THE USE OF SENTINAL SPECIES TO DETERMINE THE ENDOCRINE DISRUPTIVE ACTIVITY IN AN URBAN NATURE RESERVE

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THE USE OF SENTINAL SPECIES TO DETERMINE THE ENDOCRINE DISRUPTIVE ACTIVITY IN AN URBAN NATURE RESERVE

by

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EXECUTIVE SUMMARY

Background

There is increasing global concern over persistent bio-accumulative chemicals, their potential for bio-magnification, and, even more worrying, synergistic/additive effects of endocrine disruptor chemicals (EDCs) in mixtures. EDCs are chemicals that interfere with the structure or function of hormone-receptor complexes and may be disruptive at very low exposure levels. The damaging impact of EDCs on health is internationally no longer an issue of dispute (COMPRENDO CREDO Workshop, 2004). At the top of the food chain the aquatic species are particularly vulnerable, but effects have also been observed in terrestrial species (WHO, 2002). Colborn (1994) warned that the environmental load of EDCs has reached critical levels at which human and wildlife is at risk. The risk is not limited to reproductive health, but also general health including immunity, thyroid function, neurodevelopment and others. Recently Hennig et al. (2006) concluded that there is substantial epidemiological evidence that the pathology of cardiovascular diseases is linked in part to environmental pollution. Many environmental contaminants, and particularly persistent organic pollutants, are risk factors for the development of atherosclerosis.

The chemicals of concern for endocrine disruptive effects are not a small group of therapeutic agents, but include many compounds that are in daily use in industry, agriculture and in households (Toppari et al., 1996). Evidence of EDC exposure in South Africa includes high levels of *p*-nonylphenol (*p*-NP) in drinking water (de Jager et al., 2002) and sources similar to those reported to cause feminisation in trout (Routledge et al., 1998), leaching of *p*-NP from plastic wraps into food (De Jager et al., 1998), *p*-NP, polychlorinated biphenyls (PCBs), 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), heptachlor, endosulphan and the chlordanes (Barnhoorn et al., 2004; Fatoki et al., 2003) in selected water and sediment samples and estrogenic activity in water (Aneck-Hahn, 2002; Hurter, 2002; Timmerman, 2002). Catfish previously collected from sites in the Urban Nature Reserve (UNR) had high levels of selected EDCs in fat tissue (Barnhoorn et al., 2004). This reserve is one of the world's largest urban nature reserves, situated south of the City of Tshwane (also known as Pretoria) but still within the city limits. The Reserve is at an altitude of 1700m and is one of the very few reserves situated in the grassland biome on the central South African highveld. The stream into the Reserve receives effluent from sewage treatment plants, industries and informal settlements in the catchment areas (part of Ekurhuleni Metropolitan area). There are two dams, Dam 1 (D1) and Dam 2 (D2), interconnected with a wetland and channel.

This report summarizes the scientific background relevant to the study with emphasis on chemical residue analyses, endocrine disruptive metals (EDMs) and bio-assays for estrogenicity and dioxin and dioxin-like PCBs. It also reviews the use of possible biosentinel aquatic and terrestrial animals. General information on the UNR and the outcomes of previous projects are discussed. This is

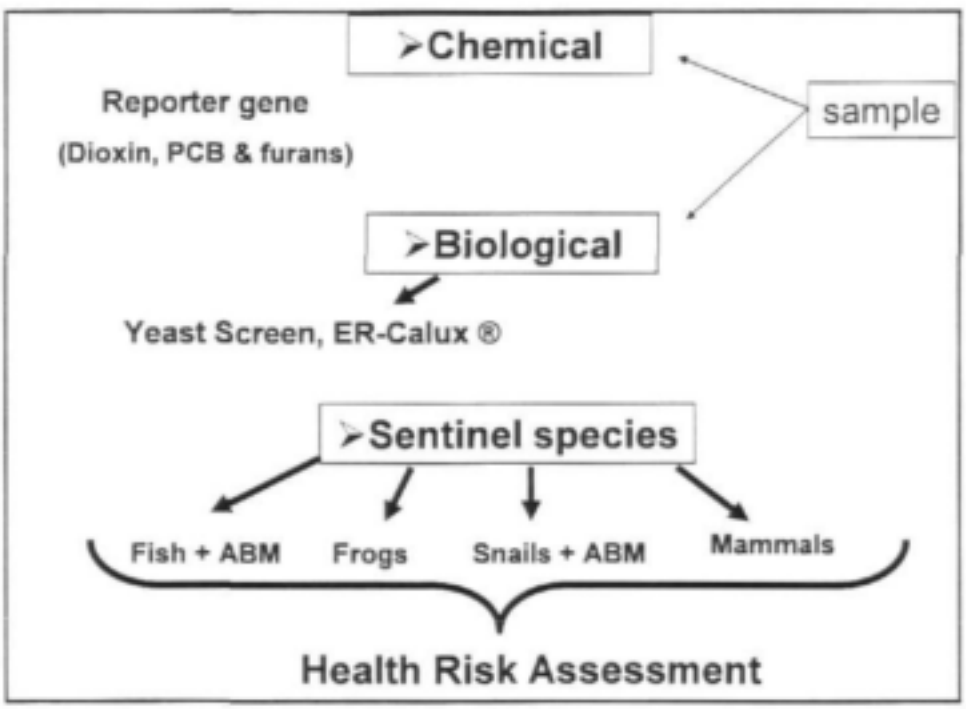
followed by separate chapters on analytical chemistry and *in vitro* bio-assays. *Clarias gariepinus* (sharptooth catfish), *Xenopus laevis* (African clawed frog), *Bulinus tropica* (freshwater snail) and *Rhabdomys pumilio* (striped mouse) were evaluated as possible biomarker species for EDC exposure. The impact of active biomonitoring (ABM) on fish and snail species in the UNR, the effect on macro-invertebrates (SASS5) and the possible role of plants in the wetlands were addressed separately. All the information gathered was integrated in a qualitative scenario-based health risk analysis and a toolkit recommended for future use.

Objective

The objective of this study was to determine whether sufficiently high levels of EDCs exist in the general environment to exert adverse health effects on aquatic or terrestrial animals or humans in and around the Urban Nature Reserve (UNR).

Methodology

Water and sediment samples were collected from D1, channel, vlei and D2 every two months over a period of two years and analysed for target chemicals and biological assessment of water for estrogenicity (Yeast assay, YES) and the ER-Calux ® reporter gene assay. Sediment samples were also analysed for dioxin- and dioxin-like chemicals (reporter gene) see in Figure below. Samples were also collected from possible 'control' sites at Lapalala Wilderness Game Reserve (LWGR) and Suikerbosrand Nature Reserve (SBR).



Schematic summary of samples and methodology.

The Urban Nature Reserve and catchment area.

Results

Chemical and biological analyses indicated that residues of polychlorinated biphenyls (PCB), octyl- (OcP) and *p*-nonylphenol (*p*-NP) were detected in water and sediment samples from the so-called 'control' sites LWGR and SBR, confirming the international concern that there is nothing like a "clean, pristine" uncontaminated site. Even more worrying were the high levels of bio-accumulated lindane, DDT and metabolites and PCB153 in fat samples collected from eland at SBR.

At the four sampling sites and in various matrices several chemical compounds and metals were present at levels of concern for human consumption. These included residues of α-BHC, lindane, endrin, heptachlor epoxide, methoxychlor, DDT and metabolites, OcP, *p*-NP, dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP). The phthalate residues were present in all samples, followed by lindane in 11 (31.4%) samples and *p,p'*-DDT in 9 (25,7%) samples. No dioxin- or dioxin-like activity was found, although cytotoxicity occurred. Cadmium levels in water from D1 and Vlei samples had levels, on average, above the target water quality range (TWQR), but at all four points above 0.3µg/L (above chronic effect value (CEV)) were also detected. In all the samples lead (Pb) levels were above 15µg/L, higher than the acute effect value (AEV) for Pb (DWAF, 1996), with D1 and Vlei having the highest levels.

At all four sampling sites in the UNR estrogenic activity was detected in the water samples. The YES assay detected estrogenicity in 9 (35%) samples ranging from 0.16 – 1.92ng/L. However, cytotoxicity was present in 69% of samples, which could lead to an underestimation of the degree of estrogenicity. This was confirmed with the ER-Calux ® assay in which the estrogenicity ranged from 0.32 – 16ng/L. 80% of the samples contained estrogenicity above 1ng/L implying that the water was posing a risk to reproductive damage in fish (Matthiessen et al., 2006).

Biosentinel animals

In sharptooth catfish, *Clarias gariepinus*, abnormalities of the sexual papilla, equivalent to a "penis" in fish, was found in 30.8% and 22.2% of catfish from D1 and D2 respectively. Testicular oocytes were observed in 63.2% of male fish collected from D1, and in 36.8% fish from D2. These figures also reflect the incidence of intersex in catfish from the UNR. With immunocytochemistry techniques the occurrence of inappropriate cell death, as well as an increased rate of cell death (apoptosis), were demonstrated in testis of D2 catfish when compared to reference site. The germ cell stage-dependent nature of apoptotic death in D2 catfish affected the androgen-sensitive stages of spermatogenesis in *C. gariepinus*.

