined to what extent the population and wild life is at risk by being exposed to these chemicals.

In the development of the strategic research plan (Volume 1), a survey was conducted on the state of the science in South Africa and existing data was evaluated. A list of priority compounds to be investigated was compiled, analytical methods for determining EDC activity and levels of individual EDC components were evaluated and selected. A survey of laboratory capability and capacity was conducted.

Research projects were identified and recommendations on a protocol for a research programme were suggested to run over three years.

During the initial stages of the planning of this programme the WRC developed the first project on EDCs for the GWRC (Global Water Research coalition). This involvement was extended through the implementation phases of the programme into participation of South African researchers in the GWRC EDC research programme .

ACKNOWLEDGEMENTS

The program was assisted internationally by the Global Water Research Coalition (GWRC) and its members (Australia, Belgium, Germany, the Netherlands, the USA) in addition to consultants from Japan and Norway. Their input, in particular their contribution to the methodology of detecting EDC activity by biological means. is much appreciated.

The author would also like to express her thanks to all participating researchers and department heads for their valuable time and worthwhile input. Please refer to the list in the Appendix

Special thanks to Prof C (Tiaan) de Jager for providing the background to this report.

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1. BACKGROUND

Endocrine Disrupting Chemicals (EDCs) are defined as chemicals that interfere with the structure or function of hormone-receptor complexes. They can cause endocrine disruptive effects at exposure levels up to a million times lower than carcinogen exposure levels of concern. Internationally, the negative impact of EDCs on health is evident and no longer an issue of dispute. (COMPREDO CREDO workshop, 2004) Examples include the increase of testicular and prostatic cancer (Toppari *et al.,* 1996; Bergstrom *et al.,* 1996; Møller,1998) the higher incidence of undescended testis and hypospadia (Møller, 1999; Skakeback 2001) the decline in male reproductive health and fertility, and the very likely causal effect on cognitive and immunological development of children (Tilson, 1998). Female reproductive health and fertility, particularly conditions like endometriosis and adenomyosis of the breast and reproductive tract cancer and possibly polycystic ovarian syndrome, seem to be mediated by EDCs (Gerhard *et al.,* 1992). Fetal exposure to EDCs was found to influence reproductive and general health. It also seems that environmental EDCs probably contribute to declines in some wildlife populations (Guilette *et al.*, 1996; Johnstone *et al.,* 1996; Vos *et al.,* 2000).

Endocrine disrupting (ED) effects are not restricted to a small group of therapeutic agents such as oral contraceptives or hormone replacements. On the contrary, many compounds that are in daily use in industry, agriculture and in households have ED effects. These include the alkylphenols, polychlorinated biphenyls, dioxins and furans, and organochlorine compounds, which are used in different forms as plasticizers, lubricants, packaging material, pesticides and insecticides. Dichloro-diphenyl trichloro-ethane (DDT) and other organochlorine pesticides are well known for their ED effects. The limited water sources of South Africa, the limited health budget, the likelihood of significant pollution by industry, the lack of proper waste control, the need to use DDT for malaria control emphasize the need for timely measures to be taken by the authorities.

Persistent organic pollutants (POPs), a subgroup of EDCs, are hazardous chemical substances that do not break down naturally, or do so extremely slowly. They accumulate in fatty tissue, becoming more concentrated higher up in the food chain over time, thereby putting the environment and human health at risk. Of all pollutants released into the environment every year by human activity, POPs are amongst the most harmful. They are highly toxic, causing an array of adverse effects, notably death, disease and birth defects, among humans and animals. Specific effects can include cancer, allergies and hypersensitivity, damage to the central and peripheral nervous systems, reproductive disorders and disruption of the immune system. They present a special risk to children because they are conveyed through the placenta and in breast milk, and can have a critical effect on the fetus and infant whose systems are at key stages of development (WHO report 2002).

The long lasting POPs travel in multiple cycles of evaporation and condensation and are transported by air to remote areas far from the source of their release, necessitating international regulation. The international POPs convention, signed in May 2001 in Stockholm, defined measures to reduce global concentration levels of the twelve identified POPs.

These POPs have been grouped in three categories:

1. Pesticides: aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex and toxaphene.
2. Industrial chemicals: hexachlorobenzene (HCB) and polychlorinated biphenyls (PCBs).
3. Unintended byproducts: dioxins and furans.

The EDCs are an even larger group of chemicals. Apart from the POPs also included in this group are:

1. Alkyl phenols and their ethoxylate: *p*-Nonylphenol (NP), *p*-Octylphenol (OP), *p*- Nonylphenol ethoxylate (NPn2EO) and *p*-*O*ctyl phenol ethoxylate (OPnEO).

2. Phthalate: butyl benzyl phthalate (BBP), di-n-butyl phthalate (DBP) and di(2-ethyl hexyl) phthalate (DEHP)
3. Plasticizers: Bisphenol A (BA)
4. Herbicides: Atrazine, Simazine, 2,4-D
5. Fungicides: Vinclozolin
6. Organophosphate pesticides: Azinphos-methyl, Parathion
7. Pharmaceuticals: Diethylstilbestrol (DES), tamoxifen and raloxifen
8. Certain heavy metals: Cd, As, Pb and Hg
9. Flame retardants: Polybromobiphenyl ethers
10. Natural and Synthetic hormones: 17β-Estradiol, Ethinyl-estradiol, estrone, estriol
11. Other industrial chemicals: Benzene, Styrene

Not all of them remain sufficiently stable to travel across international borders and to be classified as POPs. Clearly, the less stable ones are specifically harmful in the area of their release where the exposure levels are high. These, therefore, require additional local monitoring and regulations, as these are not covered by the international convention.

Public fear of EDCs can be likened to that of radioactivity and disease causing bacteria and viruses - they are not readily detectable and introduce an additional risk of unknown magnitude that government has to protect them against. On the other hand, industry is also involved and proper controls will undoubtedly be at a cost. EDC are, therefore, a politically sensitive issue. Even *perceived* threats to health and environment can be extremely precarious to the survival of the South African economy. It may be easily fall prey to manipulated or spontaneous international actions to ban export products from SA due to high levels of one or the other of the identified pollutants. The only defense against such threats is an improved knowledge of the state of our environment, by using the information to ensure that our present and future legislation is strictly enforced as far as banning - or controlled use - of certain chemicals and clean industrial production is concerned.

For this process to come into effect it is needed to establish facilities to monitor both the target chemicals and the endocrine disrupting effect.

At the first workshop held on EDCs in Pretoria during 1999, the need to conduct research on EDCs was expressed. The development of a strategic research plan was initiated as a result of this workshop. (WRC consultancy K8/447**:** *Status of Endocrine Disrupter Chemical (EDC) information in South Africa and to assess the capacity and capability of laboratories to generate data on chemical and biological analysis on these substances)*

The programme was initially developed in 4 phases:

1. Development of a strategic research plan
2. Implementation of survey and interpretation of results as well as recommendations for future research.
3. Risk assessment and remedial actions
4. Policy development for EDC pollution management.

2. AIMS AND OBJECTIVES

The aims of the EDC research programme were primarily driven by the needs of the stakeholders. All stakeholders identified the need for reliable and relevant data as well as data needed to conduct risk assessment models.

AIM OF THE FIRST PHASE:

1. To develop a strategic research plan to determine the occurrence of EDCs in South African water systems in order to determine the associated human, animal and environmental health risk in South Africa.

In order to meet the aim certain actions was needed:

1. To conduct a survey on the state of the science on EDC research in South Africa;
2. To evaluate existing data on EDC occurrence in South Africa;
3. To compile a list of EDCs relevant to South Africa that will be used as a starting point for a future monitoring programme;
4. To compile an inventory of laboratory capacity and capability in order to establish whether South Africa has the infrastructure to conduct such a programme;
5. To establish the needs of the stakeholders in the field;
6. To identify the knowledge and research gaps to satisfy the needs of the stakeholders;
7. To compile a protocol for a limited survey of selected EDCs at identified sites.

3. METHODOLOGY

3.1 Status of Research done on EDCs in South Africa

Research on Endocrine Disrupter Chemicals (EDCs) in aquatic systems in South Africa requires a multidisciplinary approach including disciplines such as chemistry, biochemistry, zoology, physiology and endocrinology. In the past research was mainly conducted by Universities and was aimed at the following:

3.1.1 Research done around biochemical and biological means of determining EDC activity in aquatic systems by biochemical and biological means: Research is focused on the total estrogenic load in water, aquatic animals and food (*In vitro* tests).

3.1.2 Research done on chemical compounds which cause EDC activity in water,environment and food by chemical analysis.

3.1.3 Research was also conducted on the determination of the estrogenic potency of the individual chemicals on aquatic life, animals and possibly humans (*In vivo* tests.)

3.1.4 Research done on the effect of endocrine disruptors on human health and the effect on wild life, with special emphasis on the reproductive system of male and females.

In order to embark on a study of the occurrence and levels of EDCs in South African water systems it was necessary to determine

a. The chemicals that needed to be studied
b. The methods by which not only these chemicals, but also EDC activity may be measured.
c. Which laboratories in the country have the capability and capacity to analyze water sediment and biological tissue for these chemicals and EDC activity.

3.2 Evaluation of existing data

3.2.1 Methodology

Data concerning chemical analysis of EDCs was collected mainly by studying publications from various researchers at Universities, publications generated by

the WRC and the Dept. of Water Affairs and from commercial laboratories generating data for monitoring and research purposes. Only data generated since 1996 was taken in consideration, because the issue of EDC contamination only became apparent in 1994 and the first studies on EDCs were conducted in South Africa in 1996.

The following Universities, Organizations and laboratories contributed to the data collection:

Water Research Commission
Department of Water Affairs
University of Pretoria
University of Stellenbosch
Northwest University
University of Fort Hare
University of Venda
CSIR Biochem/tek
SABS
Umgeni Water

3.2.2 Results

Data was generated on several of South African dams and rivers, but the aims of the studies were not the determination of EDCs. In most cases the necessary detection limits were not reached and the technology and methodology used were not suitable for the purpose of determining contaminants at extremely low levels. No data could be obtained on studies involving the EDC effect of pharmaceuticals, phytoestrogens or estrogenic mycotoxins in South Africa. It was necessary to test the credibility of the data produced according to the following criteria:

The relevancy of available data produced to EDC.
The methodology used to produce the data
The conditions under which the samples were taken and stored
The solubility of the compounds in water
The detection limits reported for the specific analysis

The relevancy of data produced about EDC effects:

Although a lot of data is available on the occurrence of contaminants such as pesticides, heavy metals, PCBs and PAHs, the data was generated for "water safety" purposes. The values obtained were measured against the ADI (Allowable Daily Intake) and ERL (Environmental Risk Level) levels. Most of these levels are set for possible carcinogenic effects and not for estrogenic effect. The estrogenic effect of some of these compounds may be up to a million times lower than the carcinogenic effect. The data produced may thus contain a number of false negative values, because the detection limits used by the various laboratories were too high.

The methodology used to generate the data:

When a sample is tested for trace levels of contaminants, it is subjected to concentration and clean up procedures. Great care must be taken that the contaminant is not lost by absorption, or be contaminated by impure solvents or contaminated glassware. This is even more crucial for EDCs since contamination can occur very easily. Analytical methods should be validated for repeatability, robustness, selectivity and sensitivity. Instruments should be regularly validated for drift and contamination. Positive results should be verified by mass spectrometer in order to avoid the reporting of false positives. Only results produced under these conditions can be credible. Very few laboratories in South Africa are accredited to conduct analysis according to these standards.

