EXECUTIVE SUMMARY

On a global scale, human, agriculture and industrial activities, historically and recently, have released large quantities of manmade chemicals into the environment. During the nineties, after several keystone papers were published on the potentially adverse effects on human and wildlife health by various environmental contaminants, for example, DDT, ethinylestradiol (contraceptive) and several other estrogen mimics, public concern increased dramatically. It has been suggested that several of these environmental chemicals may modulate the endocrine system, specifically associated with the developmental and reproductive systems of wildlife and humans. The endocrine disruption hypothesis soon emerged, stating that: "Synthetic, and some naturally occurring, chemical substances in the environment are disrupting the normal functions of the endocrine system and its hormones in humans and wildlife" (Society of Environmental Toxicology and Chemistry (SETAC) 2000). In response, international environmental agencies were mandated by their governments to identify endocrine disrupting contaminants (EDCs) by developing screening and testing programmes to supplement chemical detection of known EDCs. Subsequently, the field of EDC research received increased attention. Soon the list of potential endocrine active substances (endocrine modulators) increased.

Endocrine disruption is not an adverse effect *per se*, but rather a mode of action. Chemicals originating from a far reaching range emerged, for example, phyto (plant)-estrogens, natural hormones (e.g., 17ß-estradiol), pharmaceuticals, including synthetic hormones, industrial chemicals (e.g., PCBs in lubricants, coolants, bisphenol A and nonylphenol in polymer and plastic products), agrichemicals (e.g., pesticides, herbicides fungicides) and several consumer products (e.g. food packaging and detergents). From the literature it is clear that although research regarding the extent and effects of EDCs on the quality of life, for wildlife and humans, is progressing fast in most developed countries of the world, relatively little attention, beyond the listing of potential EDCs, has been given to the potential hazard of endocrine disruptors. Although several keystone case studies accessioned the potential health hazard to wildlife the link to human health remains controversial. Wildlife abnormalities reported, include feminization of fish when exposed to sewage effluents, altered sex determination and differentiation in reptiles, including turtles and alligators, limb deformities in amphibians, masculinization in bivalves and gastropods, DDE induced eggshell thinning in raptors, and abnormalities and declined fertility in mammal species, including small mammals, lambs, panthers and aquatic mammals.

The link to human health relates to several aspects regarding male and female reproductive biology, including low sperm counts, increased incidence of testicular cancer and reproductive abnormalities of the male reproductive system (e.g., cryptorchidism, hypospadias), increased risk for breast cancer, ovarian abnormalities and abnormalities of the female reproductive system.

South Africa, although a developing country, but being a user of most of the potential EDCs, including several persistent organic pollutants (POPs), the most well-known being DDT, did not escape this concern. The first WRC funded project, mainly a literature review, regarding estrogen and estrogen mimicking substances in the water environment was published in 2000.

This study recommended that screening and testing methodology be developed or optimized, *in vitro* and *in vivo* bioassays as well as analytical methods for the specific detection of suspected EDCs in the aquatic environment and consumer products. Several reports also mentioned disruption of normal reproductive hormone profiles in several wildlife species, although in a South African context, few studies were available reporting normal values for local wildlife species, including the well studied African clawed frog, *X. laevis*. Another obvious shortfall was the lack of validated biomarkers to assess the disruption of the androgenic system (e.g., anti-androgenic effects).

It was against this backdrop that this research evolved, on the one hand with the focus on learning more about potential endpoints/biomarker systems as well as potential endogenous bio-indicator species on the other hand, but also to explore the available validated endpoints using internationally recognized bio-indicator species to begin to assess the extent of EDC activity in South African water resources. Most of the initial international focus was on the disruption of the male and female reproductive systems, but it was soon recognized that the disruption of the thyroid system should also be a major concern, specifically during early development of organisms. *Xenopus laevis*, was internationally selected (USA-EPA, Japan-EPA, EU and OECD) as a model system, using the control of the metamorphosis (tadpole to frog) phenomenon by the thyroid system as potential biomarker complex. Although the use of local wildlife species in toxicological assessment of the local aquatic health has a long history in South Africa, few studies and reports refer to the non-lethal, subtle effects, mainly because of a general lack of well researched biomarker systems in local species.