After extensive trapping (total 2400 cumulated trap hours) only 7 male and 13 females African Clawed Frog, *Xenopus laevis* were collected at D2, and the sex ratio was M:F=1:4. No *Xenopus* was found in D1.

Freshwater snail, *Bulinus tropicus* was the major species found at the UNR and reflects the drastic decline in species diversity since 1996. The ratio of sheath:preputium of snails from D2 was significantly different when compared with similar measurements from both live-caught and preserved specimens. In six snails from D2 no penis was present.

Active biomonitoring (ABM) using *M. tuberculata* and *C. gariepinus* indicated that the ABM study interfered with energy metabolism, specifically the energy consumption (E_a).

All 24 eland, *Tragelaphus oryx* had testes in scrotal position and no overt epididymal lesions or cystic dilatations were noted. Macroscopically, focal white gritty areas were observed in testes of all eland which, on sectioning, appeared as grey-white, calcified areas varying in size, dispersed throughout testes without a specific distribution pattern. Microscopic examination of testicular sections containing these white gritty areas revealed loci of seminiferous tubules with sperm stasis and dystrophic calcifications but the degree and extent of mineralization varied between samples.

In several sections in which mediastinum testis was present, lesions of the rete testis characterized by epithelial hypertrophy and adenomatous proliferation were observed. The proliferative lesions of the rete testis were also associated with seminiferous epithelial degeneration in segments of the tubules proximal to the affected rete. The degenerative lesions included vacuolization of Sertoli cells, and death and desquamation of differentiating germ cells. Although full complement of spermatogenesis was observed in focal areas, spermatogenesis was generally impaired consequent to progression of degenerative changes in seminiferous epithelium. The progression of degenerative process was manifested by a spectrum of lesions including complete sloughing of seminiferous epithelium, calcification of exfoliated detritus (microlithiasis), granulomatous reaction and, ultimately, fibrosis of lobules of seminiferous tubules. Although a few atypical germ cells were encountered in eland testes, detailed morphological evaluation ascertaining carcinoma in situ (CIS) was not possible because of limitations of tissue fixation and processing.

In Striped Mouse (*Rhabdomys pumilio*) the mean cauda epididymal sperm count for the mice was relatively low 22.1 x 10^6/ml, but more importantly two animals were without any sperm (azoospermia). Spermatogenis was incomplete on histology of the testis and clear evidence of possible toxin damage was observed. These include apical sloughing of the germinal epithelium, degeneration of spermatogonia, vacuolization and seminiferous tubule shrinkage. Sperm motion parameters were successfully analyzed with the CASA system.

Health risk assessment

The assessment indicates that if untreated water from D1 and D2 is used for domestic purposes, unacceptable health risks (carcinogenic as well as toxic effects) could be anticipated. The greatest health concern would be if this water is used for irrigation of vegetables with a hypothetical risk of developing cancer calculated to be 2 in 1 000 for D2 water and 1 in 1 000 for D1. The risks of dermal absorption was in the order of 5 and 4 in 10 000 for D1 and D2, respectively. Lindane, DDT, and DEHP were the chemicals causing this risk in D2, and DEHP in D1. Domestic use for consumption of water had an estimated risk of 3 in 10 000 and 4 in 10 000 risk of developing cancer for D2 and D1 respectively. DEHP is the chemical responsible for this particular risk. Adding the possible impact of endocrine disruptive metals (EDMs), hazard quotients were 27 to >450 times higher than that assumed to be safe for a life time consumption.

Discussion of results

The most probable sources of lindane were the chicken farm and landfill site, but since lindane is used as an insecticide on animals and animal premises, the other farming activities should be seriously considered. The slope from the farm favours the run-off of water towards the Channel and D2. The water from D1 had low lindane levels and, therefore, the possibility that lindane was coming from the

stream into the UNR, seems negligible. The slope from the landfill site and run-off water will also only affect the D2 values. On the other hand, contamination of ground water and underground connections with D2 seems a possibility, but this does not apply to the Channel because of the building construction.

Lindane in surface waters and soils is taken up and bioconcentrated by terrestrial and aquatic organisms (Just et al., 1990; Matsumura and Benezet, 1973; Ramamoorthy, 1985; Verma and Pillai, 1991; Viswanathan et al., 1988) and accumulates in the food chain (Szokolay et al., 1977). This process also applied to the UNR because of the high lindane levels in fish collected from all sites and sampling events.

The major component of technical grade DDT is the *p,p'*-DDT (85%) isomer, but it also contains *o,p*-DDT (15%), and *o,o'*-DDT (trace amounts). Technical grade DDT may also contain DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane) and DDE as contaminants. DDD was used previously as pesticide, but to a far lesser extent than DDT. Both DDD and DDE are breakdown products of DDT (ATSDR, 2002).

The DDT residues detected at the UNR could be a result of past use in farming activities. It may also still be released from the landfill site north of D2, as observed in the United States of America (USA) (ATSDR, 2002) or it may also be released into the atmosphere from areas in South Africa where DDT is still being used for malaria vector control (Limpopo, Mpumalanga and KwaZulu-Natal). DDT and its metabolites also enter the atmosphere through the volatilization of residues in soil and surface water, albeit a result of past use. These chemicals will be deposited on land and in surface water as a result of dry and wet deposition. The process of volatilization and deposition may be repeated many times, and is referred to as a 'global distillation' from warm source areas to cold Polar Regions. Consequently, these chemicals have been found in snow, and animals in the Arctic and Antarctic regions where DDT was never used (ATSDR, 2002). It may also be the case with the UNR that the DDT residues were the result of environmental distillation. However, the possibility of illegal use and dumping of DDT into water could not be excluded. The finding of almost similar levels at all the sites, except the higher levels at D2 in November 2004, is in favor of precipitation from polluted air. For the reason of the extensive past use of DDT worldwide and the persistence of DDT and its metabolites, these chemicals are now virtually ubiquitous and are continually being transformed and redistributed in the environment.

In surface water, DDT will bind to particles in the water, settle, and be deposited in the sediment from where it is taken up by small organisms and fish in the water. DDT, DDD, and DDE accumulate in fatty tissues and from the aquatic food webs may enter higher trophic levels of the food chain. The

impact of bioaccumulation on fish was emphasized by the very high DDT and metabolite concentration in fat.

Of even greater concern is the possible impact of the high levels of *p,p'*-DDE, the persistent metabolite of DDT on the living organism due to its anti-androgenic properties (Kelce et al., 1995). Adverse reproductive system effects associated with *in utero* DDT or DDE exposure in male animals include, amongst others, abnormal development of ovarian tissue (Fry and Toone, 1981), reduced penile size (Guillette and Guillette, 1996), hypospadias (Gray et al., 2001) and cryptorchidism (Facemire et al., 1995; Gray et al., 2001). Although the high values were found in male catfish there seems to be no reason why females should not have similar fat concentrations. If that is the case, the high DDT and metabolites will affect both oogenesis and spermatogenesis and may contribute to the defects seen in the sharptooth catfish. However, estrogenic and anti-androgenic chemicals in the aquatic environment water are most likely to affect the developing embryo after fertilization, especially if the free swimming larvae are male. This may contribute to the "feminization" of the male urogenital papilla (UGP) as well as the development of testicular oocytes and intersex. In this study altogether 28 of 97 (28.9%) intersex males were collected from the UNR over the two year period, which is extremely high. Although the chemical insult occurs during early embryogenesis, the effect will only become evident in later life stages.

Although tissue concentrations of target chemicals were not measured in snails, frogs and small mammals, it is hypothesized that in D2 snails both the smaller penile sheath (containing the penis) and prepuce could result from feminizing compounds such as estrogenics and anti-androgenics. This possibility was supported by the novel finding of penile agenesis in six snails, which has not been reported in either *B. tropicus* or South African waters before. Similar effects were reported by Tillmann et al. (2001) in *Marisa cornuarites*, a freshwater gastropod, after exposure to antiandrogens. Furthermore, the absence of mayflies and stoneflies and the abundance of highly tolerant organisms were, amongst others, signs of poor water quality in the system generally indicating industrial pollution (Graham and Dickens, 2002; Williams and Feltmate, 1992). The reduced biodiversity in the snail and macroinvertebrate population at the UNR is alarming and further supports the concern on the magnitude of environmental pollution. This aspect has to be studied in greater detail.

Although the frogs appeared to do fine in this system the skewed sex ratio observed in the main stream is of concern. The sample size is too small to come to a conclusive answer, but despite various extensive attempts, only a limited number of frogs could be found. This was attributed to the predatory sharptooth catfish, however, the possible impact of chemical pollution could not be excluded. The ratio of 4 females for each male is abnormal and of concern as the normal picture for

Xenopus is a 50-50 ratio ± 10% (Du Preez et al., 2005). It is known that pollutants in water can cause demasculinization or complete sex reversal in frogs (Clark et al., 1999) and is a matter of concern.