Sampling and storage conditions:

Sampling should be done by professional samplers under strict guidelines. Some of the pesticides may absorb on plastic containers and will not be detected. Plastic containers may posses EDC activity because of the chemical composition of the plastic and may thus lead to a false positive result. Samples should be taken in clean glass containers, transported to the various laboratories under cool dark conditions and analyzed as soon as possible after arrival. Most of the EDCs are persistent in the environment and stable for a considerable time period, but great care

should be taken that contamination and breakdown do not take place, which may lead to either false negatives or false positives. Samples taken for heavy metal analysis should be slightly acidified.

Solubility in water:

Some of the values reported exceeded the solubility of the specific compound in water. This may have been due to calculation errors. This data was discarded.

Detection limits used for specific analysis:

As stated above the data was not generated to meet the low levels of EDC contamination. Therefore the detection limits reported for various methods were too high. This may lead to reporting of false negatives.

3.2.3 Conclusion:

Very little of the data obtained from the various sources could be used for EDC evaluation. This may explain the reason why estrogenicity could be detected by biological means, but no chemical contamination could be indicated. It was also indicated in literature that chemical contamination has an additive effect and that the total load of the contaminants may have produced the EDC effect. Two studies conducted in water and sediment of South African rivers and dams (Meintjies *et al.,* 2000) produced good data, but at the time when these studies were done the low effect levels of the various compounds were not yet known and the detection limits used by the laboratories conducting the chemical analysis were too high.

It is of the greatest importance that biological and chemical analysis be conducted on the same sample. The sensitivity of both biological monitoring and chemical analysis (detection levels and methodology) should be of such a nature to meet the needs of the study. It is also important that the extraction procedure used for biological assay do not remove the chemical compounds, which may cause estrogenic effects.

Because so little data could be used no conclusion could be made of the status of EDC contamination of South African rivers and dams.

3.3 Selection of relevant EDCs for South Africa

Taking the detrimental influence of EDCs on human and animal health into account, it is important for a country such as South Africa to determine the occurrence and levels of EDCs as well as other toxicants which do not have Endocrine disruptive (ED) effects in drinking water in order to take the necessary steps to protect its population. It must also be remembered that a large portion of the population does not have access to purified water and still relies on surface water for drinking water purposes. Any study on EDCs in water should therefore also include the occurrence and levels of EDCs in surface water.

It is estimated that more the 4 000 chemicals might have EDC properties and the list is expanding daily. The majority of these compounds are also regarded as toxicants. The cost of monitoring all these compounds individually would be enormous.

EDCs are defined as any chemical that may have a detrimental effect on the endocrine system. This includes the reproductive system, the neurological system, the thyroid function and the immune system (Weber, RF.*et al.,* 2000; Krsteyska-Konstantinova, M., *et al.,* 2001; Ferguson, SA., et al., 2000; Aoki, Y., *et al*., 2001) The effect of the contamination during the first weeks of pregnancy on the foetus is irreversible (Tilson,1998). It is not only the potential human health aspects of EDCs and toxicants which are of concern to the country but also the fact that EDC and toxicant contamination may limit the export of produce from South Africa. This is especially true of the POPs contamination. It is therefore important when compiling a list of EDCs and toxicants to be monitored that the lists and priorities of South Africa's main trading partners also be taken into account. Information was thus gathered from the following countries:

United Kingdom (UK)
USA (EPA)
Germany
France

Australia
The Netherlands
Japan
UNEP – (United Nations Environmental Program)

Although Germany, the Netherlands and France are all part of the European Union (EU) different compounds are monitored in these countries.

3.3.1 Chemicals considered

It was decided to collect data on the occurrence in water and sediment of the following substances:

a. Organochlorine pesticides (DDE, DDT, DDD, alpha BHC, gamma BHC, beta BHC, Endosulphan, Dieldrin, Toxaphene Heptachlor)
b. Organophosphates (Azinphos methyl and Chlorpirifos)
c. Herbicides (Atrazine, Simazine, Terbutylazine, 2'4-D and Metoxychlor)
d. Pyrethroids (Deltamethrin)
e. Fungicides (Vinclozolin)
f. Polychlorinated Biphenyls (PCB) (1254 and 1260)
g. Alkylphenols (*p*- Nonyl phenol and *p*-Octylphenol and their ethoxylates)
h. Phthalates (DEHP, DPB)
i. Plasticizers (Bisphenol A)
j. Heavy metals (Cadmium, Mercury, Arsenic and Lead)
k. Metals (Zinc and Calcium)
l. Phytoestrogens (genistein and daidzin which are isoflavones and coumosterol)
m. Estrogenic mycotoxins (Zearalenone and deoxynevalenol(DON))
n. Pharmaceutical substances
o. Commercial antioxidants: butylated hydroxyanisole (BHA)
p. Natural and synthetic hormones (17β-Estradiol, Estriol, Estrone, 17α-Ethinylestradiol)
q. Flame retardants: Polybromo diphenyl ethers (PBDEs)

Organochlorine pesticides (OCs) are still widely used in South Africa especially in areas where malaria still prevails. DDT, DDE and DDD are detected in the rivers running through the eastern parts of the country as well as in the Western Cape and the Free State. Most of the OCs is also listed as POPs. They have long life times and may persist in the environment for up to 50 years. The OCs are lipophilic and tend to accumulate in the fatty tissue of fish, animals and humans. It is therefore not the amount to which a mother is exposed during pregnancy that is critical but rather her lifetime exposure and total body burden that will determine the level of exposure of the fetus and the breast-fed infant. These compounds have been listed by several authors as EDCs (Toppari *et al.,* 1996; Facemire *et al.,* 1995).

Organophosphate Pesticides (OPs) are utilized as Insecticides on a large variety of crops. In contrast to the OCs, they have short half-lives in the environment. Studies done by Dr. Reinecke of Stellenbosch University indicated that these chemicals may have a devastating influence on the environment. These compounds are rarely detected in water and environmental samples and only 2 have been selected for further study: Azinphos methyl and Chlorpirifos. Stellenbosch University in collaboration with the University Braunschweig, Germany, is currently conducting a study on the influence of these chemicals on the environment in the Western Cape (Schultz *et al.,* 2001*)*.

Herbicides

- Atrazine
- Simazine
- Propazine
- 2,4-D

Atrazine is widely used in South Africa as a herbicide especially in the maize producing areas. Simazine, Terbutylazine and Propazine, members of the Triazine family of herbicides, are also used in considerable quantities in the country. The Triazine family of herbicides is

relatively persistent to biotic breakdown (Solomon *et al*., 1996; Kahn *et al,.*1976). Both Atrazine and Simazine as well as 2,4-D are reported to be estrogenic (Short *et al.,*1999; Sanderson, 2001). Glyphosate have been used to control the water Hyacinth infestation in several dams in the country (Findley *et al.,* 1996*).* It was also proposed as herbicide in young pine plantations. (Zwolinski *et al.,*1996*.)*. Up to date no confirmation could be obtained on the EDC effect of Glyphosate, although the formulation "Roundup" has EDC effects (Walsh *et al.*, 2000). Certain herbicides are water-soluble and although they readily broken down by soil bacteria, they may leach into rivers and dams. Both Atrazine and 2,4-D are endocrine disruptors (Short and Colborn,1999)

Pyrethroids: Deltametrin (DM) has been used extensively during the past years for mosquito control in Malaria areas. It is reported to have weak estrogenicity.

Pyrethroids are xenoestrogens, which can increase the estrogenic load in the body (Garey *et al.*, 1998) and increase the levels of estrogen in breast cancer cells (Chen *et al.*, 2002). DM exposure studies showed decreased semen quality and impaired reproductive function in laboratory animals at 1.0 and 2.0 mg/kg (El-Aziz *et al.*, 1994), as well as increased frequencies of chromosome abnormalities in mice (Bhunya and Pati, 1990). While a number of international studies have been performed to assess the potential biophysical and human health impacts of synthetic pyrethroids, no studies have been performed in South Africa. Because of the different climate conditions in South Africa, neither the rate of breakdown, nor the impact on the environment of the insecticides is known (Tren, 2000). Therefore data on exposure levels in South Africa is not available, but the Acceptable Daily Intake of DM allowed by the WHO is 0.01 mg/kg (FAO/WHO, 1992). Evidence has shown that synthetic pyrethroids have an effect on the neuro-transmitters of animals and there is potential for synergism between DDT and pyrethroids. Synergism has been found between DDT and synthetic pyrethroids. However the clinical significance of this synergism has not been studied (Tren, 2000).

This is particularly important since DDT is still being sprayed in selected areas and substitution of DDT with DM in some malaria infected areas, where background levels of DDT already exist in the environment, will result in concomitant exposure of humans. A baby fed entirely by breast milk in South Africa exceeds the allowable daily intake for DDT (0.02 mg/kg, FAO/WHO 1985), by 5 to 18 times (Curtis, 1994). Technical-grade DDT (used as an indoor spray) is a mixture of three isomers, of which the *p,p'*-DDT isomer has estrogenic actions and the *o,p'*-isomer can inhibit endogenous ligand binding to the estrogen and progesterone receptors (EPA report, 1997; Toppari *et al.*, 1996). Anti-androgenic (demasculizing) and estrogenic (feminizing) effects can exhibit themselves in the same way, but through different receptors. The discovery therefore that p,p'-DDE (dichlorodiphenylchloroethane) is a anti-androgen may explain some of the estrogenic effects observed in the environment – it may be due to the anti-androgenic effects of xenoestrogens (Gray, 1999; Toppari *et al.*, 1996).

Fungicides: Vinclozolin is a dicarboximide fungicide with Androgen Receptor (AR) antagonism. Vinclozolin metabolites M1 and M2 competitively inhibit the binding of androgens to the mammalian AR. M1 and M2 also inhibit DHT-induced transcriptional activity in the cells transferred with human AR (Kelce *et al*.,1997), which may lead to malformed male offspring (Monosson *et al.,* 1999).

Polychlorinated biphenyls (PCBs):

PCBs are normally associated with industrial pollution. They were extensively used since 1929 as heat transfer and hydraulic fluids, adhesives, flame retardants, dielectric fluids for capacitors and transformers and waxes. PCBs consist of 209 congeners, which are found in different mixtures in commercial products. Biological effects caused by the various congeners differ, not only in potency but also qualitatively. Indications are that some of the congeners produce modifications in the reproductive functioning of male fish and although the

mechanism is unknown, elevated PCB concentrations are associated with a decrease in plasma testosterone concentrations in small mammals. (Toppari *et al.,* 1996).

It is reported that specifically the following congeners have EDC effect:

- 2', 5' dichloro-4-hydroxybiphenyl
- 2', 4', 6'-trichloro-4-hydroxybiphenyl
- 2', 3', 4', 5'-tetrachloro-4-hydroxybiphenyl (Soto et *al* 1995*.,* Toppari *et al*, 1996*.,* Facemire *et al.,*1995).

PCBs are very stable in the environment and may remain in a contaminated area for up to 50 years. It is reported that PCBs not only affect the reproductive system in humans, but also have an effect on the neurological system and the thyroid function (WHO Report, 2002).

Alkyl phenols (APEs) and the Ethoxylates:

p-Nonylphenol (NP) and *p*-Octylphenol (OP) and the ethoxylates (NPnEO and OPnEO) have proven to possess weakly estrogenic properties. They are all consumed in large annual volumes throughout the world. Over 300 million kilograms of APEs are produced annually. NPnOP and OPnEO are predominantly used as emulsifying agents and surfactants in cleaning and washing products.(Nordic Council of Ministries Report,1996) After sewage treatment, approximately 60% of the APEs are released into the aquatic environment as short chain APEs (NP2EO), alkylphenol carboxylic acids (NP1EC) and alkylphenols (NP).(Toppari *et al.,*1996.) Documented estrogenic effects for above mentioned compounds have been shown *in vitro* (human breast cancer cell lines and rainbow trout hopatocyte bioassay). The estrogenic potency increases with decreasing length of the ethoxylates side chain in NPEO and OPEO (Nordic Council of Ministries Report, 1996).