Most of the research reported on here, is the culmination of a long and difficult process that started with studies to understand the basic reproductive biology of the aquatic amphibian, *X. laevis* collected from its natural aquatic environment as well as other potential bio-indicator species (e.g. turtles and crocodiles). As the study proceeded, analytical methods had to be developed and new biomarkers identified and studied. The main aim being to eventually establish or contribute towards a battery of EDC tests that could be used in a cost-effective way in South Africa as part of the envisaged toxicology monitoring programme mandated by the National Water Act (Act No 36 of 1998) and employed in local ecological impact assessment. In order to also serve the needs of water suppliers the development of *in vitro* tests were included, as well as, set-up and validation of internationally used bioassays using exotic species under local conditions. This report must be seen against the background of being part of the initial South African EDC initiative when hardly any research had been conducted in this field, specifically, research regarding novel bioassays using indigenous wildlife species and being cost-effective for a developing country.

The project, WRC 926: "An assessment of the extent of estrogenic activity in Western Cape water resources" was followed by project, WRC 1253: "Endocrine Disrupting contaminants (EDCs) in South African water resources: Development and validation of *in vitro* and *in vivo* bioassays to detect endocrine interaction and characterize physiological disruption in non-mammalian animals". Since the research associated with these projects link very closely, it was decided to combine the reports.

The assessment of chemical interaction with the vertebrate endocrine system, especially the reproductive system, including female hormone, estrogen, and male (androgenic) hormones rely on relevant bio-indicator species and specific and sensitive biomarker systems reflecting the levels of such interaction. This research represents a first attempt to utilize local endemic vertebrate species as bio-indicators investigating the potential of specific biomarkers such as the hepatic produced yolk precursor, vitellogenin (Vtg) to determine environmental estrogenic activity.The estrogen (female hormone) inducible proteins (EIP), including the yolk precursor, vitellogenin (Vtg), produced in the liver under control of female hormone, estrogen, was studied as potential end-point for estrogen activity. The African clawed frog (*Xenopus laevis*), internationally used as a model species, was selected as bio-indicator. In this study, Vtg was isolated and anti-Vtg polyclonal as well as anti-EIP monoclonal antibodies produced in rabbits and mice. These antibodies were validated and used to set-up ELISA bioassays to quantitatively estimate Vtg induction in *X. laevis* frogs.

To understand the dynamics of the natural reproductive cycles in potential local bio-indicator fresh water species, including *Xenopus laevis* and the turtle, *Pelomedusa subrufa*, seasonal studies were conducted for the first time on these species.

These base-line seasonal data are a prerequisite for using the selected local species in environmental monitoring programmes. The results show that although *X. laevis* may breed throughout the year a peak in reproductive activity occur in spring and summer months. Reproduction in the fresh water turtles is a seasonal phenomenon and male and female cycles may not be well synchronised. The potential of using local fresh water turtles as bio-indicators of historical EDC exposures and effects of sex determination was confirmed.

Although males don't normally exhibit high levels of circulating estrogens, they have estrogen receptors in the liver and therefore could be used in Vtg induction studies. The development of ELISAs for *Xenopus* Vtg and total EIPs for the first time allowed that frogs could be used as biomonitors for estrogen induction by environmental chemicals. Subsequently, *X. laevis* frogs was used in, *in situ* (caged) studies (21 day exposure) in natural water bodies in three different agricultural areas (vine, fruit and wheat) in the Western Cape to assess possible estrogenic activity in these water resources. In the grape producing area limited estrogen activity was measured along the river as well as on a seasonal basis (4-5 times throughout the year). In the fruit producing area cages were mostly placed in man-made water bodies (dams) receiving run-off from orchards. Limited estrogenicity was measured over the study period. However, in the wheat producing area estrogenicity was measured on several occasions in several sites. Because these laboratory exposures require large numbers of male frogs and to limit the number of live male frog needed to *in situ* caged exposure studies, a *in vitro* liver slice culture bioassay methodology was developed and validated, in which the liver of a single male was used in a tissue culture exposure set-up to study the induction of EIPs and Vtg.. Increased sensitivity was obtained by using liver tissue of males pre-exposed to estrogen in the laboratory. This assay was extensively used to study estrogen activity in water collected from the same agricultural areas as well as other water resources, including sewage effluents, and drinking water.