Not only aquatic animals at the UNR seemed to be affected, but also terrestrial animals. Barnhoorn et al. (2002) found macroscopic calcification in testes of eland (*Tragelaphus oryx)*. On microscopy epithelial hypertrophy and adenomatous proliferation of the rete testis were observed as well as degeneration of the seminiferous epithelium. The degenerative lesions included vacuolization of Sertoli cells, and death and desquamation of differentiating germ cells. Although full complement of spermatogenesis was observed in focal areas, spermatogenesis was generally impaired consequent to progression of degenerative changes in seminiferous epithelium. The presence of adenomatous lesions of the rete testis in the eland was similar to diethylstilbestrol-induced rete adenocarcinoma in laboratory animals (Newbold et al. 1985; Newbold 2000) and was, therefore, indicative of possible chronic estrogenic exposure. The findings in eland (Bornman et al., submitted) are similar to the testicular dysgenesis syndrome in humans attributed to developmental exposures to chemicals (Skakkeback et al., 2001). The fat samples of UNR eland contained high levels of the estrogenic compound, *p*-NP.

The finding of similar macroscopic and microscopic appearances in eland testes collected from SBR was completely unexpected. Where the UNR eland fat samples contained *p*-NP, at SBR not only *p*-NP, but also OcP and high levels of PCB153 and DDT. Both alkylphenols and the persistent organochlorine pesticide DDT (both isomers and their metabolites DDE and DDD) have estrogenic activity (Sonnenschein and Soto, 1998; Sonneveld et al., 2005). Thus, although the possibility of simultaneous exposure to estrogenic agents such as phytoestrogens and estrogenic mycotoxins cannot be ruled out, considering the spectrum of pollutants present in the body fat, the testicular lesions observed in eland could have been caused by chronic impingement of these chemicals. In experimental paradigms, it has been demonstrated that a variety of xenobiotics released from fat during fasting produce estrogenic effects (Bigsby et al, 1997). The magnitude of concentration of estrogenic pollutants in body fat indicates that EDCs bio-concentrate in terrestrial mammals as in aquatic life (Barnhoorn et al., 2004). The levels of *p*-NP were even higher than those found in fat collected from catfish inhabiting the dams in the UNR. Those male fish had signs of feminization as well as a high prevalence of intersex. Water and sediment samples had estrogenic activity on the yeast screen test (Aneck-Hahn, 2002). The eland were dependent on these water sources in the Reserve.

Exposure *in utero* to *p,p'-DDT* or its metabolite *p-p'-DDE* or OcP induced atypical germ cells resembling carcinoma in situ (CIS) in rabbits (Veeramachaneni, 2000; Veeramachaneni, 2006). Human testicular cancer arises from CIS cells, which are suspected to originate from primordial germ cells that escaped normal differentiation *in utero* (Skakkebæk et al., 1987; Rajpert-De Meyts et al.,

1998). The first cases in animals of atypical germ cells resembling CIS cells of human testis were reported in a subfertile, unilaterally cryptorchid stallion (Veeramachaneni and Sawyer, 1998) and an infertile rabbit (Veeramachaneni and VandeWoude, 1999). Although a few atypical germ cells were encountered in eland testes, detailed morphological evaluation ascertaining CIS was not possible because of limitations of tissue fixation and processing. Interestingly, a variety of testicular tumors including rete adenocarcinoma and seminoma along with microlithiasis and CIS were found in Sitka black-tailed deer suspected to have been developmentally exposed to an environmental estrogenic agent(s) (Veeramachaneni et al., 2006). For these reasons, the novel findings in eland may be the first evidence that non-aquatic wildlife are also being impacted by environmental pollution of EDCs in South Africa.

The findings of Sertoli cell vacuolization and sloughing of the epithelium observed in eland were similar to those observed in rats following experimental exposure to *p*-NP (De Jager et al., 1999a), and also similar to findings in small mammals. In the striped mouse the testicular histopathology showed various degrees of degeneration including apical sloughing, degeneration of spermatogonia, vacuolization and shrinking of the seminiferous tubules. These findings are typical of exposure to chemicals such as DDT, PCBs and other EDCs (De Jager et al., 1999a). In another terrestrial species from the UNR, Bornman et al., (submitted) found in eland generally impaired spermatogenesis, Sertoli cell vacuolization and sloughing of the seminiferous epithelium. Moreover, adenomatous changes of the rete testis, reflective of possible chronic estrogenic exposure, were found. Although the number of mice studied was small, the low sperm count and absence of any sperm in two mice are reasons for concern. A decrease in sperm count is the most common manifestation of testicular dysgenesis (Bay et al., 2006), and although tissue levels of target EDCs were not measured, it seems likely that these chemicals could have had an effect. The finding of impaired spermatogenesis in these terrestrial animals raises the question what there source and route of contamination would have been. This could imply a much wider and more serious level of environmental contamination in the UNR than previously anticipated. These aspects should be addressed in future projects.

The inappropriate occurrence of cell death in D2 catfish emphasized the effect of chemicals on the testis. The water (per L) contained 14.04ug lindane, 3.18mg DEP, 3.93mg DMP and 0.46mg DEHP respectively, was toxic and had estrogenic activity. Moreover, the catfish fat samples bioconcentrated these chemicals and contained lindane, aldrin, PCB153 and DDT and metabolites. The concentrations in fat reflect the body burden of these chemicals, and therefore provide an indication of the exposure at a cellular level. The catfish had a significantly higher incidence of caspase 3-dependent germ cell apoptosis, compared to laboratory-reared catfish, both in terms of number of caspase-positive cells and the number of spermatogenic tubules containing at least one cluster of apoptotic cells, all of which may be the molecular basis of testicular regression and/or demasculinisation. The apoptotic events

occured predominantly in primary and secondary spermatocytes, germ cell stages that are exquisitely androgen-dependent. The findings of this study will form the basis of future endeavours in which *C. gariepinus* will serve as a sentinel species to study the effects of endocrine disruptors on male reproduction.

Not only agrochemicals were present in the water and sediment samples, but also industrial chemicals including OcP, *p*-NP and the phthalate esters. The compounds were present at all four sites, albeit in higher concentrations in D1. Phthalates are a family of chemicals that are produced in the millions of tons annually worldwide, and are a principal component of many diverse products such as flexible polyvinyl chloride plastic (PVC), cosmetics and other personal care goods, pesticides, building materials, lubricants, adhesives, and film, among other items. Okubo et al. (2003) found that various phthalate esters suggested anti-estrogenic activities *in vitro*. Both DBP and DEHP affect particularly the developing male reproductive tract, although effects on the liver, kidneys, lungs, and blood clotting are also of concern (DiGangi et al., 2002). Phthalates are ubiquitous in the environment and it is possible that humans are continuously exposed to them. The most possible source is from the industrial activity higher up in the catchment, also because the levels in D1 were higher than at the other sites.

It is important to note that not all effects of EDCs are mediated via the cell receptor pathway (Pretorius and Bornman, 2005). Chemicals like *p*-NP, lindane and others may use non-receptor-mediated mechanisms and disrupt the cell ion pumps, such as Ca^{2+}. Disruption of Ca^{2+}-pumps will also disrupt the endoplasmic reticulum Ca^{2+} pumps and function (Hughes et al., 2000), as well as inhibit the inositol triphosphate (IP_3)-sensitive Ca^{2+} channels. These molecular events are crucial in particularly the Sertoli cell (Khan et al., 2003). Each Sertoli cells supports a specific number of spermatogonia and developing sperm during spermatogenesis. The Sertoli cells also forms the blood-testis barrier and controls the transport of substances to the developing sperm. Impairing Sertoli cell function will negatively impact on the development of especially germ cells that are sequestered behind the blood-testis barrier, such as spermatids and sperm (Bergmann et al., 1984).

Not only EDCs were present in the water and sediment, but the four endocrine disruptive metals (EDMs) were also detected in water from all four sites. Cadmium (Cd) levels were high in D1 and Vlei, but, although lower at other sites, still above the chronic effect value (CEV). Lead (Pb) occurred in all samples at levels higher than the acute effect value (AEV) (DWAF, 1996). Mercury (Hg) was only once detected (>AEV) and arsenic (As) was high in D2. These metals may be endocrine disruptive, or add to the disruption in a synergistic or additive way. Irrespective of the precise mechanism, the EDMs most probably impact negatively to an already overloaded organic system.

A *quantitative* health risk assessment for known carcinogenic and toxic effects was carried out. Where limited data and information was available, a *qualitative* health risk assessment approach was used. Hypothetical communities were evaluated; one exposed to D1 water for domestic, agricultural and irrigation purposes, as well as consumption of fish, and one community exposed to D2 water, for domestic, agricultural and irrigation purposes, as well as consumption of fish. The USEPA health risk assessment method was used comprising hazard-, dose-response-, and exposure-assessment, followed by risk characterisation.

The health risk assessment indicates that if untreated water from D1 and D2 is used for domestic purposes, unacceptable health risks (carcinogenic as well as toxic effects) could be anticipated. The greatest health concern would be if this water is used for irrigation of vegetables with a hypothetical risk of developing cancer calculated to be 2 in 1 000 for D2 water and 1 in 1 000 for D2! Any risk over 1 in 100 000 is considered by the World Health Organisation (1993) to be unacceptable.

This risk is followed closely by dermal absorption of the chemicals through direct contact, with risks of developing cancer in the order of 5 and 4 in 10 000 for D1 and D2, respectively. Lindane, DDT, and DEHP were the chemicals causing this risk in D2, and DEHP in D1.