Phthalates:

butyl benzyl phthalate (BBP)

di-n-butyl phthalate (DBP)

di-(2-ethhylhexyl)phthalate (DEHP)

These chemicals are mainly contained in product types such as paints, lacquers, joint materials, glues and printing inks. (Nordic Council of Ministries Report, 1996) It is reported that these substances have EDC properties and that they may leach into water systems. (Fawell *et al.,* 2001).

Bisphenol A (BPA):

BPA is mainly used as an intermediate in the production of epoxy and polycarbonate resins, as a stabilizer for plasticizers in PVC and as an antioxidant in rubber and plastics (Nordic Council of Ministries Report, 1996; Findley, 1996). It is also used in the lacquer coating of food cans that are often heated for sterilization purposes. (Toppari *et al.,*1996) BPA was shown to have an estrogenic effect on MCF-7 breast cancer cells (Fawell *et al*., 2001).

Heavy metals: Cadmium and Mercury.

There is sufficient evidence from animal studies supporting the disruptive effects of mercurials, but more studies are needed to extrapolate this data to humans. (Zhu *et al.,* 2000: Facemire *et al.,*1995). In certain regions of South Africa mercury and cadmium levels are very high and therefore the analysis of mercury and cadmium should be included in this survey.

Metals: Zinc and Calcium

Indications are that Zinc and Calcium have a synergistic effect with heavy metals such as Cadmium as far as estrogenicity is concerned and it is for this reason that these two metals are included in the survey.

Phytoestrogens:

The isoflavones genistein and daidzin have both weak estrogenic effects. They occur in plant material such as wheat, cabbage and soybeans and are of great concern because soybean protein are utilized in the manufacture of baby formulas and soybeans are one of the staple foods for vegetarians (Toppari *et al.,*1996).

Estrogenic mycotoxins:

Zearalenone and deoxynevalenol (DON) are both very potent estrogens. The ratio between these two mycotoxins determines the potency of the compounds. These mycotoxins are associated with low grade maize, which are utilized as animal feed, but sometimes also in poverty stricken rural areas. It may enter the human food chain via the consumption of meat from bovine or porcine origin. Zearalenone has the same estrogenic potency as 17β-Estradiol (Soto *et al.,* 1995).

Pharmaceutical substances:

The growth hormone zearanol has the same potency as 17β-estradiol. It is a very popular substance used in cattle farming. (Leffers *et al*., 2001} Apart from diethylstilbestrol (DES), tamoxifen and raloxifen are also reported to have estrogenic properties. (Witorsch *et al*., 2000) A large number of drugs are used in animal husbandry as growth promoters. Most of these substances are hormones. They may enter the human body via the food chain, but may also enter the aquatic system via excretion of animals.

Commercial antioxidants:

BHA (Butylated hydroxyanisole) is used widely in food products, especially in fatty products such as cooking oils and margarines. It is reported to have estrogenic effect (Fawell *et al.,* 2001).

Natural and synthetic hormones:

17β-Estradiol, Estriol, Estrone and Ethinyl-estradiol may enter the water sources due to human excretion.

Flame retardants:

PBDEs leach into the air and sewage from plastics in alliances and computers, foam in upholstery and fabric from carpets and draperies. The complete toxic profile is much like PCBs that cause birth defects, thyroid imbalances and neurological damage in animals and people (Meerts *et al.,* 2000; Zhou *et al.*, 2001)

Great care needs to be taken in selecting substances that are suspected endocrine disrupters. The EU recommends that only those substances that are listed as possessing EDC activity *in vivo* would be appropriate. However, in terms of the precautionary principle, it seems crucial that the EDC effect of compounds tested *in vitro* should be further investigated to determine whether they possess *in vivo* effect in humans. A list of compounds was compiled using the following criteria.

3.3.2. Criteria for including a compound on the EDC priority list:

1. The substance should have indication of *in* vivo EDC effect. The effect of EDCs on the thyroid function and immune system should also be taken into account. Any of the biochemical tests and/or animal studies may be utilized to establish these effects. At this stage the relative potency to 17β-estradiol should not be taken as the only guideline, because some research still needs to be done on this subject.

2. Usage: There must be proof of past or present use. (Banned substances should not be excluded from the list). Some of the persistent organic pollutants such as organochlorine pesticides (DDT, Dieldrin) are banned, but are still used in Malaria-infected areas. The use of PCB compounds has

been phased out, but because of the long half-life of these compounds, they are still present in the environment.

3. Persistency: There must be proof that the substance is persistent in the environment and/or accumulates in the body (organism). There are, however, indications that frequent exposure to non-persistent chemicals may have the same detrimental effect as chemicals that persist in the environment. It is therefore recommended that data on frequency of use in the case of a non-persistent compound should be taken into account as well as the fate of the substance in the environment and/or purification plant. The mechanism of action should also be investigated to determine at which level the compound would be active. There are indications that some of the breakdown components of a chemical may have more potent EDC effects than the mother compound. (NP and OP are products from APEs)

4. Substances to be included could be naturally occurring, synthetic or anthropogenic. Some researchers define an EDC as a synthetic compound. It would be unwise to disregard compounds such as hormones, phytoestrogens and heavy metals because they are not synthetic compounds.

5. With respect to monitoring of **drinking water** an additional criteria regarding water solubility/mobility could be added.

   Water solubility/mobility: The water solubility/mobility of compounds should be taken into account especially when monitoring drinking/purified water. The lipophilic compounds such as organochlorine pesticides and PCBs adhere to solid particles and may be found in large amounts in environmental water and sewage sludge, but are easily removed in drinking water treatment processes.

   However, these compounds have long half-lives and also tend to accumulate in fatty tissue of animals and humans and could therefore not be eliminated from the overall list.

Additional limitations to be considered

1. Both the effect on humans and/or animals should be taken into account. Disruption in wildlife is no longer disputed and should be a warning signal for human health. Because of the difficulty in extrapolating the EDC potency from one species to another, it seems adamant at this stage not to concentrate on the effect on humans only. Potency is mostly determined by *in vitro* methods and is dependant on the mode of action and the receptor binding. Great differences are observed when using different methods of determination as shown in the following table.

Table 1. Relative EDC potency (17βEstradiol=1) of ER-agonistic compounds determined as ED_{50} –values from dose response curves (Gutendorf *et al,* 2001).

Compound	MVLN cells	HGELN cells	E-Screen	Binding to ER-α	Binding to ER-β
17β-Estradiol	1	1	1	1	1
Estriol	0.083	0.4	0.071	0.07	0.26
Estrone	0.01	0.058	0.01	0.007	0.065
Ethinylestradiol	1.25	5.71	1.25	1.16	1.44
Diethylstilbesterol	1.25	8.0	2.5	1.75	1.3
4-Nonylphenol	1.25×10^{-5}	8.0×10^{-5}	1.3×10^{-5}	1.75×10^{-4}	2.3×10^{-3}
4-Octylphenol	8.33×10^{-5}	8.0×10^{-4}	1.0×10^{-4}	7.0×10^{-4}	6.5×10^{-3}
Bisphenol A	2.5×10^{-5}	1.9×10^{-4}	2.5×10^{-5}	2.3×10^{-4}	2.6×10^{-3}
Genistein	1.32×10^{-4}	8.0×10^{-4}	1.3×10^{-5}	1.0×10^{-4}	0.032
β-Sitosterol	1.0×10^{-4}	7.3×10^{-4}	9.6×10^{-5}	8.75×10^{-4}	0.016
Coumosterol	1.25×10^{-4}	1.0×10^{-3}	1.1×10^{-4}	1.17×10^{-3}	0.022
Tamoxifen	8.33×10^{-6}	7.1×10^{-7}	4.0×10^{-5}	0.023	0.054

2. The ability to control the substance in the environment should not be used as a reason to include or exclude a substance from being on the list. Some substances such as heavy metals and phytoestrogens occur naturally in the environment and may be virtually impossible to control. Procedures however, exist to minimize these compounds in purification systems.

Apart from their EDC properties, the heavy metals are also toxic and should be monitored in drinking water.

3. The unavailability of an analytical procedure to analyze for the substance at the necessary low detection limit should not be an obstacle to include the substance on the list. Some chemicals show EDC effect at levels a thousand to a million times lower than the ADI (allowable daily intake). The majority of analytical procedures were developed to meet the toxic endpoints of a compound (ADI, NOEL (No effect level values)). These methods will not necessarily meet the low detection limits needed for EDC analysis. In these cases it is suggested that new methodology be developed rather that excluding a substance from the list, because there is not at present an appropriate analytical method. A list of EDC substances was compiled taking in consideration the usage in South Africa (Present and Past) and adding to these compounds which may be considered as important for monitoring to the main trading partners of South Africa. Information regarding manufacture and usage of pesticides in South Africa was obtained from the Department of Agriculture and Department of Health. Very limited information regarding the tonnages of industrial chemicals manufactured and used in the country could be obtained as this information is not freely available.

3.3.3 Suggested list of EDC compounds to be monitored

Table 2: Suggested list of priority EDC compounds to be monitored

Compound	Chemical class	Relative potency to 17β-estradiol
17β-Estradiol Estriol Estrone 17α-Ethinylestradiol	Hormone	1 0.08-0.8 0.09-1 0.9-1.2
p-Nonylphenol Nonylphenol ethoxylates *p*-Octylphenol Octylphenol ethoxylates	Alkylphenols	$7x10^{-3}$-$1x10^{-5}$ $1x10^{-5}$ $1.5x10^{-3}$-$1x10^{-4}$
PCBs	Polychlorinated biphenyls	$1x10^{-2}$-$1x10^{-4}$
DDT, DDE, DDD Dieldrin, Aldrin, Endrin α-Endosulfan, β-Endosulfan, Endosulfan-sulphate Heptachlor, Heptachlor epoxide Lindane (γ-BHC)	Organochlorine pesticides	$1x10^{-7}$
Chlorpirifos Azinfos-methyl Parathion	Organophosphate pesticides	
Deltamethrin	Pyrethroid pesticide	
Atrazine Simazine Terbutylazine 2,4-D, 2,4,5-T Metoxychlor	Herbicides	 $1x10^{-4}$
DEHP DBP Bisphenol A	Plasticizer Raw material for resins	$1x10^{-5}$ $1x10^{-5}$
Dioxins, Dibenzofurans	Dioxins/furans	
Tributyltin, Cyhexitin	Organo-tin compounds	
Lead, Cadmium, Mercury, Arsenic	Toxic heavy metals	
Vinclozolin	Bactericide	

3.3.4 Additional compounds:

Compounds suspected of having EDC properties are listed in Table 3. More information needs to be obtained concerning the *in vivo* EDC activity, mode of action and potency before they can be included in the Priority List.

Table 3: Additional compounds to be considered

Pesticides, Herbicides and Bactericides	**Plasticizers**
Aldicarb	Di-n-butyl phthalate
Amitrole	Dicyclohexyl phthalate
Benomyl	Diethyl phthalate
Carbaryl	Diethylhexyl adipate
Chlorfenvinphos	Dihexyl phthalate
Cypermethrin	Dipentyl phthalate
1,2-Dibromo-3-chloropropane	Dipropyl phthalate
Esfenvalerate	
Fenvalerate	
Hexachlorobenzene (HCB)	
Isoproturon	**Fire retardants**
Malathion	
Mancozeb	
Maneb	Polybromobiphenyl ethers
Methomyl	
Metiram	
Metribuzin	***Other***
Nitrofen	
Pentachlorophenol	
Permethrin	Benzo(a) perene
Trans-nonachlor	n-butyl benzene
Trifluralin	Styrene
Triphenyltin	
Zineb	Dichlorophenol *(Dye intermediate)*
Ziram	Benzophenone *(Raw material in medical products)*
	4- nitrotoluene *(2,4-dinitrotoluene intermediate)*
	Octachlorostyrene *(Byproduct of organochlorine compounds)*

Many elements do not have direct EDC activity, but they do have an impact on toxicokinetics and toxicodynamics of those which have disruptive activity. Their presence may either enhance or diminish the EDC effect of certain compounds.