Limited estrogenic activity was found in several sites, although not in a consistent pattern. However the potential of using the *in vitro* frog liver culture assay as a screening tool for estrogenic activity has been demonstrated.

In order to expand the battery of tests, additional biomarkers for estrogen activity were investigated. For example, estrogen inducible lipo-proteins in *X. laevis* and estrogen induced proteins in cultured chicken cell-line. To expand the use of Vtg as biomarker for estrogen activity, an ELISA determining Vtg (using commercial Vtg antibodies) in tilapia (*Oreochromis mossambicus*) was set-up and validated. In addition a novel universal Vtg antibody was validated and used in a universal ELISA (UniVtg) that could be used for several Vertebrate species to study Vtg induction. This assay was used in several fish species, the frog, *X. laevis*, the chicken, crocodiles and fresh water turtles to measure plasma Vtg levels. This assay eliminates the expensive production of species specific anti-Vtg antibodies for ELISA determinations.

Since endocrine disruption also includes modulation of the male reproductive system, including anti-androgenic activity and thyroid system, bioassays to asses the effect of potential EDCs on these systems were initiated. For this, the androgenic control and histology of the breeding glands in the skin of male *X. laevis* frogs were studied. An initial controlled exposure study, including known anti-androgenic compounds as well as suspected ones, was conducted to evaluate the use of these glands as biomarkers for androgenic effects.

In this study, however, only histological changes proved to be reliable bio-markers of anti-androgenic effects, rather than biochemical aspect of the secretion produced by these structures. The exact nature of the secretion of these glands could not be characterized and needs further study.

Following C18 solid phase extraction protocol of environmental water samples, liver slices were exposed to a range of different water samples, including drinking water, water from bore-holes, rivers and dams in urban and rural agriculture areas, final sewage effluents, and several synthetic chemicals suspected to have estrogenic activity. Results show that if cytotoxic effects are eliminated the liver slice bioassay is a relatively sensitive screen for estrogenic activity in water samples. Although this assay only represents a screening tool, several water samples were found to be positive, in that significant increases in Vtg production by liver slices were measured after a six day incubation period. This bioassay was used in a study regarding the effectiveness of treatment in the Windhoek sewage reclamation plant (direct system) in Namibia. It was found that the activated charcoal filters removed most of the estrogenic activity detected in the untreated sewage. Results also showed some estrogen activity in the source water originating from bore-holes, suggesting some ground water contamination.

These projects were the first to investigate the potential of several biomarkers for estrogenic, anti-androgenic and thyroid related EDC activity using local and international bio-indicator wildlife models. The studies confirmed that male breeding glands in *X. laevis* are under control of androgens and not affected by gonadotrophic activity directly. It was also confirmed that compounds like, DDE and the fungicide, vinclozolin, indeed have anti-androgenic effects in male frogs, including the possible inhibition of breeding behaviour.

Preliminary results also confirmed that metamorphosis of *X. laevis* tadpoles (under control of thyroid hormones) has potential to be used as biomarker to assess EDC activities related to disrupting the thyroid system.

Although the recently revised National Water Act (Act No 36 of 1998), passed by the South African government, is designed to protect our natural water resources, specifically catchments areas, no regulations regarding EDC activity are in place and this research contributes towards the establishment of a battery of tests to assess EDC activity in local water resources or chemicals known to contaminate natural water resources. Moreover, this programme also successfully highlighted the use of biomarkers related to the male androgenic system as well as the sex determination and differentiation developmental systems. In addition, the potential of using the functional involvement of the thyroid endocrine gland in the early developmental and metamorphosis programmes in amphibians as biomarker system for studying the interaction with the thyroid systems proved valuable.

APPENDIX

The following research outputs generated by these projects are available in the literature or on request. The peer reviewed scientific articles submitted for publication, in press or published in local or international scientific journals contain further details on the work summarized in this report.