Domestic use for consumption of water was also found to result in unacceptably high risks with an estimated 3 in 10 000 and 4 in 10 000 risk of developing cancer for D2 and D1 respectively. DEHP is the chemical responsible for this particular risk.

Risks of toxic effects were unacceptably high, particularly for the use of untreated water for vegetable watering (caused by DDT, DEHP and DBP). Risk of toxic effects was also high through dermal absorption for D2 and by DBP for D1.

Adding the possible impact of EDMs, hazard quotients were 27 to >450 times higher than that assumed to be safe for a life time consumption. The cancer risk for As was calculated to be close to 1 in a 100.

Risks were also calculated for the organic chemicals assuming 80% reduction as a result of going through a treatment process. This is the assumed removal of estrogen mimicking compounds going through a water treatment process using inactivated carbon (Snyders et al., 2003). It appears that the treated water is safe to use, provided that the process remains functional and there is no massive dumping of chemicals.

A qualitative health risk assessment making use of both ecological data and the concentration of natural and synthetic hormones in D2 was also undertaken. The results indicated that the risk for endocrine disruption, focussing on reproductive effects, is high. This assessment is based on the effects observed in many different levels of animals and includes the fish and snails, and more relevantly, the small and large mammals. The hormones found in D2 in 2002-2003 (Burger et al., 2006 *in press*) were at concentrations in the order of 25 – 350ng/L with estrogenic activity measuring at 0.16 ng/L estradiol equivalents. These levels are up to 10 000× higher than those known to cause initiate activity in breast cancer cells. Even assuming that the cells are far more sensitive at inducing estrogen mimicking effects, one can still assume that these effects can be anticipated in a human population based on both the hormone and ecological results.

In summary, one can expect that untreated water from both D2 and D1 used for domestic, agricultural or recreational activities would result in unacceptably high human health risks. These risks include carcinogenic risks, toxic effects and endocrine disruption.

Conclusion

Although no method is available to calculate the long-term risk of EDCs and EDMs on the ecosystem of the UNR, the findings of high chemical residue levels in water, sediment and tissue, skewed sex ratios, reduced biodiversity, gonadal malformations in sharptooth catfish and freshwater snails, intersex in catfish, histological impacts on spermatogenesis in catfish and striped mouse are matters of serious concern. It is highly unlikely, if at all possible that such a diversity of effects in a range of biosentinel animals, could be coincidental.

Recommendations for future research

1. Conduct pollutant source inventory in immediate catchment, involving DWAF to identify point and diffuse sources, either current or latent (buried or underground) sources.
2. *In vivo* exposure studies on fish using alkylphenols, DDT, lindane, Pb and Cd.
3. Further investigation on *Bulinus tropicus* as EDC biomarker.
4. Further development of small mammals as EDC sentinels.
5. Investigate for EDC effects in food chains, both aquatic (yellow fish), terrestrial (fox/meerkat), and wetland (frogs and birds).
6. Environmental contamination and biodiversity of the fish population at the UNR.
7. Chemical and bio-assays of tap drinking water in the Metropolitan area.
8. Determine water contact patterns of people using the UNR water, as well as people in the catchments.

Recommendations from Workshop held at the Urban Nature Reserve 24 January 2007

1. Risk assessment of the pollutants in the catchment area should be executed by engineers using Risk Based Environmental Management System (RBEEMS) and Discretized Source Pathway Receptor Element Model (DISPREM).
2. The results should be reported to other catchment management forums throughout the country.
3. The information generated during this project should be used in the further development and refinement of the South African Water Quality Guidelines and in the National Monitoring Programme.

A. CONFERENCE PRESENTATIONS

Aneck-Hahn NH, de Jager C, Barnhoorn IEJ and Bornman MS. Endocrine disruptors and health: should South Africa be concerned? Conference Proceedings WISA, Biennial Conference and Exhibition. Durban International Convention Centre, Durban, South Africa, 21-25 May 2006.

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Bornman MS. Diseases of Sexual Development in Humans and Wildlife - a South African Environmental Health Perspective. Department of Biomedical Sciences. Seminar held at the Department of Biomedical Sciences, Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins Co, USA 31 January 2007

van Dyk JC, Barnhoorn IEJ, Marchand MJ, Pieterse GM, Bornman MS and van Vuren JHJ. A histo-morphological study of the testes of *Clarias gariepinus* and *Oreochromis mossambicus* from endocrine disrupting chemical polluted waters in south Africa. Poster presentation SETAC Europe 17th Annual meeting, 20-24 May 2007, Porto, Portugal.

Marchand MJ, Pieterse GM, Barnhoorn IEJ, and Bornman MS. Histopathological alterations in the gills of *Clarias gariepinus* as an indicator of exposure to environmental toxicants. Poster presentation SETAC Europe 17th Annual meeting, 20-24 May 2007, Porto, Portugal.

Van Vuren JHJ, Bornman MS and Wepener V. Bio-indicators in the assessment of the effects of chemicalson aquatic species in an Urban Nature Reserve, South Africa. Platform presentation SETAC Europe 17th Annual meeting, 20-24 May 2007, Porto, Portugal.

Pieterse GM, Van Dyk JC, Marchand MJ, Van Vuren JHJ, Barnhoorn IEJ, Bornman MS. A quatitative and qualitative histological assessment to determine fish health in polluted water in South Africa. Poster presentation SETAC Europe 17th Annual meeting, 20-24 May 2007, Porto, Portugal.

Bornman MS ,Barnhoorn IEJ, Dreyer L, Veeramachaneni DNR. Testicular microlithiasis and Neoplastic lesions in eland (*Tragelaphus oryx*) in South Africa: Sequelae of exposure to environmental pollutants. Poster presentation SETAC Europe 17th Annual meeting, 20-24 May 2007, Porto, Portugal.

The same topics listed under Publications will be prepared for presentation at various meetings.

B. PUBLICATIONS TO BE EMANATING FROM THIS PROJECT

McClusky LM, Matlala E, Barnhoorn IEJ, Bornman MS, van Dyk JC. 2007. Increased caspase 3-dependent testicular apoptosis in feral sharptooth catfish (*Clarias gariepinus*) inhabiting xenoestrogen-polluted water sources. (submitted)

Kruger T, Barnhoorn IEJ, van Vuren JHJ, Bornman MS. The effects of synthetic estrogens on the secondary sexual urogenital papilla of feral Sharptooth Catfish (*Clarias gariepinus*) from an estrogen polluted source, Gauteng, South Africa.

Moolman L, Kruger T, Shaddock B, Barnhoorn IEJ, van Vuren JHJ, Bornman MS. Evaluation of the use of active biomonitoring (ABM) and the biomarker methodology as a measure of the water quality status of an Urban Nature Reserve. (submitted)

Barnhoorn IEJ, Bornman MS, van Vuren JHJ, Kruger T. The use of the sharptooth catfish *Clarias gariepinus* as a sentinel species to indicate possible endocrine disrupting chemical (EDC) pollution in freshwater systems of South Africa.

Wolmarans CT, de Kock KN, Bouwman H. Possible effects on the reproductive organs of *Bulinus tropicus* from the Urban Nature Reserve, South Africa.

Bornman MS, van Vuren JHJ, Bouwman H, de Jager C, Genthe B, Barnhoorn IEJ. Endocrine disruptive activity and the potential health risk in an Urban Nature Reserve.

Bornman MS, Barnhoorn IEJ, Dreyer L, de Jager C, Veeramachaneni DNR. Testicular microlithiasis and neoplastic lesions in eland *(Tragelaphus oryx)* in South Africa: Sequelae of exposure to environmental pollutants?

Aneck-Hahn NH, van Zijl C, Bornman MS: Estrogenic contamination of water sources in an Urban Nature Reserve.(submitted)

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D. KNOWLEDGE DISSEMINATION

A workshop and information session were held at the Urban Nature Reserve on 24 January 2007. Recommendations and future projects have been proposed (Refer to p. xiii)

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Water Research Commission Project Steering Committee

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LIST OF ABBREVIATIONS

°C	degrees Celsius
11-KT	11-ketotestosterone
A	alleles per locus
ABM	active biomonitoring
AEV	acute effect value
Ah-I	aryl-hydrocarbon-immunoassay
ALH	amplitude of later head displacement
ALP	alkali-labile phosphate
amsl	above mean sea level
APEOs	Alkylphenol polyethoxylates
APs	alkylphenols
AR	Androgen receptor
ARC	Agriculture Research Council
Arnt	Ah receptor nuclear translocator
As	Arsenic
ASPT	average score per taxa
ATCC	American Type Culture Collection
ATSDR	Agency for Toxic Substances and Disease Registry
BCF	beat-cross frequency
BHC	benzene hexachloride or gamma-HCH
BMWP	Biological Monitoring Working Party
BNF	buffered neutral formalin
Bo	Maximum binding
BPA	Bisphenol A
BUBE	Butyl benzoate
CALUX	Chemically Activated Luciferase eXpression
CASA	Computer-Assisted Sperm Analysis
CAT	Catalase
Cd	Cadmium
CDC	Centre for Disease Control and Prevention