Elements of concern are

Zinc, Calcium, Selenium, Chromium, Fluoride, Bromide, Boron, Copper, Iodine, Molybdenum, Strontium and Vanadium. These elements may all be determined in an ICP-AES scan to determine their presence and then be individually quantified when necessary.

3.3.5 Toxicants:

Not all toxicants are EDCs. A list of compounds which are regarded as toxicants in water, but without proven EDC activity is given in Table 4.

Table 4: Additional toxicants to be considered

Compound	Chemical class
2,4,5-Trichlorophenol	Disinfection byproduct
2.4-Dichlorophenol	
2-chlorophenol	
Benzene	
Bromate	
Bromodichloromethane	
Bromoform	
Chlorite	
Chloroform	
Cyanogen chloride	
Dibromoacetonitrile	
Dibromochloromethane	
Dichloroacetic acid	
Dichloroacetonitrile	
Formaldehide	
Trichloroacetaldehide(chloral-hydrate)	
Trichloroacetic acid	
Trichloroacetonitrile	
Microcystin-LR	Algal Toxin
Anabaena-a	Algal Toxin
saxitoxins	
cylindrospermopsin	

3.4 Selection of methods to determine EDCs activity and occurrence

EDC activity is mostly determined by biochemical (*in vitro)* methods, EDC effect is determined by biological (*in vivo)* means and occurrence of individual chemicals is determined by chemical analysis. The selection of the appropriate and relevant method is of crucial importance when conducting research on EDCs.

3.4.1 Determination of EDC activity

Bioassays are a valuable tool to measure total activity (estrogenic as well as androgenic) resulting from all of the EDCs present including unknowns. The assessment of EDC activity is made by *in vitro* and *in vivo* methods. *In vitro* methods are mostly based on the interaction of a chemical with the endocrine system at cellular level using cell cultures. They are based on the binding of the EDC to specific receptor on the test cell.

EDC effects in *in vivo* experiments are measured in the whole animal by a measuring a variety of endpoints such as the increase in uterus weight. *In vivo* tests have the major advantage because they take into account absorption, metabolism and excretion, but they are expensive and time consuming. (GWRC report: Endocrine Disrupting Compounds: An overview of sources and biological methods for measuring EDC, Sept 2003)

3.4.1.1 *In vitro tests*

(GWRC report: Endocrine Disrupter Compounds: An overview of source and Biological methods for measuring EDC, Sept 2003)

The Yeast Estrogen screen (YES) assay

The yeast estrogen screen (YES) (Routledge and Sumpter,1996) is a recombinant reporter gene assay that uses yeast cells stably transfected with human ER-α cDNA and an ERE-regulated expression plasmid (*lac-Z*). Interaction of an estrogenic compound with the estrogen receptor results in expression of the reporter gene *lac-Z* and secretion of the enzyme β-galactosidase in the yeast medium. β-galactosidase transforms the yellow substrate chlorophenol red–β-D-galactopyranoside (CPRG) present in the

medium to red. This is measured spectrophotometrically, (Vethaak *et al*., 2002). The EC_{50}, that is the concentration causing 50 % of the maximum response amounts to 100 pmol 17β-estradiol. Quanrud reported EC_{50} of 6 – 9 nmol 17β-estradiol (Quanrud *et al*., 2002). This method was also evaluated by Coldham.*et al.,*1997 and found suitable for EDC activity determination..

Two-hybrid recombinant yeast cell bioassay TRCBA

This bioassay is used in Japan to measure the estrogenic activity in surface water, process water and drinking water (Kobuke *et al.*, 2002).
After filtration with glass fibre filters to remove solid particles, the water samples were then passed to a C-18 solid phase cartridge. Residual water was removed under a nitrogen stream. The adsorbed organic compounds were desorbed with 2 ml methanol. Estrogenic activity of the concentrated samples was determined by use of Two-hybrid recombinant yeast cell bioassay TRCBA
Estrogenic activity was measured in surface water and effluents of wastewater treatment plants. Poor elimination of estrogenic activity by biological treatment at the sewage treatment plant resulted in the occurrence of estrogenic activity in the source water of drinking water treatment plants. Coagulation, flocculation and filtration removed about 50 % of the estrogenic activity. Chlorination removed the estrogenic activity to the measured blank level.

Kawagoshi *et al*., (2002) simplified the original method described by Nishikawa and used the simplified method for screening estrogenic effects in leachate from a landfill. The sensitivity of the method was about a factor 10 times higher than the original method. EDCs were extracted from the water samples by extraction with dichloromethane at pH 7 and pH 3. The extract was concentrated nearly to dryness and dissolved in methanol. With this method the estrogenic activity of leachate was in the order of 0.1 nmol of 17β-estradiol. The main contribution to the activity could be explained by measured concentrations of bisphenol A and alkylphenols.

Estrogen Receptor (ER) binding assay

This assay used for screening the estrogenic potency of water samples is described by Murk *et al*., (2002). This test examines the binding capacity of a compound to the estrogen receptor (ER). The sensitivity of this assay expressed as the 50 % response (EC_{50}) amounts to 6-9 nM 17β-estradiol. In this assay both agonists and antagonists give an estrogenic response resulting in higher 17β-estradiol equivalency (EEQ) levels for water samples compared to the YES- and ER-CALUX® assay.

Quanrud *et al.* (2002) concluded that the ER- binding assay is the least physiologically significant, least sensitive test, and tends to show higher concentrations of estrogenic compounds in water samples than the other tests. Reasons for these reported higher concentrations are that the test additively detects both estrogens and anti-estrogens and that the test does not account for the barriers for transporting the chemicals across the cell wall.

Enzyme linked immuno-assay (ELISA)

An ultra-sensitive immunoassay has been developed and tested for the quantification of 17β-estradiol at low levels (1.5 ng/L) in water samples without any sample pre-concentration [Rubio *et al*., 2002]. The precision varies between 2 and 16 %. The specificity is good, other hormones show a cross-reactivity ranging from < 0.01 to 0.7 % (estrone 0.7%, estriol 0.3 %). The average recovery for 17β-estradiol added to groundwater samples is 95%. Interference by nitrate, magnesium, calcium, sulphate, phosphate was not observed at levels up to 20,000 ppm.

E-screen cell proliferation assay

The E-screen MCF-7 cell proliferation assay is widely used to detect weakly estrogenic compounds. A dose-related increase in cell numbers in treated cultures is taken as evidence of estrogenicity of the test compound. MCF-7 estrogen-sensitive human cancer cells are used and cell proliferation measured (Soto *et al*., 1995). The estrogenic activity of a test compound is evaluated by determination of the relative effect to cell proliferation. The sensitivity, expressed as 50 % response (EC_{50}) amounts 1 pM 17β-estradiol (Quanrud *et al*., 2002). There is no standardized protocol for this assay, and various test regimes and

cell sub-lines have been used. As a result, considerable variability has been reported in published results.

ER-Calux® assay

The estrogen receptor (ER)-mediated '*Chemically activated luciferace gene expression*' (ER-CALUX®) bioassay (Legler *et al*., 1999) is a recombinant reporter gene assay which offers highly sensitive measurement of the trans-activation of the ER following exposure to xeno-estrogens. The ER-CALUX® comprises genetically modified $T_{47}D$ human breast adenocarcinoma cells containing endogenous ER-α and ER-β. An ER-mediated luciferace reporter gene construct containing three estrogen response elements (EREs) was introduced stably and integrated in the genome of the $T_{47}D$ cells. Exposure of cells to (xeno-) estrogens results in diffusion of chemicals through the cell membrane, binding to the endogenous ER, activation of the receptor, and consequently, binding of the ligand-receptor complex to EREs present in the promotor region of the luciferace gene. Luciferace protein is then induced, and is easily measured by lysing the cells, adding luciferin substrate, and measuring photon production. The amount of light produced is proportional to the amount of ligand-ER binding, which can be related to estradiol equivalents, EEQs (Vethaak *et al*., 2002). The test is extremely sensitive and capable of detecting femtograms of estrogenic substances. This allows small sample sizes. Using 96-well test-systems allows rapid analysis of large numbers of samples.

However, before measuring the estrogenic activity, extraction techniques like liquid-liquid extraction or solid phase extraction (ESP) have to be used to isolate EDCs from the matrix.

The EC_{50} of the ER-CALUX® assay amounts 6 pmol 17β-estradiol and is more sensitive compared to the YES assay and CARP-HEP assay.

DR-CALUX® assay

Derived from studying the toxicological mode of action of dioxins an in vitro bioassay has been developed as a screening method for the detection of dioxins and/or dioxin-like compounds.

The Dioxin Responsive Chemically Activated Luciferase Expression (DR-CALUX®) bioassay comprises a genetically modified H4IIE rat hepatoma cell-line, incorporating the firefly luciferase gene coupled to DREs as a reporter gene for the presence of dioxins and /or dioxin-like compounds. Cells that are exposed to dioxins and /or dioxin like compounds not only express proteins and enzymes that are, under normal circumstances associated with the DRE, but also luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-AhR binding and related to 2,3,7,8-TCDD toxic equivalents (TEQs).

The method is extremely sensitive and capable of detecting femtogram of dioxins. However, before applying this bioassay the dioxins have to be extracted from the matrix (water, suspended matter or sludge).

Carp-HEP assay

The carp hepatocyte (CARP-HEP) assay (Smeets *et al*., 1999) allows measurement of estrogenic activity in natural untransformed cells. It measures vitellogenin which is the precursor of yolk proteins and which is secreted by liver cells in response to exposure to estrogens. Vitellogenin is produced in liver cells of female oviparous vertebrates. The vitellogenin gene is also present in male oviparous vertebrates but under normal circumstances the estrogen levels are too low to induce vitellogenin production. However, exposure to estrogens can induce vitellogenin in males.

The CARP-HEP assay is less sensitive compared to the YES- and ER-CALUX®-assay, for example the EC_{50} for 17β-estradiol is 100 nmol (Vethaak *et al*., 2002).

T47D-KBluc cell line (EPA approved)

This is a newly developed cell line that is specific and sensitive for screening chemicals for estrogenic and antiestrogenic activities. T47D human breast cancer cells, which naturally express estrogen receptor (ER) alpha and beta were stably transfected with a triplet ERE (estrogen-responsive elements) – promoter – luciferase reporter gene construct. The reporter gene construct consists of three ERE upstream from the TATA box that regulates the expression

of a luciferase reporter gene. Stable transfection of this promoter-reporter construct into T47D cells resulted in a sensitive, responsive clone.
In very simple terms, compounds enter the cell; estrogen receptor ligands bind to the ER; two ligand-bound receptors dimerize and bind coactivators; then the dimmer binds to the ERE on the reporter gene construct and activates the luciferase reporter gene. The presence of the luciferase enzyme can then be assayed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The amount of light produced is relative to the degree of estrogenic activity of the test chemical. Model compounds, with well-defined mechanisms of action, were used to evaluate the utility of this cell line, T47D-KBluc, and those results are presented here. When testing chemicals using the T47D-Kbluc cells, an estrogen was defined as a chemical that induced dosedependent luciferase activity, which could be specifically inhibited by the antiestrogen ICI. Plans include making these cells widely available by depositing the cell line with ATCC for maintenance and distribution (Wilson *et al.,* 2004).