List of Abbreviations continues

CDCH	caudodorsal cell hormone
CEA	cellular energy allocation
CEV	chronic effect value
cf-VTG	catfish vitellogenin
CIS	carcinoma in situ
Ck	creatine kinase
COC	Committee on Carcinogenicity
CPA	cyproterone acetate
D1	Dam 1
D2	Dam 2
D2P	Dam 2 Population
D1P	Dam 1 Population
DBP	di-*n*-butyl phthalate
DCM	Dichloromethane
DDD	1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane
DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene
DDT	1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane
DEHP	di-(2-ethylhexyl) phthalate
delta ALAD	delta amino levulinic acid dehydratase
DEP	diethyl phthalate
DES	Diethylstilbestrol
DL	detection limit
DEX	dexamethasone
DHEA	Dehydroepiandrosterone
DHHS	Department of Health and Human Services
DHT	Dihydrotestosterone
DM	deltamethrin
DMP	Dimethylphthalate
DNA	Deoxyribose Nucleic Acid
DWAF	Department of Water Affairs and Forestry
E_2	17-beta-estradiol
E_a	Available energy reserves

List of Abbreviations continued

E_c	Energy consumption
EC_{50}	Effective concentration in 50% of exposed animals
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EDCs	endocrine disrupting chemicals
EDMs	endocrine disruptive metals
EDSTAC	Endocrine Disruptor Screening and Testing Committee
EE	estradiol equivalents
EE_2	Ethinylestradiol
EE-Max	maximum estradiol equivalents
Eh	potential
ELISA	enzyme linked immunosorbent assays
EPA	Environmental Protection Agency
EPCB	Arochlor 1:1:1
ER	estrogen receptors (α and β isoforms)
ERE	estrogen-responsive elements
ER-α and ER-β	estrogen receptor -alfa and ER-beta
ESHRE	European Society for Human Reproduction and Embryology
Est	Esterase
ETS	electron transport activity
Fe	Iron
FETAX	Frog Embryo Teratogenesis Assay – Xenopus
FQPA	Food Quality Protection Act
G3pdh-1 and –2	phosphate dehydrogenase
gamma-HCH	gamma-HCH (lindane)
GC	Gaschromatography
GC-MS	gas chromatography mass spectrometry
GnRH	gondadotropin releasing hormone
Gpi-A and –B	glucose-6-phosphatase isomerase
GPS	Global Positioning System
GPx	Peroxidase
GSH	Glutathione

List of Abbreviations continued

GSI	gonado-somatic index
GWRC	Global Water Research Coalition
HAI	health assessment index
HCH	hexachlorocyclohexane
HE	expected average heterozygosity
HF	High-flow sampling
Hg	Mercury
HPLC	High performance liquid chromatography
HRA	Health risk assessment
HSP 70	Heatshock Protein 70
I	Intersex
IARC	International Agency for Research on Cancer
IGRs	insect growth regulators
IHAS	integrated habitat assessment
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
ISNT	*in situ* nick translation
IUPAC	International Union of Pure and Applied Chemistry
IVOS	Integrated Visual Optical System
L	Ligand
LC_{50}	Lethal dose in 50% of exposed animals
Ldh-A and B	L-lactate dehydrogenase
LF	Low-flow sampling
LIN	linearity
LOAEL	lowest-observed-adverse effect-level
LWGR	Lapalala Wilderness Game Reserve
MDA	Malondialdehyde
Mdh-1, -2 and –3	Malate dehydrogenase
Mn	Manganese
MSA	multi system approach
N	nitrogen
N/A	Not available

List of Abbreviations continued

NADP+ Idh	isocitrate dehydrogenase
NADP+ Mdhp	Malate dehydrogenase
NCP	Northern Cape population
NOAEL	no-observed-adverse-effect-level
NSB	Non-specific binding
NSC	National Snail collection
NWU	North-West University
OcP	octylphenol
OCs	Organochlorines
OD	Optical density
OECD	Organization for Economic Cooperation and Development
OHA	11-β-hydroxyandrostenedione
OHF	hydroxyflutamide
p-NP	*p*-nonylphenol, 4-nonylphenol
P	phosphorous
P = polymorphic loci	P = polymorphic loci
PAHs	polycyclic aromatic hydrocarbons
Pb	Lead
PCBs	Polychlorinated biphenyls
PCDD	chlorinated dibenzo-p-dioxins
PCDF	polychlorinated dibenzofurans
Pep-S1 and –2	substrate (leucyl tryrosine)
Pgdh	phosphogluconate dehydrogenase
Pgm	phosphoglucomutase
POPs	Persistent organic pollutants
RfC	reference concentration
RfD	reference dose
RIA	Steroid radioimmunoassay
ROL	radial oxygen loss
SASS	South African Scoring System
SBR	Suikerbosrand Nature Reserve
SD	standard deviation

List of Abbreviations continued

SDWA	Safe Drinking Water Act
SE	Standard error
SHSPH	School of Health Systems and Public Health (UP)
SOD	superoxide dismutase
SPE	solid phase extraction
STP	sewage treatment plants
T1S	Tier – 1 screening battery
T_4	thyroxin
TBT	tributyltin
TCDD	2,3,7,8-tetrachloro dibenzo-*p*-dioxin
TDI	tolerable daily intake
TDS	total dissolved solids
TERA	Toxicology Excellence for Risk Assessment
t-NP	technical nonylphenol
Tributyltin	TBT
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay
TWQR	target water quality range
UGP	urogenital papilla
UGPL	urogenital papilla length
UGPLI	urogenital papilla length index
UJ	University of Johannesburg
UK	United Kingdom
UP	University of Pretoria
UNRP	Urban Nature Reserve Population
UNR	Urban Nature Reserve
US	United States (of America)
US EPA	United States Environmental Protection Agency
USA	United States of America
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight-line velocity

List of Abbreviations continued

VTG	vitellogenin
VZ	vinclozolin
WHO	World Health Organization
WP	wild population
WRC	Water Research Commission
YES	yeast estrogen bioassay
Zn	zinc

CHAPTER 1

1.1 Introduction and rationale

There is growing international concern over persistent bio-accumulative chemicals, their potential for bio-magnification, and, even more perturbing, synergistic/additive effects of endocrine disruptor chemicals (EDCs) in mixtures. EDCs are chemicals that interfere with the structure or function of hormone-receptor complexes. These EDCs can be disruptive at very low exposure levels. Effects are not restricted to a small group of therapeutic agents, but include many compounds that are in daily use in industry, agriculture and in households (Toppari et al., 1996). The Water Research Commission (WRC) compiled a Priority List of Chemicals for Residue Analysis in South African waters (Table 4.1, EDC Workshop, Morgenhof), including Priority Areas for EDC monitoring such as the Urban Nature Reserve (UNR), with anthropogenic, industrial and agricultural activities in the area (Burger, 2005). Evidence of EDC exposure in South Africa include levels of *p*-nonylphenol (*p*-NP) in drinking water and sources equal to those reported to cause feminisation in trout (Routledge et al., 1998), leaching of *p*-NP from plastic wraps into food (De Jager et al., 1998), *p*-NP, polychlorinated biphenyls (PCBs), *1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT)* and *metabolites, heptachlor,* endosulphan and the chlordanes (Barnhoorn et al., 2003; Fatoki and Awofula, 2003a) in selected water and sediment samples and estrogenic activity in water (Aneck-Hahn, 2002; Hurter, 2002; Timmerman, 2002). Catfish collected from some water sources in SA had significant levels of selected EDCs in fatty tissue (Barnhoorn et al., 2003).

In South Africa, the production and uncontrolled disposal of high volumes of industrial chemicals and unmonitored occupational exposure may contribute to considerable environmental pollution and adverse health effects in humans and wildlife. The scientific and public awareness on the possible implications of EDC exposure in South Africa is very restricted, and research capacity on EDCs is very limited. Few of the exposure routes and potential EDCs, and hardly any of the suspected health effects have been investigated.

1.2 Aim

The objective of this study was to determine whether sufficiently high levels of EDCs exist in the general environment to exert adverse health effects on aquatic or terrestrial animals or humans.

1.3 Study area

The study was performed in the UNR which is one of the world's largest urban nature reserves, situated south of the City of Tshwane (also known as Pretoria) but still within the city limits. The Reserve is at an altitude of 1700m and is one of the very few reserves situated in the grassland biome on the central South African highveld. The stream into the Reserve receives effluent from sewage

treatment plants, industries and informal settlements in the catchment areas. There are two dams, Dam 1 (D1) and Dam 2 (D2), interconnected with a wetland and channel.

1.4 Report outlay

This report summarizes the scientific background relevant to the study with emphasis on chemical residue analyses, endocrine disruptive metals (EDMs) and bio-assays for estrogenicity and dioxin and dioxin-like PCBs. It also reviews the use of possible biosentinel aquatic and terrestrial animals. General information on the UNR and the outcomes of previous projects are discussed.