3.4.1.2 *In vivo* assays

(GWRC report: Endocrine Disrupter Compounds: An overview of source and Biological methods for measuring EDC, Sept 2003)

The OECD proposed the development of guidelines for testing the estrogenic effects *in vivo* with fish. Three types of tests based on an *in vivo* test were distinguished:

a. 'Juvenile Growth Test' OECD 204 with vitellogenin induction as endpoint and histopathological examination of the gonads of the larvae at the end of the testing period;
b. 'Early Life Stage test' OECD 210 with vitellogenin induction as endpoint and effects on development of the gonads;
c. 'Fish full life cycle test'.

Since that time different tests have been developed with a range of different fish species: (transgenic) Zebra fish, Fathead Minnow and Japanese Medaka.

In situ exposure studies with rainbow trout, salmon, carp, bream and flounder have been successfully carried out for the examination of estrogenic activity.

Genetic differences in sensitivity to endocrine disruption by estrogen have been reported. Bioassays using selected animals may underestimate the potential effects in sensitive populations. These differences in sensitivity must be accounted for when a specific animal model is used for EDC effects (Bornman, 2000).

Estrogenic effects in fish:
Hatching and survival of embryo's, larvae and (juvenile) fish in ELS-test;
General condition: condition factor for liver weight and gonad weight;
Sex ratio of fish in ELS-tests and wildlife;
Ovotestis in male fish;
Vitellogenin production measured in blood plasma of male fish or in whole body homogenates.

Vitellogenin production in serum of male Mature Medeka has been used to evaluate the estrogenicity of EDCs such as nonylphenol (NP), bisphenol-A (BPA) or 17β-estradiol (E2). Vitellogenin was not detected in 53 negative controls (exposure time 0 - 5 weeks, except in three cases: levels of 330, 889 and 3150 ng E2/ml). Test solutions of 100 µg/l p-NP, 1000 µg/l BPA and 0,05 µg/l E2 showed vitellogenin production. The relative potencies decreased in the order E2 >> p-NP >> BPA. In the same experiments after chlorination of these test solutions for 24 hours the estrogenic activity was reduced drastically (Kobuke *et al*., 2002).

High concentrations of vitellogenin have been found in male bream together with a high number of ovotestis (33-43%) in surface water in the Netherlands (Vethaak *et al*., 2002). This surface water receives effluents of domestic sewage treatment plants, but is also influenced by industry and agriculture.

Laboratory tests with zebrafish confirmed the estrogenic activity measured in effluents with the '*in vitro*' ER-CALUX® test.

3.4.2 Evaluation of bio-assays for determining EDC activity in aquatic Systems

Not all above mentioned methods are applied in South Africa. During a workshop held in Pretoria on May 2004 some of these methods were evaluated on issues such as sensitivity, repeatability, time of analysis, ease of use and cost. A summary of the results is given below.

Methodology

Presentations were given by delegates on the following assays after which the selection procedure took place.

In vitro assays

The Yeast- screen assay

The E-screen assay

The MVLN assay

The liver culture ELISA assay

In vivo assays

Zebra fish assay for determining estrogenic effect

Xenopus lavis assay for determining estrogenic effect

XEMA test for determining thyroid function

Selection: When considering the applicability of any analytical procedure for monitoring purposes certain issues have to be taken in consideration.

a. The method must allow for high numbers of samples to be analyzed.

b There must be no restrictions on chemicals or any other components such as yeast strains which are important in the execution of the assay.

c. The training level and experience of the analysts are of greatest importance because complicated and difficult procedures need to be done by experienced analysts and results obtained evaluated by highly trained scientists.

After presentation each assay was evaluated on the following criteria

Sensitivity

Repeatability

Robustness

Time of analysis

Optimum batch size

Cost

Ease of use

Type of analyst needed to do analysis

Evaluation of the methods were categorized in the following categories

Applicability: This was evaluated on the sensitivity, robustness, repeatability, batch size and ease of use of the method.

Time: The time of the analyses were divided into

a. Total time of analytical procedure.
b. Labour time (Time spent by analyst on the bench handling the samples).
c. Non-labour time (Time spent by samples in incubators, water tanks ect.)

Cost: The cost was calculated on the following basis:

a. Labour cost calculated as the cost of an experienced analyst for analysis and the cost of a highly qualified scientist to supervise the procedure and evaluate the analytical results (±R300 per hour).
b. Non-labour cost, which includes non-labour time and overhead costs (±R150 per hour non labour time). Cost of chemicals is not included in this calculation.
c. Because of the high cost of running a single sample the cost was calculated as the average cost per sample in an optimum batch size.

Results

Results of evaluation are given in Table 5 and time and cost evaluation is given in Table 6.

Table 5: Evaluation of bio-assays

Bio-assay	Sensi-tivity	Repeat-ability	Robust-ness	Total analytical time	Batch size	Volume required	Analyst training level	Critical Factor
In vitro								
Yeast screen	1.3ng/l	S<1	G	10-14 days	8	4litres	B.Tek B.Sc 2 years exp	Yeast cell line
E-screen	1.5ng/l	S<1	W	10-14 days	8	4litres	B.Tek B.Sc 2 years exp	MCF7 cell line
MVLN assay	0.1ng/l	ND	G	10-14 days	8	1 litre	B.Tek B.Sc 2 years exp	MVLN cell line
Xenopus liver screen	10 ng/l	S<5	G	7 days	8	200 ml	B.Tek B.Sc 2 years exp	Xenopus
In vivo								
Zebra fish ELISA	10µg/l	ND	G	14 days	3	500 ml	B.Tek B.Sc 2 years exp	Zebra fish
Xenopus ELISA	10µg/l	ND	G	14 days	3	500 ml	B.Tek B.Sc 2 years exp	Xenopus

17β-Estradiol active at 0.03ng/l G = Good ND = Not Done

S = Standard deviation W = Weak

After discussion by the delegates on the scientific merits of the above mentioned assays it was decided to discard the E-screen assay because of the weak robustness of the method and other difficulties in executing the procedure. A presentation on the XEMA bio-assay for the determination of Thyroid activity was presented by Stellenbosch University. No further evaluation on this method was done because it does not address the estrogenic effect as was stipulated in the terms of reference of the workshop. The cost analysis indicates that there is little difference in the cost of the *In vivo* assays. These assays are more expensive than the *In vitro* methods and the batch sizes are smaller. Development in this area is urgently needed if these procedures are to be implemented in field studies. The *Xenopus* liver culture assay described in the *In vitro* assays seems to be more cost effective and takes less time than the Yeast-screen and MVLN screens, but the sensitivity does not meet the present criteria of 0.1ng/l. More development is needed to include this method as a standard method for a monitoring program. Only the Yeast screen is presently conducted by more than one laboratory in South Africa. Because only a few methods were reviewed no scoring was done and it was decided by consensus that the Yeast screen will be proposed as the first step in selecting methods for monitoring purposes.

Table 6: Time and cost analysis of bio-assays (Cost of chemicals excluded)

Bio- assay	**Labour time**	**Non labour time**	**Labour cost**	**Non labour cost**	**Total cost per batch**	**Cost per sample when done in optimum batch**
In vitro						
Yeast screen	16 hours	40 hours	R4 800	R6 000	R10 800	R1350
MVLN screen	16 hours	40 hours	R4 800	R6 000	R10 800	R1350
Xenopus liver screen	8 hours	20 hours	R2 400	R3 000	R 5 400	R 675
In vivo						
Zebra fish assay	12 hours	40 hours	R3 600	R6 000	R9 600	R3 200
Xenopus assay	12 hours	40 hours	R3 600	R6 000	R9 600	R3 200

Conclusions and recommendations

1. Only three *in vitro* assays are at present suitable for monitoring of estrogen effect in water systems namely the Yeast screen, The MVLN assay and the Xenopus liver culture assay. Two laboratories (CSIR and University of Pretoria) are able to conduct this assay. Only Stellenbosch University has presently the capability to conduct the Xenopus liver assay and only University of Pretoria is able to conduct MVLN assay. It would be wise at this stage for at least one other facility to develop the Yeast screen so that inter laboratory tests may be conducted to evaluate the quality of the analysis
2 Two *in vivo* assays were presented at the workshop. These two assays only differ in the organism that produces the Vitellogenin, which is the indicator of estrogen production. These assays are both expensive and time consuming. At present only three samples can be done in a period of 14 days. Further research is needed in this field. The assays can only be done at University of Stellenbosch

3.4.3 Determination of individual EDCs

Analytical chemistry methods are normally used to determine the occurrence and levels of EDCs in Aquatic systems. These methods usually consist of an extraction procedure, followed by instrumental detection by GC, GC-MS, HPLC or HPLC-MS.

Extraction and clean-up

Various extraction procedures have been suggested such as liquid-liquid (LL) extraction, Solid phase extraction (SPE) and super critical fluid Extraction (SCFE). All these procedures have merit depending on the specific matrix (Water, waste water, sediment or biological tissue). It is necessary to conduct recovery studies to determine the efficiency of the specific extraction procedure. In some cases multi-step clean-up will be necessary.

Instrumental detection

Depending on the volatility and other properties of the specific compounds, levels of EDCs may be determined by Gas Chromatography (GC) or High Performance Liquid Chromatography (HPLC). Confirmation of the identity of a specific compound is normally done by GC-MS (Gas chromatography-Mass spectrometry) or HPLC-MS, but because of the extremely low levels at which these compounds are active it is often very difficult to achieve this confirmation due to the limits of the MS (Mass spectrometer). The properties of some compounds such as volatility, polarity and solubility also limit the scope of analysis. It is, therefore, practically impossible to analyze for all EDCs in one run.

Elements and minerals are normally detected by Atomic Absorption (AA) or AA-ICP methods

3.5 Survey of laboratory capacity and capability

Criteria were set for laboratories that would be considered to be capable of conducting EDC analysis:

1. The laboratory should have an approved quality assurance system in place such as SANAS accreditation. Alternatively they may qualify if they participate in regular inter-laboratory tests with good results.
2. In the case of laboratories conducting chemical analysis they should have the necessary equipment (or have access to this equipment) to conduct ultra trace analysis. Some EDCs have to be analyzed at low ng/l levels.
3 There must be proof that the equipment used in the laboratory is in good working order and is regularly serviced and tuned.
4 Sophisticated methods are used for ultra trace analysis and the analysts conducting these analyses should be well trained and supervised.
5 The laboratory should have a good system of record keeping in order ensuring the trace ability of the results produced.
6 The analytical methods used in the laboratory must be validated or at least verified (When using published methods). Alternatively established methods such as AOAC methods may be used.

7 There must be proof of continuity in staff especially in the case of supervisors.

8 The laboratories must be able to present proof of annual training and review and updating of methods.

A summary of laboratories conducting chemical analysis is given in Table 7.

There are a number of analytical facilities in South Africa that undertake toxicity tests (Table 8). The majority of these facilities are geared for acute and chronic toxicity tests in water.

Volatile Organic compounds (VOC) and semi-volatiles are also conducted by Northwest University and CSIR (Bio/Chemtek). Analysis of F, SO_4, NO_3, NO_2 and PO_4 are also undertaken by the Northwest University.