This is followed by separate chapters (including Introduction, Materials and Methods, Results, Discussion and Conclusions) on analytical chemistry and *in vitro* bio-assays. *Clarias gariepinus* (sharptooth catfish), *Xenopus laevis* (African clawed frog), *Bulinus tropica* (freshwater snail) and *Rhabdomys pumilio* (striped mouse) were evaluated as possible biomarker species for EDC exposure. The impact of active biomonitoring (ABM) on fish and snail species in the UNR, the effect on macro-invertebrates (SASS) and the possible role of plants in the wetlands were addressed separately. All the information gathered was integrated in a qualitative scenario-based health risk analysis and a toolkit recommended for future use.

CHAPTER 2
LITERATURE SURVEY

2.1 Introduction

2.1.1 Endocrine system

The endocrine system provides the key communication and control link between the nervous system and bodily functions such as reproduction, immunity, energy control, metabolism and behaviour (e.g. fight or flight response) and growth and development.

The endocrine system is made up of three components, namely

- endocrine glands,
- hormones and
- receptors.

The endocrine glands secrete hormones, which circulate around the body via the blood stream and modulate cellular or organ functions by binding with receptors in the target cells. Lastly the receptors in the target cells, once activated by binding of the hormone, regulate the functions and processes in the tissue through interactions with the cell's DNA or other complex intracellular signalling processes.

The biological actions of hormones, including estrogens, androgens, progesterone, thyroxine and the neurosteroids pregnenolone and dehydroepiandrosterone (DHEA), are mediated via high affinity protein receptors inside the target cells. Steroids circulate in the blood stream bound to carrier proteins or to serum albumin. They are fat-soluble and readily cross the cell membrane, interacting with dimeric receptor proteins, which in the case of estrogens are estrogen receptor (ER)-α and ER-β. Recent findings support a third class, putative-ER or ER-γ in fish and possibly mammals (Dodge et al., 1996; McLachlan, 2001). The steroid-receptor complex binds to target regions of DNA termed "response elements", which activate the cascade of reactions, and the response to the steroids (Waring and Harris, 2005). Naturally, EDCs have been viewed to exert their effects exclusively by genomic mechanisms, acting as steroid agonists and binding to the receptor. Recent evidence, however, indicates that some estrogenic compounds do not only have estrogen-receptor mediated effects, but non-genomic pathways may be significant in the target cell response (Pretorius and Bornman, 2005).

The endocrine system regulates processes as diverse as blood pressure, smooth muscle contraction, fluid balance and bone-resorption. For many of the systems the setup is programmed during fetal

development and an abnormal environment during this critical stage can result in permanent misprogramming (World Health Organization (WHO), 2002).

Table 2.1 summarises the major glands and hormones involved in the endocrine system.

Table 2.1: The endocrine systems' glands, hormones and their functions.

Gland	Hormones	Functions
Hypothalamus	Releasing hormones	Stimulate pituitary activity
Pituitary	Trophic (stimulating) hormones	Stimulate thyroid, adrenal, gonadal and pancreatic activity
Thyroid	Thyroid hormones	Regulate metabolism, growth and development, behaviour and puberty
Adrenal	Corticosteroid hormones Catecholamines	Regulate metabolism Regulate behaviour
Pancreas	Insulin and glucagons	Regulate blood sugar levels
Gonads	Sex steroid hormones (androgens and estrogens)	Regulate development & growth, reproduction, immunity, onset of puberty and behaviour

A similar, but not identical, endocrine system to that of humans is found in nearly all vertebrates including other mammals, fish, amphibians, reptiles and birds, although the precise structures and roles of the various organs and hormones differ between different groups (EUROPA EU, 2004). Invertebrates also have endocrine systems that control a similar range of body functions although these have evolved along markedly different lines to those of vertebrates (WHO, 2002; EUROPA EU, 2004).

2.1.2 Endocrine disruption

The phenomenon of endocrine disruption is dealing with a mechanism that is so complex that there is no consensus yet on the definition. Some refer to it as endocrine modulators, some to endocrine active substances and others prefer to call them endocrine disruptors.

In 1996, Kavlock and colleagues defined an endocrine disrupting chemical (EDC) as:

"An exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes."

For purposes of this report, the United States Environmental Protection Agency's (US EPA) definition of EDCs has been used as *"exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations"* (EPA, 1997).

They may be naturally occurring, such as the phytoestrogenic flavonoids in fruits and vegetables, natural and synthetic hormones, or may be industrial chemicals such as some types of plasticizers, lubricants, packaging materials, or pesticides, insecticides etc. in agriculture. These environmental estrogens can enhance (an agonist) or inhibit (an antagonist) the action of endogenous hormones. In some instances this group of substances can act as both an agonist and an antagonist, depending on the target tissue (Kroes et al., 2000). Cross talk may occur between different systems and may affect systems other than the anticipated one. The cross talk phenomenon means that even when an environmental chemical is shown to have a steroidal hormone activity, it may also possess other relevant activities. For example, some endocrine disruptors such as 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) isomers and certain phthalates may have both anti-androgenic as well as estrogenic effects. It is therefore very important to be cautious in extrapolating *in vitro* hormone activity detected to the *in vivo* situation (WHO, 2002).

Endocrine disruption is by now an established phenomenon in most, if not all first world countries (WHO, 2002). Information from underdeveloped countries is extremely limited, but there seems to be no logical reason why these should not be equally affected, albeit it by different classes of chemicals.

2.1.3 Types of adverse effects expected from EDCs

Of the 80 000 chemicals that are registered for commercial use there is a need to establish how many have endocrine disrupting activity. A number of chemicals on this list are suspected of having these endocrine disrupting effects. General classes of chemicals considered to be endocrine disrupting include insecticides, herbicides, fumigants and fungicides used in agriculture as well as in the home, detergents, resins, and plasticisers. The Centre for Disease Control and Prevention (CDC) have classified 48 chemicals as EDCs (Choi et al, 2004), whereas the Japanese have listed 67 chemicals as suspected (Tohyama et al., 2000).

Various field and laboratory studies reported that exposure to certain EDCs has contributed to adverse effects in some wildlife species and populations. Most of the data were collected in Europe and the United Stated of America (USA). Observations ranged from subtle changes in basic physiology and sexual behavior of species to permanently altered sexual differentiation. A wide range of species, from crustaceans, fish, birds through to mammals and man, have been

reported as being affected by EDCs (Waring and Harris, 2005). At the top of the food chain the aquatic species are particularly vulnerable, but effects have also been observed in terrestrial species (WHO, 2002). The negative impact of EDCs on health is internationally no longer an issue of dispute (COMPRENDO CREDO Workshop, 2004).

At least four major categories of adverse biological effects may be linked to exposure to endocrine disruptors, namely:

- cancer,
- reproductive and developmental alterations,
- neurological effects and
- immunological effects.

Endocrine systems that may be involved include the thyroid, adrenal, pituitary, and gonadal system. This includes cognitive effects, which have been observed in animals and humans (Schantz and Widholm, 2001).

The hypothesis that EDCs can cause cancer in humans is based largely on the clear association between exposure of females *in utero* to diethylstilbestrol (DES), a potent synthetic estrogen taken by pregnant women to avoid miscarriage, and subsequent onset of reproductive organ cancers (Silbergeld et al., 2002). In addition, it has been established that some other xenobiotics such as DDT, PCBs and tetrachloro t-dioxin are unequivocally carcinogenic (Tsuda et al., 2003).

All aspects of reproductive function are controlled by various endocrine communicating systems that employ a large number of protein/peptide and steroid hormones, growth factors and other signaling molecules that affect target gene cell expression and or protein synthesis. The developing foetus may be more sensitive to the effects of exposure to environmental chemicals than the adult system. However, effects may not manifest until adulthood (WHO, 2003).

The majority of the studies examining endocrine disruption have focused on reproductive health effects. A number of adverse reproductive health effects have been observed in which endocrine disrupters could play a role, namely:

- Declining sperm counts: Some studies in certain western countries have reported decreases in sperm numbers over the last 50 years. However, other studies in different regions have failed to detect such changes.
- Congenital malformations in children: In recent years there has been an increase in the incidence of hypospadias (a congenital abnormality of the urethra in the penis) and cryptorchidism

(undescended testes) in humans. However, no causal association with chemical exposure has yet been established.

- Delayed or premature sexual development: A few reports have been published suggesting that adolescents in polluted areas may develop puberty earlier especially in girls, while other take longer to reach puberty. However, the potential mode of action of any such effect is still not clear.

It has been suggested that the apparent decline in male reproductive health might be caused by an excess of estrogenic compounds (Sharpe, 1994). These EDCs end up in the environment where animals and humans are exposed through various routes. In South Africa the vector control program for malaria uses both DDT and deltamethrin (DM) for indoor residual spraying. DDT exposure has negative reproductive health effects on humans and biota (Gray, 1992; Facemire et al., 1995; Sumpter, 1995; De Jager et al., 2006). Synthetic pyrethroids such as DM were developed to limit the use of DDT (Sharp, 1999; Tren, 2000), but pyrethroids are also EDCs (Garey and Wolff, 1998). 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, 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 (dichloro-diphenylchloroethane), the stable metabolite of DDT is an anti-androgen, may explain some of the estrogenic effects observed in the environment – it may be due to the anti-androgenic effects of EDCs (Gray et al., 1999; Toppari et al., 1996).