Table 7: Summary of laboratories conducting chemical analysis on EDCs

Laboratory	Hormones	Alkylphenols	PCBs	Pesticides and Herbicides	PAHs	Heavy metals
SABS [1] [2]		X	X	X	X	X
ARC(PPRI) [2]				X		X
Ampath [2]	X	X	X	X	X	X
CSIR[1] (Bio/chemtek)			X		X	
TWR/UJo						
Pentech			X	X	X	
Rand Water[1]			X	X		X
Umgeni Water[1]			X	X		X
ERWAT[2]		X				X
UCT				X		
City lab of Cape Town			X	X		
Northwest University[2]			X	X	X	
Durban Institute of Technology			X	X		X
Waterlab[2]						X
University of Johannesburg (Zoology) [2]						X
Johannesburg Water[1]		X	X	X		X

Where: [1] Laboratory is accredited, [2] Laboratory complies with GLP

Table 8: Summary of laboratories conducting Toxicant tests

Laboratory	No. of qualified analysts	Routine capabilities	Matrix analyzed	Acute toxicity	Chronic toxicity	Algal toxins
Rand Water[1]	6	X	W,S	X	X	X
Umgeni Water[1]	4	X	W,S,F	X	X	X
Northwest University[2]	7		W,S, B	X		
Waterlab[2]	10	X	W,S	X	X	X
CSIR Biotoxicology Laboratory (Environmentek) [2]	5		W,S,B	X	X	
RAU (Zoology)	8	X	W,S,B	X	X	
Ondersterpoort Veterinary Institute (Residue Laboratory)[1]	7		W,S,B			
Unilever Center for Environmental Water Quality[2]	5	X	W	X	X	
Water and Environmental Technology, Sasol Technology R&D[2]	3		W,S	X	X	
Johannesburg Water[1]	25	X	W,S,F,D	X		

Where: [1] Laboratory is accredited, [2] Laboratory complies with GLP, W = water, S = sediment,

B = biological tissues, F =food, D = dairy

At the time of writing no facility existed in the country for the chemical determination of Dioxins and Furans. Northwest University, however offers a bioassay to determine dioxins, dibenzofurans and coplanar PCBs in extracts (sediment, soil, water and air). The procedure provides an integrated I-TEQ value, using the H4IIE cell-line (semi- quantitative). They have a quality control arrangement with the University of Michigan. (This assay, however, is not available on a commercial basis). These substances are of the most toxic substances known to man. The creation of a chemical facility to determine these compounds in South Africa should have high national priority.

A list of laboratories conducting EDC activity tests is given in Table 9.

Table 9: Laboratories conducting EDC Activity Tests

Laboratory	Type of test
University of Pretoria	E-screen, Yeast screen, MVLN reporter analysis, Uterotrophic, Herschberger test *Catfish VTG analysis end 2003*
CSIR (Environmentek)	Yeast screen, AIMS test, Daphnia test, Anti-androgen test, urease enzyme tests, mammalian cell cloning efficiency, Ames Salmonella mutagenicity tests, frog embryo teratogenicity test.
Stellenbosch University	Vitellogenin, ELISA, Thyroid function *Xenopus* assays
Northwest University	Dioxin,dibenzofuran,PCB bioassay, *MVLN Cell-line 2003, MDA cell-line anti-androgenic activity 2004, EDC Xenopus testing*
DWAF	*Daphnia pulex, Poecilia reticulate, Oreochromis mossambicus*
DIT	*YEAST screen to be developed*

3.6 Needs of stake holders and identification of knowledge and research gaps

In order to determine the needs of the stakeholders in this field a workshop was held in Stellenbosch during 2003. The needs were determined and the knowledge gaps to satisfy these needs were identified.

The delegates to the workshop were divided in separate groups:

The government Departments
The water suppliers
The environmentalists
The group concerning human health
The industry

Each group was given the task of determining the specific needs of the section. It was also required of them to list the gaps in the knowledge and propose research projects to fill these gaps.

3.6.1 Government Departments (Department of Water Affairs and Forestry).

Need:

The need of this Department is to put regulations in place to ensure the safety of drinking water in South Africa.

Gaps:

a. Credible data on the occurrence, levels and potency of EDCs and toxicants in South African water sources.
b. Cost effective analytical procedures to determine the EDC effect and to chemically analyze chemicals in water, sediment and aquatic animals
c. Risk assessment to determine the risk to the population
d. Training of personnel to conduct the studies.

3.6.2 The Water suppliers

Need:

To conform to the government regulations regarding the levels of EDCs and Toxicants in water systems with special emphasis on purified water.

Gaps:

a. Reliable and credible data on the occurrence, levels and potency of EDCs and toxicants.
b. The lack of cost effective analytical procedures to determine the chemicals at the low levels required in water.
c. Models to predict contamination in water systems
d. Knowledge on the fate and behavior of EDCs and toxicants in water purification systems with special emphasis on the natural and synthetic hormones.
e. Well trained technicians and scientists

3.6.3 The environmentalists

Need:

To manage the problem of occurrence and effect of EDCs and toxicants in the environment in order to minimize the effect on wild life.

Gaps:

a. Reliable and credible data on the occurrence, source and potency of EDCs and toxicants in rivers, dams, estuaries and wet lands.
b. Analytical procedures to establish EDC activity in rivers, dams, estuaries and wet lands. (Continuous flow samplers and bio-markers)
c. Knowledge about the fate and behavior of EDCs and toxicants in the environment with special emphasis on wet lands.
d. Environmental risk assessment models to determine the risk to wild life.

3.6.4 The Human Health Group

Needs:

a. To determine the effect of exposure to EDCs on human reproduction, thyroid function, nervous system and immune system.

b. To do a human health risk assessment on the exposure to EDCs and toxicants.

Gaps:

a. Reliable and credible data on the levels of EDCs and toxicants in water.

b. Knowledge on the fate and behavior of EDCs and toxicants in the water systems as well as in humans.

c. Cost effective and reliable analytical methods to determine EDC activity in water as well as quick analytical chemical methods to determine levels of EDCs and toxicants in water and biological tissue.

3.6.5 The Industry

Needs:

To conform to government regulations

To manage the contamination of EDCs and OTs in effluent.

Gaps:

a. No regulations are in place.

b. Knowledge of fate and behavior of chemicals in water systems.

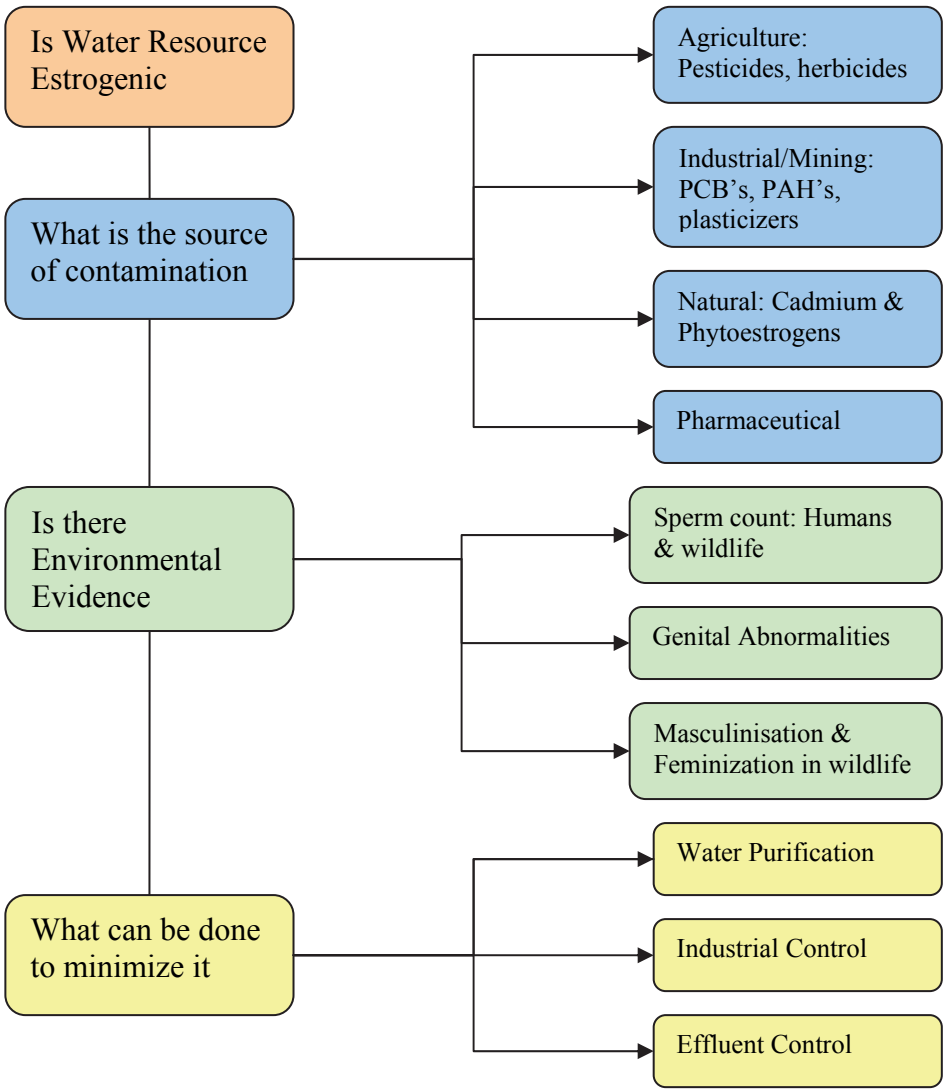
4. RECOMMENDATIONS AND PROPOSALS FOR A LIMITED STUDY ON EDC CONTAMINATION IN SOUTH AFRICA

4.1 Aims and Objectives

The long-term objective of this study is to obtain sufficient information to enable authorities to put a management plan in action to minimize the effect of possible pollution on the population of South Africa. In planning the study on the status of EDC contamination in South African aquatic systems the following questions should be asked:

1. **Are the aquatic systems contaminated with EDCs. (Does the water or sediment show estrogenic properties)?**
2. **What is the source of the contamination?**
   a. Agricultural contamination: Contamination from the usage of pesticides, herbicides, veterinarian drugs or other agricultural chemicals.
   b. Industrial contamination: Contamination from industrial activity resulting in deposition of chemicals such as PCBs, PAHs, Alkyl phenols and plasticizers.
   c. Contamination from natural sources: Some of the estrogenic phytosterols may leach into rivers and dams and cause estrogenic effect.
   d. Pharmaceutical contamination: In areas where wastewater may leach into rivers the water may become contaminated by pharmaceutical products or the breakdown products of these compounds, which also have estrogenic properties. Many of these compounds are hormones or have hormone-like properties that have an effect on the endocrine system.
   e. Contamination due to human excretion: Water may be polluted by urine of women on the "pill". This may cause large amounts of Ethynylestradiol, estrone and estriol to enter the water systems. The potency of these compounds is similar to that of 17β-Estradiol and it may have a large impact on people dependant on surface water for drinking purposes.

3. **Is there evidence in humans and wild life that they were exposed to these chemicals?**
4. **What can be done to manage the contamination in order to minimize the effect on humans and wild life?**



The study is proposed in two phases: The first part of the proposed study will only concentrate on answering the first two questions, namely determining whether some the water sources of South Africa are contaminated with EDCs and the chemical analysis of the water which give indications of being estrogenic, in order to determine the specific chemicals responsible for the contamination and possibly the concentrations of these chemicals.

A follow up study will have to be conducted to determine the effect of the EDCs on humans and wild life in the areas where positive estrogenicity can be detected.

This will involve an epidemiological study on a large scale, which will require substantial funding. . Funding and resources will have to be available to embark on such an exercise.

In order to develop a management system to manage the problem of contamination, a risk assessment study will have to be conducted. The present models for risk assessment do not comply with EDC exposure, because they are aimed at an endpoint for onset of cancer. EDC exposure has no endpoint, only an effect. Risk assessment models will have to be developed for EDC exposure.

4.2 Problems concerning EDC research in South Africa

Some of the difficulties surrounding a major project on EDCs are discussed below:

4.2.1 Geographical diversity:

The geographical diversity of South Africa poses certain challenges in this field of research. A large variety of crops are planted in different regions of the country. Every crop has its own set of agricultural pests and therefore its own pesticide contamination problem. For instance, Atrazine is extensively used on maize crops, while Organophosphate pesticides are used in large amounts on the fruit and vegetable growing areas of the Western Cape. In some of the rural areas, where no industrial activity takes place it may seem pointless to monitor for industrial pollution such

as PCBs and Phthalates, but pilot studies indications were that contamination seems possible. It would be of great importance to establish the pesticide load and industrial waste pollution in the sites chosen for any comprehensive study on EDC contamination.