Gray et al. (1999) suggested that administration of potentially anti-androgenic pesticides and toxic substances during sexual differentiation alter male sexual differentiation via different mechanisms. Longnecker and co-workers (2001) found that exposure to DDT used increased preterm births, which is a major contributor to infant mortality. An experiment done by Hauser et al. (2002) showed trends that were suggestive of an association between PCBs and *p,p'*-DDE and abnormal sperm count, motility and morphology.

Table 2.2 provides a list of the more common endocrine disruptors, their uses, mechanisms of action and health effects. Most of the effects listed below are reproductive health effects as most studies have focused on this endpoint.

There is much debate about the significance of evidence that endocrine disruptors are affecting the health of the general population. Epidemiological studies are almost nonexistent. However, surveillance of possible hormone related conditions show an increase in these conditions. Table

2.3 provides an overview of the potential endocrine disrupting effects with regards to human reproductive development.

PCBs are a family of organochlorine chemicals that are chemically stable, fire resistant and do not generate vapours easily. They are practically insoluble in water, but are soluble in oils and fatty substances. Hauser et al. (2003) studied the relationship between human semen parameters and environmental exposure to PCBs and *p,p'*-DDE. They determined sperm count, motility and percentage normal sperm in a sample of men who visited an infertility clinic. At the time of enrolment, it was not known whether the couple's infertility was due to male or female impairment. A blood sample was taken the same day they obtained sperm samples and analysed the serum for *p,p'*-DDE and 57 different PCB congeners. One PCB congener, PCB138, showed a strong dose-response relationship with sperm motility and sperm morphology and a lesser trend for sperm count. Men with higher levels of PCB138 were more likely to have sperm parameters below reference values. These and other results add weight to prior findings indicating that PCB levels may affect sperm parameters adversely, at levels many animals and humans are being exposed to.

Animals and humans are also daily exposed to many other xenoestrogens. *p*-NP belongs to a group of alkylphenols polyethoxylates. These surfactants are used in detergents, paints, herbicides, pesticides and cosmetics and are relatively persistent and bioaccumulate in the lipids of living organisms. *p*-NP, a known estrogen, has been found to have an adverse effect on the testis and epididymis of rodents in reproductive toxicology studies (Lee et al., 1999). In South Africa, *p*-NP was detected in selected drinking water samples (De Jager et al., 2002). In another study significant levels *p*-NP were found in fatty samples of eland, sediment and fish fatty tissue (Barnhoorn et al., 2002). This confirmed that wildlife in South Africa is being exposed to environmental EDCs. Histological parameters including germ cell necrosis, apical sloughing and vacuolisation increased with p-NP concentration and these negative effects were enhanced after maternal exposure in laboratory rats (De Jager et al., 1999a,b).

Table 2.2: Endocrine disruptors and their mechanisms of action (Solomon and Schettler, 2000).

Chemical	Use	Mechanism	Health Effect
DES	Synthetic estrogen	Estrogen receptor agonist	Humans (prenatal exposure): vaginal cancer, reproductive tract abnormalities (females); cryptorchidism, hypospadias, semen abnormalities (males)
Methoxychlor	Insecticide	Metabolite is an estrogen receptor antagonist	Rodents: accelerated puberty, abnormal ovarian cycling (females), aggressive behaviour (males)
DDT	Insecticide	Metabolite (DDE) is an androgen receptor antagonist	Rodents (males): delayed puberty, reduced sex accessory gland size
Vinclozolin	Fungicide	Androgen receptor antagonist	Rodents (males): feminization, nipple development, hypospadias
PCBs	Production stopped; still in electrical transformers, capacitors, toxic waste sites, food chain	Accelerated T_4 metabolism, decreased T_4 levels, elevated TSH levels (high doses: thyromimetic)	Humans (*in utero* exposure): delayed neurological development; IQ deficits
Atrazine	Herbicide	Reduces gonadotropin-releasing hormone from hypothalamus, reduces pituitary LH levels, interferes with metabolism of estradiol, blocks estrogen receptor binding	Rodents (females):mammary tumours, abnormal ovarian cycling. Humans: some evidence of breast and ovarian tumours
Dioxin	By-product of industrial processes including waste incineration; food containment	Aral hydrocarbon receptor agonist; increases estrogen metabolism, decreases estrogen mediated gene transcription, decrease estrogen levels, decreases testosterone levels by interfering with HPG axis	Rodents (*in utero* exposure): delayed puberty, increased susceptibility to mammary cancer (females), decreased testosterone, hypospadiasis, hypospermia, delayed testicular descent, feminized sexual behaviour (males) Humans: decreased T_3 and T_4 levels decreased testosterone levels * cancer*

Table 2.3: Trends in human health effects potentially related to endocrine function (Solomon and Schettler, 2000).

End point	Trend	Degree of change
Hypospadias	Increasing	3.3 - 4.3% per year
Cryptorchidism	Increasing	1.6 - 3.5% per year
Sperm count	Decreasing	-5.3 to -0.7%/mL per year
Testicular cancer	Increasing	2.1 – 5.2% per year
Prostate cancer	Increasing	3 - 5.3% per year
Breast cancer	Increasing	1.9 - 3.3% per year
Sex ratio	Shift toward females	-0.5 to -1.0males/10 000 per year
Age at breast development	Shifting earlier	11.2-9.96 years in white US population

Phytoestrogens on the other hand are natural compounds present in plants and are ingested daily in milligram quantities. The active substances are isoflavones (genistein), coumestans (coumesterol) and fungal metabolites (mycotoxins), such as zearalenone. Phytoestrogens possess estrogenic effects *in vitro* and *in vivo* and have been responsible for reproductive disorders in some animal species (Toppari et al., 1996; Santti et al., 1998). Neonatal exposure of genistein inhibited the growth and proliferation of testicular cells in rats (Diaka et al., 1998). All these support the concerns that phytoestrogens in combination with other EDCs may have serious implications for reproductive health of human and animals and that further research is needed in South Africa (De Jager et al., 2004).

Most of the existing literature on the toxicological properties of phthalates focuses on the traditional approach in toxicology: high-level exposure for cancer endpoint and occupational exposure leading to adult infertility. Over the past several years, the US EPA (Gray et al., 1999), turned attention to the impact of low-dose toxicity of phthalates during crucial exposure windows during fetal development. While high doses of phthalates do constitute risks in the sense of traditional toxicology, these low doses changed the stakes dramatically. The authors showed that male reproductive development is acutely sensitive to some phthalates. Dibutyl phthalate (DBP) and diethylhexyl phthalate (DEHP) produced dramatic changes in male sexual characteristics when exposure took place *in utero* at levels far beneath those of previous toxicological concern. These changes included increases in the rates of hypospadias and other indications of demasculinisation (Gray et al., 1999).

It is important to understand the effects of these compounds because they may persist in the body for prolonged periods of time. Dose, body burden, timing and duration of exposure at critical periods of life are important for assessing the effects of an endocrine disruptor. These effects may be reversible or irreversible, immediate/acute or latent and not expressed for a period of time (EPA, 1997). Other complicating factors of exposure assessment include time lags between exposure and effect (i.e. transgenerational effects). Seasonality may impact on the sensitive reproductive stages in wildlife, and seasonal rainfall, storm runoff, and water releases change the aquatic environment. Furthermore, multiple chemical exposures occur in real life and have the potential to modify the effect (e.g., synergy, additivity, or antagonism).

The endocrine system includes a number of central nervous system-pituitary-target organ feedback pathways in regulating bodily functions and maintaining homeostasis. Therefore, there are several target organ sites at which an environmental agent could disrupt endocrine function.

2.2 Chemical residue analysis for estrogenic substances

Validated exposure data is a key component for assessing the causal relationships between exposure to EDCs and health effects (WHO, 2002). The list of chemicals with possible endocrine disruptive effects are structurally very diverse and include natural and synthetic hormones, phytoestrogens, pesticides, and various industrial chemicals and by-products. It is, therefore, not possible to define a "typical" EDC or to suspect possible endocrine disruptive effects on the structure of the compounds.

During the first workshop of the WRC on EDCs in 1999, the need to investigate EDCs in the South African context was emphasized and a strategic research plan was subsequently initiated. Little of the existing analytical data generated in South Africa was useful for EDC evaluation and, although estrogenic activity could be detected, it was not supported by chemical contamination. It was concluded that it is crucial that both biological and chemical analysis be conducted on the same sample at detection levels relevant to the study. It was also emphasized that care should be taken that the extraction procedures be appropriate and not indeed removing those compounds with estrogenic activity. Proper and strict guidelines are essential for sampling, sample handling, storage and transport, extraction and clean-up procedures (Burger, 2005). Unless these are followed diligently, the interpretation of the analytical work may not only be jeopardized, but become of little, if any, value.

For a detailed discussion on chemical groups with endocrine disruptive activity, the reader is referred to Burger, 2005. From that report a list of priority EDC compounds for monitoring purposes in South African water was compiled (Table 2.4). During a workshop held at

Morgenhof, Stellenbosch in May 2003 four sites across the country were selected for a limited surveillance study. One of them was the Urban Nature Reserve near Pretoria which was included because of the agricultural, industrial and anthropogenic activities in the inflow or surroundings (Burger et al 2006., *in press*).