Laboratory facilities are available in most of the areas where possible contamination may occur.

4.2.2 Laboratory capability and capacity:

The cost of running an analytical laboratory is very high. Most of the laboratories investigated for this study have scaled down or have concentrated on certain specialized fields. Trained personnel are hard to find and it takes up to a year to train an analyst to operate some of the sophisticated instrumentation needed for trace analysis at very low detection limits. In order to give the results generated in the study international credibility, data should ideally be produced in accredited laboratories. Very few commercial laboratories (SABS and CSIR) are SANAS accredited. However, the survey conducted earlier on in this report indicates that South Africa has the capability to undertake a study as proposed, bur few laboratories have the capacity to handle large numbers of samples, especially in the field of activity testing. The capability is not centered in a single organization, but spread over several laboratories and institutions. Cooperation amongst these organizations will be a necessity for the success of the project. The only family of compounds that cannot at present be analyzed is dioxins. South Africa has the scientific capability to analyze these substances, but the necessary funding is not at present available to set up a laboratory for the purpose of determining dioxins.

4.2.3 Lack of funding for research projects and cost of analysis:

The traditional institutions where research used to be done, such as Universities and parastatal organizations (CSIR and ARC), are continually short of funds. The Science Councils have been partially privatized and concentrate on projects, which are profitable.

An additional problem arises because universities depend to a large extent on postgraduate students to conduct research and these students can normally only handle a small project. Students are also only available during midterm when they are not busy with exams or on holiday. An additional problem is that of continuity: At the end of an academic year students may leave the university and a new group of students has to be trained to continue with a project. With limited funding, universities are not able to afford the services of the commercial laboratories.

4.2.4 Lack of coordination between researchers and projects:

Universities and Technikons run small projects which are seldom coordinated, resulting in fragmentation and duplication of work in certain areas. A few working groups have been established in an effort to overcome this problem. (Inter University Workgroup on EDC Research)

4.2.5 Politics:

It is difficult to obtain permission to conduct research projects in certain rural areas. Due to our previous history, the local people are wary and suspicious. In certain cases it takes almost a year to obtain permission from all concerned to work in a specified location or site. It would be advisable to employ someone in each community to liaise between the research team and the relevant communities.

In certain industrial areas the industrial companies will do everything possible to prevent a survey on pollution to be conducted. The cooperation of certain industries, especially the chemical industries, will be of the utmost importance.

4.2.6 Lack of data on EDC research:

Although a vast amount of data exists on contamination of rivers, dams and dumping sites, this data was not generated with EDCs in mind, but rather to establish the “health” of rivers and aquatic systems. Risk assessments were done by using ADIs for exposure to carcinogenic

substances. Very little toxicity data is available for estrogenicity. It will be impossible to conduct a risk assessment until this data is available.

4.2.7 Incompatibility of methods used for determining EDC contamination.

Methodology to determine total estrogenic load in aquatic systems is still in the developing phase. Whilst very sensitive methods exist for certain chemicals, others may not be detected by the specific method. For instance the Atrazine family of chemicals can not be determined by the receptor binding analytical methods such as the E-screen and yeast cell tests, but indications are that they are estrogenic (Sanderson, *et al.,* 2001). It will therefore be necessary to use a bank of different tests, depending on different principles, in order not to obtain false negative results. Most of the traditional chemical analytical methods will have to be modified to meet the very low detection limits required for EDC contamination. The vast number of different EDC chemicals will also elevate the analytical cost to impossibly high levels should they be analyzed individually. New methods of detection by GC-TOF MS may be a solution, but method development in this case will also be necessary. Novel techniques to analyze non-volatile compounds need also to be developed such as LC-MS-MS and molecular interaction analysis (MIA).

4.2.8 The logistics of the project

Samples should ideally be analyzed as soon after sampling as possible. Very large volumes of water will be needed to obtain the necessary concentration for both biological and chemical analysis. This will mean the sampling, transport and storage of large amounts of water contained in glass containers over long distances.

5. PROTOCOL FOR CONDUCTING A LIMITED MONITORING STUDY FOR ENDOCRINE DISRUPTERS IN SELECTED SITES IN SOUTH AFRICAN AQUATIC SYSTEMS

In order to address all the difficulties mentioned above it was proposed that the following steps be followed in this study:

- **Step 1: Preparation and planning and training**
- **Step 2: Pilot study**
- **Step 3: Main study**
- **Step 4: Final assessment**

A schematic representation of the proposed protocol of the project is given .

5.1 STEP 1: Preparation and training

a) A training program for all interested laboratories in the following disciplines:

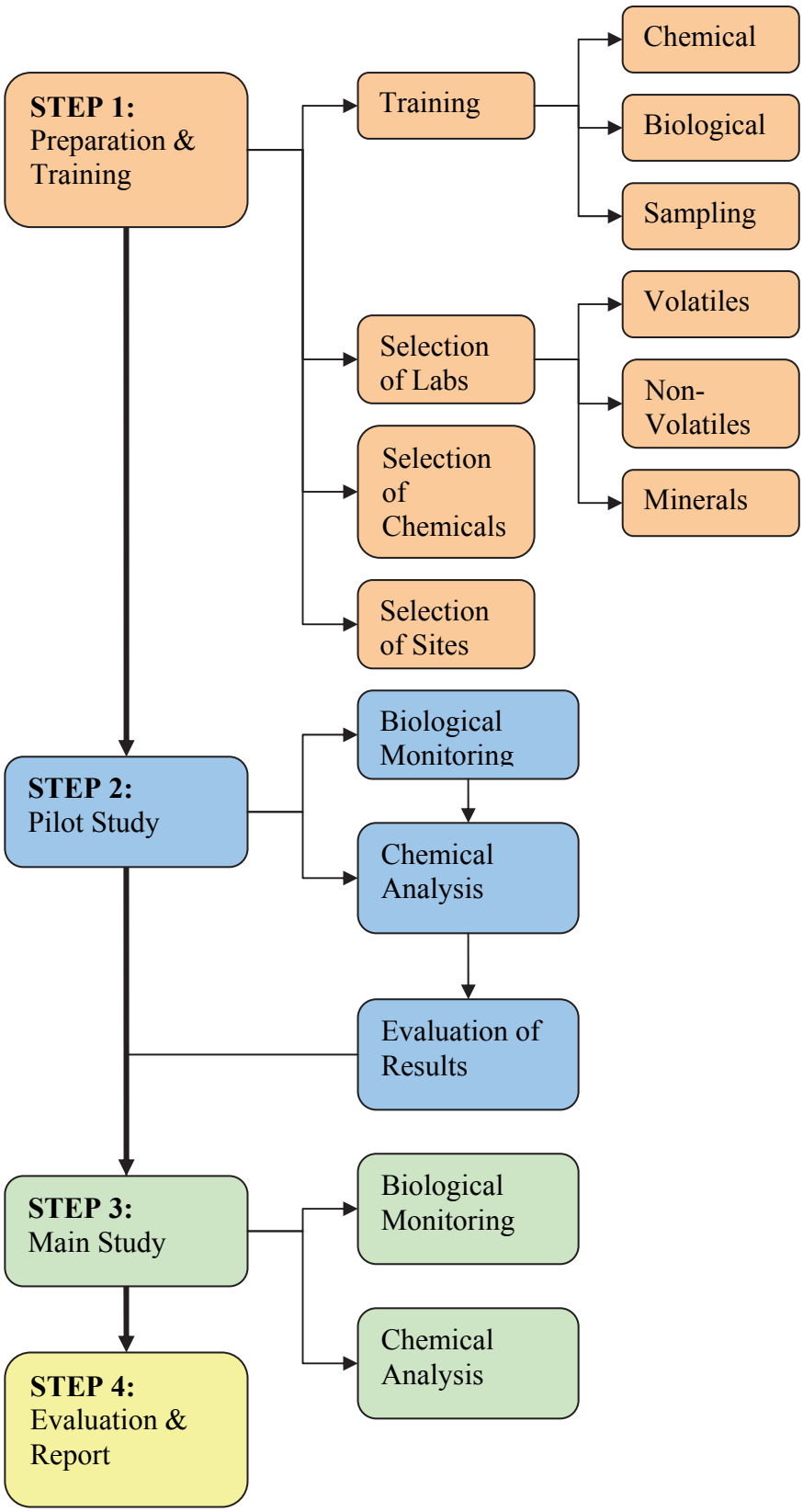
- sampling procedures for water, sediment and tissue and sampling for both biological and chemical analysis.
- combination of biological tests for determining total estrogenic load and estrogenic concentration in water, sediment and wildlife.
- Training for chemical analysis which should include:
  - ✓ **Extraction procedures** both liquid–liquid and solid phase extraction.
  - ✓ **GC operation** on capillary columns and ECD detector
  - ✓ **GC-MS operation**
  - ✓ **Calculation** and evaluation of results
  - ✓ **Detection limits**
  - ✓ **Quality Control** procedures to be followed
  - ✓ **Storage and transport** of samples
- Training of field personnel in:

✓ Sampling on site

✓ On site extraction

✓ Storage and transport of samples

The training will be done in-house as well as by means of workshops


STEP 1:
Preparation &
Training
Training
Chemical
Biological
Sampling
Selection
of Labs
Volatiles
Non-
Volatiles
Minerals
Selection
of
Chemicals
Selection
of Sites
STEP 2:
Pilot Study
Biological
Monitoring
Chemical
Analysis
Evaluation of
Results
STEP 3:
Main Study
Biological
Monitoring
Chemical
Analysis
STEP 4:
Evaluation &
Report

b) Selection of laboratories and sampling organizations

It is proposed the professional sampling organizations be used to do the sampling. Two organizations (SABS and Dept. of Water Affairs and Forestry) have expressed willingness to be of help in this regard. The sampling process is the most critical if all procedures in a study such as this and it is of utmost importance that it should be executed in the correct way.

After attending the training session for chemical analysis it is proposed that an inter-laboratory test be done in order to select the laboratories that will take part in the monitoring. It is essential that the same extraction and detection procedures be followed by all the laboratories in order to have comparable results.

c) Selection of sites and obtaining permission to work in the selected areas:

Although several sites have been proposed by the main researchers, it is at this stage difficult to determine where the so called “hotspots” are in the country. It would be sensible to consider the following guide lines in selecting the sites:

- Sites where it is known that industrial pollution has been detected.
- Sites where considerable agriculture activity takes place
- Sites where actions such as Malaria control are taking place
- Sites where effects on humans and wildlife have been noted
- Sites where research is already being done. (This may add to the information).

It is suggested that sites be selected at a workshop where all role players in the field will be present. Once the sites have been selected by the project team, the next step will be to obtain permission from the local authorities and communities to sample and conduct studies in the various areas. The input goodwill and support of the communities is essential to avoid suspicion, misunderstanding and sabotage of the project. It will be wise to make use of a facilitator in the different communities to explain to the communities what the project is about.

d) Choice of chemical compounds to be analyzed:

The project team should select the compounds to be analyzed by the different laboratories. Over 400 chemicals are reported to have EDC effect. With limited funding it will be impossible to analyze for all of these compounds. A selection process should be followed.

It suggested that the following guidelines be implemented in the selection process:

- Does the chemical or group of chemicals have proven estrogenic properties?
- What is the estrogenic potency (In comparison to17 *beta*-estradiol)?
- What may be the source of the pollution: agriculture, industrial, natural or pharmaceutical?
- What are the estimated quantities used or produced in the country and what is the estimated load on the environment?
- By which mechanism can the pollutant enter the aquatic system?
- Is the pollutant persistent or does the pollutant enter the environment on a continual basis?
- Has the pollutant been detected in the environment?

The various classes of chemicals have been investigated earlier in this document. It is suggested that the following classes of chemicals be investigated:

- ✓ Organochlorine pesticides (OCs)
- ✓ Polychlorinated biphenyls (PCBs)
- ✓ Poly aromatic hydrocarbons (PAHs)
- ✓ Selected fungicides
- ✓ Alkyl phenols and Ethoxylates
- ✓ Pyrethroids
- ✓ Triazine family of herbicides
- ✓ Phthalates
- ✓ Certain plasticizers
- ✓ Heavy metals Cd and Hg
- ✓ Selected pharmaceuticals
- ✓ Natural and synthetic hormones
- ✓ Flame retardents

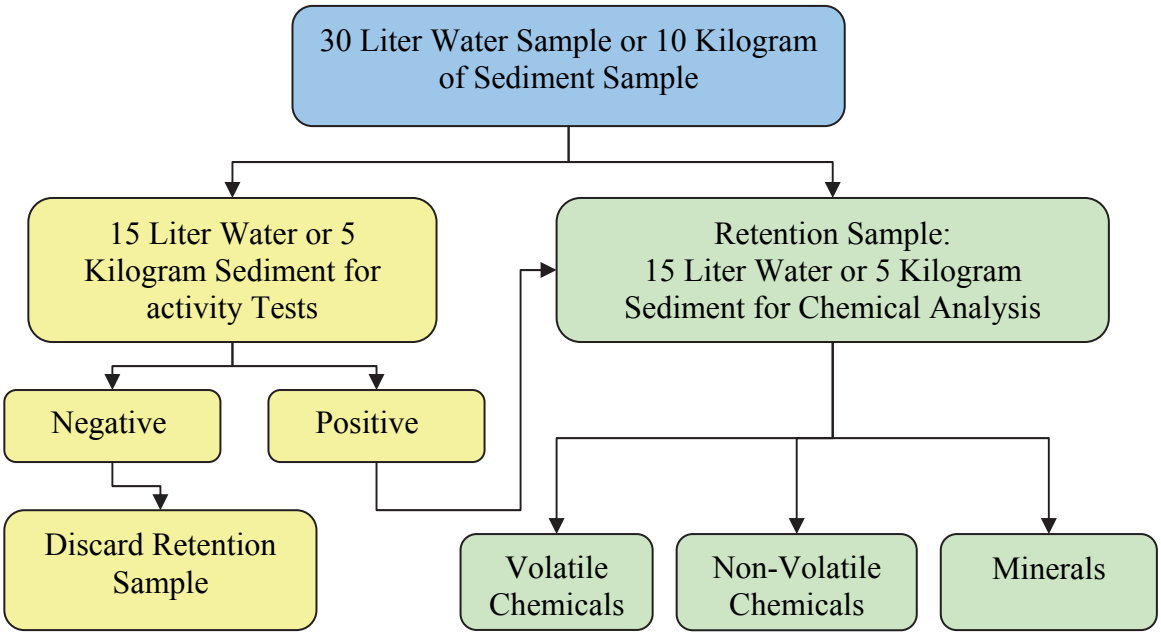
e) Selection of methods used for biological and chemical analysis

It is important that methods be used in the study that will provide data, which will be recognized internationally. Ideally methods of the AOAC and RIVM should be used for the chemical analysis. However such methods may not yet be available when new techniques such as GC-TOF and MIA are utilized. It is recommended that a subcommittee consisting of the laboratory heads and chief scientists is formed to evaluate methods before they are implemented in this study. It may be necessary to conduct inter-laboratory tests to establish the best method to be used.

It will also be necessary to conduct recovery studies on the clean up procedures used in the biological assays in order to establish whether chemicals are not lost during the procedures.

5.2 STEP 2: Pilot study

It is recommended to do a pilot study before embarking on a major monitoring exercise to ensure that all systems are in place, the laboratory personnel trained and methods for biological and chemical analysis validated. It is suggested that the pilot study be done as follows:



1. A limited number of samples (2 water and 2 sediment samples) are to be taken from each of the selected sites. Enough of the sample material should be taken to be utilized for both the biological and chemical analysis. The volume needed is estimated at 30 liters and the mass of sediment estimated at 10 Kg.
2. Each sample taken should be divided into two separate samples of which the first half is to be taken for biological assay to establish whether the sample gives any indication of estrogenicity. The second half of the sample should be extracted and kept as a retention sample. **Care should be taken that the extraction procedure is compatible for all types of chemical analysis i.e. For GC, HPLC, Heavy metal and ELISA analysis.**
3. Should the first sample be positive, the retention half of the same sample should then be taken for chemical analysis. It may be necessary to divide this sample in three separate sub samples for analysis of volatile, non-volatile and mineral analysis.
4. Results of both studies should be submitted to the project manager and project team for evaluation.
5. Depending on the results of the investigation the project team should then decide whether to proceed to the Main Study.

5.3 STEP 3: Main study

It is suggested that the main monitoring study take place over a time period of at least two years. It should follow the same protocol as the pilot study but samples should be taken at least four times a year. The number of samples to be taken at each site will depend on the funding available in the project. It is also recommended that tissue samples of aquatic animals be included in the main study. Most of the persistent chemicals accumulate in the fatty tissue of the fish and other amphibians. The residues found in these animals give a good indication of the historic conditions in the system.

5.4 STEP 4: Evaluation and interpretation of the results

The results of the study should be submitted to the project team, which should evaluate these results and submit a final report

5.5 Proposed project team

It is proposed that the project team consists of the following:

- Two members of WRC of which one should be project manager.
- Two members of the Dept. of Water Affairs and Forestry.
- The heads of the laboratories taking part in the investigation.
- The main researchers from Technikons and universities who may take part in the study.

6. CONCLUSIONS

1. The research done on EDCs in South Africa is of very high standard and is recognized by peer groups overseas. The research is mainly conducted at universities and parastatal research organizations. Each organization works on its own field of expertise with very little co-operation between the researchers. A need exists for a well coordinated research programme to address the problem of EDC and toxicant research in South African water systems.
2. EDC research requires high level of expertise conducted by well qualified and experienced scientists. Most of the research is conducted by post graduate students at universities. On the whole it may be stated that these scientists have the capability to conduct the research. These students, however, can only handle a limited number of samples. Building capacity at Universities may not be a long term solution because of the continuous change in work force.
3. Very little of existing data could be used. The data collected on occurrence of pesticides in the water systems were aimed at toxicity. The EDC effect is observed at levels sometimes a million times lower than the toxic effect. (This is normally measured at the level where cancer may be the result of the contamination). The limits of detection in the methods used in the analysis were therefore not appropriate and false negatives were probably reported.
4. No data existed on some of the most potent EDCs, because these compounds were not in the past regarded as toxicants (natural and synthetic hormones, phthalates and alkylphenols).
5. Although laboratory capability existed to analyze for most of the EDCs there were no facility to conduct analysis of the dioxins and dibenzo furans. The facilities to conduct analysis on hormones were limited to laboratories conducting these tests on blood and urine. These laboratories were unwilling to extend their work to water analysis because their capacity was already stretched to the limit.

6. There is no single test which addresses all the aspects of EDC activity in water. Internationally research is still ongoing in this field. The South African researchers are working in cooperation with overseas institutions to overcome this problem in taking part in the EDC programme of the GWRC, the OECD and other projects.
7. The need for a national programme to monitor EDC and toxicant pollution in water was expressed by the stakeholders during the needs and gaps analysis. For decision making and risk assessment reliable and relevant data is needed. In order to produce this data good facilities and reliable analytical methods are of utmost importance.

7. RECOMMENDATIONS

1. It is recommended that a national programme for monitoring EDCs and toxicants be introduced to generate data which may be used by the authorities to manage the problem of pollution of the water resources of the country.
2. It is of great importance that a facility(s) be created where EDCs and toxicants may be determined. This should be an independent and accredited facility. The international scientific community will not easily accept analytical results that are not generated in such a facility. The implication of this is that universities are not suitable institutions for such a laboratory because it is virtually impossible to get accreditation for a university. It is therefore recommended that such a laboratory be created within one of the state department facilities. The laboratory should have the capability and capacity to conduct chemical analysis for individual EDCs and toxicants and also have the facilities to determine EDC activity by biochemical/biological means. The ideal situation will be that all state departments (DWAF, DEAT, Dept of Health, Dept of Agriculture and DTI) concerned in this issue have a stake in this facility.
3. More research is needed to refine the chemical analysis of EDCs and toxicants, especially the analysis of hormones, flame retardants and dioxins. In the field of activity testing more research is needed in an effort to get a suitable method to determine the effects of EDCs on the nervous system, the immune system and the thyroid function of humans and animals.
4. A human health risk assessment model should be developed to assess the risk of EDC contamination to humans. The classical risk assessment models are not suitable because they take the onset of cancer as an endpoint.
5. A training program should be put in place to train scientists and students in this field.There is also a need for a facility where these scientists may find jobs after completing their studies.

8. CONTACT DETAILS OF RESEARCHERS AND LABORATORY MANAGERS:

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- Bornman, Prof. Riana, University Pretoria tel: 012 3542377
- Brown, Mrs.Liesl, Medunsa, tel: 012 5215841
- Dark, Me Stevie, Cape Town City Lab tel: 021 6841059
- De Jager, Prof. Tiaan, University Pretoria tel: 012 8412684
- Fatoki, Prof. O.S. University Venda tel: 015 9628504
- Garbers, Mr Hein,SABS tel: 012 4286446
- Govender, Mr. P, Rand Water Board, tel: 016 4215150
- Jagals, Prof, .Paul, University of Johannesburg tel: 011 4062608
- Kahn, Dr. Abie, Umgeni Water, tel: 033 3411150
- London, Dr Leslie, University Cape Town tel: 021 4066524
- Meinardt, Mr. Ralph, ARC tel: 082 7763808
- Naidoo, Mr. Clive, tel: 021 4428940.
- Okonkwo, Dr O.J., Pretoria Technikon tel: 015 9628572
- Peall, Mrs. Sue, UCT tel: 021 4428940
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BL Sereda; HR Meinhardt

The agricultural use of pyrethroids and other insecticides registered for crop protection increased in the emergent farmer sectors in KwaZulu-Natal. These areas are protected by the malaria control programme in which DDT and delmethrin are used for indoor spraying of dwellings and constitute a worst–case scenario potential contamination of the water environment. Cases of pyrethroid and organophosphate resistance in the malaria vector Anopheles species were detected. Insecticide resistance in malaria vectors can originate from the selection of mosquitoes to agricultural pesticides.

The research objectives were to establish the patterns of agricultural pesticide used by emergent farmers in two areas, namely Ubombo and Ingwavuma to develop a protocol for sampling, analysis of insecticides and interpretation whereby areas at risk can be investigated for resistance potential within a short period. The residues of the insecticides in water sources near the communities were determined and selected for resistance in malaria vector larvae.

The study area was selected as being the most polluted with agricultural insecticides and anti-malaria chemicals. A questionnaire was used to gather information on a list of pesticides used in the area so as to select the pesticides for residue analysis. Water and sediment samples were collected and analysed for the residues.

Results of the analyses show insecticide contamination of the water environment in the investigation area from pyrethroid, organophosphate, organochloride and carbamate chemical groups. Game reserves selected for control areas supposedly not contaminated were found to be contaminated.

It is believed that the major selection pressure for the development of mosquito resistance exists in the study area and it is crucial to ascertain the relative contribution of the different insecticide classes to the development of resistance. The identification of pyrethroid and organophoshate resistance reported in the study area is of great concern and poses severe consequences in designing an efficient mosquito vector programme. The detection of DDT in the samples shows contamination of the water sources resulting from anti-malaria control interventions and is a serious health threat not only in South Africa, but also internationally, taking into account that DDT is on the Stockholm Convention (2001) list of the 12 POP pollutants that circulate globally through air and water.

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