Table 2.4: Suggested list of priority EDC compounds to be monitored (Burger, 2005).

Compound	Chemical class
17β-Estradiol Estriol Estrone 17α-Ethinylestradiol	Hormone
p-Nonyl phenol Nonylphenol ethoxylates *p*-Octylphenol Octylphenol ethoxylates	Alkylphenols
PCBs	Polychlorinated biphenyls
DDT, DDE, DDD Dieldrin, Aldrin, Endrin α-Endosulfan, β-Endosulfan, Endosulfan-sulphate Heptachlor, Heptachlor epoxide Lindane (γ-BHC)	Organochlorine pesticides
Chlorpirifos Azinfos-methyl Parathion	Organophosphate pesticides
Deltamethrin	Pyrethroid pesticide
Atrazine Simazine Terbutylazine 2,4-D, 2,4,5-T Metoxychlor	Herbicides
di-(2-ethylhexyl) phthalate (DEHP dibutyl phthalate (DBP) Bisphenol A	Plasticizer Raw material for resins
Dioxins, Dibenzofurans	Dioxins/furans
Tributyltin, Cyhexitin	Organo-tin compounds
Lead, Cadmium, Mercury, Arsenic	Toxic heavy metals
Vinclozolin	Bactericide

Various extraction and clean-up procedures may be used including liquid-liquid extraction and solid phase extraction (SPE), depending on the specific matrix (water, sediment, biological tissue etc). Recovery studies are also necessary to determine the efficiency of the extraction procedure. It is impossible to analyze EDCs on one run, but levels may be determined by gas chromatography (GC) or high performance liquid chromatography (HPLC). Confirmation of the identity of a compound is done using gas chromatography mass spectrometry (GC-MS) or HPLC-MS. The extremely low levels at which these substances are active may sometimes be beyond the limitations of the MS (Burger, 2005).

2.3 Endocrine disruptive metals (EDMs)

Metals are natural elements and some are essential for normal growth and development. Therefore, low levels of metals and metal salts in the environment are unlikely to affect the general human or wildlife population by disrupting endocrine function. Metals are therefore listed as EDCs via their secondary/even tertiary effects and their ability to cause adverse reproductive and developmental effects at high doses, as a direct insult on the organ such as degeneration, necrosis, inflammation and edema (Colborn et al., 1993).

In South Africa no research has been done on the endocrine disrupting effects of metals on aquatic wildlife or humans. Although most studies have been done on the accumulation of selected metals in different aquatic species the possible endocrine effects were not considered. Metal contamination of aquatic ecosystems have been recognised and accepted as a pollution problem in South Africa (Department of Water Affairs and Forestry (DWAF), 1986; van Vuren et al., 1999).

According to Heath and Claassen (1999) the first bioaccumulation study in fish was undertaken in 1977 and from then on metal bioaccumulation studies increased rapidly. Literature on the metal levels in freshwater fish species of South Africa is abundant (Bezuidenhout et al., 1990; Seymore, 1994; Barnhoorn, 1996; Robinson and Avenant-Oldewage, 1997; Kotze et al., 1999; Nussey et al., 2000; Coetzee et al., 2002) and apart from fish, information is available on metal levels in water (Fatoki et al., 2002; Fatoki and Awofolu, 2003b), sediment (Seymore et al., 1994; Sanders et al., 1999; Binning and Baird, 2001), aquatic birds (van Eeden, 2003) and the freshwater river crab *Potamonautes warreni* (Steenkamp et al., 1994; Sanders, 1999; de Kock, 2003). Most of the abovementioned studies included the accumulated values of Cd, As and Pb in the organs, tissues, sediment and water measured in different fish species from a range of rivers and dams in South Africa. South African waters are also known for the high concentrations of metals present (Heath and Claassen, 1999).

2.3.1 Cadmium (Cd)

Cd is a non-essential, widely distributed metal that is extremely toxic in relatively low concentrations (Anoop et al., 2003). Cd is one of the most readily absorbed and accumulated elements in plants grown on contaminated soil. Cd levels in water and sediment result from anthropogenic sources such as mining and metal refining industries (Baldisserotto et al., 2004). The cadmium divalent forms including free cadmium (II) ion, cadmium chloride and cadmium carbonate usually occurs in freshwater systems and the Cd^{2+} are readily taken up by aquatic organisms (DWAF, 1996). Contaminated drinking water thus may expose people and have adverse effects on health.

Locally a number of papers present data of the bioaccumulation of Cd in the organs and tissues of different fish species (Barnhoorn, 1996; Coetzee, 1996; Kotze, 1997; Van Vuren et al., 1999). A literature review by Heath and Claassen (1999) revealed that Cd bioaccumulation levels was the highest in the liver of all the species monitored, but it was also present in the gonads, fat, muscle, gills and intestine. Although the authors came to the conclusions that the occurrence of trace metals in African Aquatic systems is not excessive when compared to other areas of the world, the possible implications change considerably if the concern about endocrine disruption is included (Tilton et al., 2003).

Stoica et al. (2000) indicated that Cd activates the ER-α in humans and blocks the binding of E_2 to ER-α in a non-competitive manner, suggesting that Cd interacts with the hormone binding domain of the receptor. Cd is frequently associated with reproductive disorders in mammals and fish. Furness (1996) listed numerous effects of Cd and impairments included altered behaviour, lower egg production, testicular damage and kidney damage. Cd treated female rats showed hyperplasia and hypertrophy of the uterus (Johnson et al., 2003) indicating that Cd has potent estrogen-like activity *in vivo*. In other mammals testicular necrosis, ovarian bleeding and delayed embryo implantation was also reported (De et al., 1993).

Cd induced alteration of the hormonal balance in rainbow trout testes *in vitro* (Kime, 1984) and impaired control of ovarian maturation and steroidogenesis by pituitary hormone in common carp (Mukherjee et al., 1994). It also inhibits egg development in the fry stage of brown carp. Impaired vitellogenin (VTG) production has been demonstrated in winter flounder populations collected from contaminated areas or undergoing experimental exposure to cadmium during early vitellogenesis. Cd also decreased plasma VTG levels in rainbow trout (Haux et al., 1988). Apart from mammals and fish, Au et al. (2001) found that exposure to Cd reduced the quality of gametes in the sea urchin. The males were, however, more sensitive than the females to chronic exposure. Cd caused developmental malformation at numerous concentrations in studies of embryo toxicity

in amphibians (Kotyzova and Sunderman, 1998). Lienesch et al. (2000) found in *Xenopis laevis*, the percentage of oocytes at all stages of oogenesis was decreased and the population of atretic oocytes increased significantly after exposure to daily cadmium chloride doses.

2.3.2 Arsenic (As)

As is a naturally occurring element widely distributed in the crusts of the earth (Basu et al., 2001) and one of the most important global environmental toxicants (Gebel, 2000). Arsenic is found in two oxidation states in aquatic systems namely arsenate [As(V)] and arsenite [As(III)] (Suhendrayatna et al., 2004). Inorganic arsenic compounds are mainly used to preserve wood while organic arsenic compounds are used as pesticide primers on cotton plants. As is primarily absorbed by ingestion, inhalation and transcutaneously through food, drinking water, breathing air containing As and breathing sawdust or burning smoke from wood treated with As (Basu et al., 2001). Contaminated drinking water is, however, the main route for chronic human As intoxication (Gebel, 2000). The principal sources of As in ambient air are the burning of fossil fuels, metal production, agricultural use and waste incineration. Arsenic is introduced into water through the dissolution of minerals and ores, from industrial effluents and via atmospheric deposition. Inorganic As is completely stable and does not break down. This leads to accumulative levels in the soil, which may affect a wide range of organisms for long periods. A lot of debate is currently focusing on As in children (Tsuji et al., 2004). Although As compounds were formerly used as medications (Hathaway et al., 1991) poisoning episodes have been reported all over the world (Mandal et al., 2002).

In the South African environment As has not been monitored as part of metal monitoring studies by most researchers (Heath and Claassen, 1999). Before 1999, the highest water As value recorded was 15µg/L, which falls within the target water quality range (TWQR) (DWAF, 1996). Background As concentrations in groundwater from most countries are less than 10µg/L or lower. The drinking water guideline from the EPA is 50µg/L, while the WHO suggests ≤10µg/L and the maximum permissible limit accepted is 50µg/L. Although the environmental heavy metal concentrations are not comparable to the rest of the world (Heath and Claassen, 1999), the existence of these metals in the environment has led to concerns about their possible accumulation in plants and animals (Fatoki et al., 2002).

No literature is available on the toxicological effects of As on any fish species in South Africa. Very little data is available on the bioaccumulative values of As in local fish species. Fish can accumulate As but the effect is mostly not harmful. Worldwide it has been reported to have adverse effects on vertebrate and invertebrate aquatic organisms and depended on the life stage of the organism (DWAF 1996). Very low doses of As disrupt the function of the glucocorticoid

receptor, a steroid hormone receptor that regulates a wide range of biological processes. Subsequently it has an effect on embryo development, stress, blood glucose levels, blood vessel function, lung and skin development and cancer development (Kaltreider et al., 2001). Aquatic organisms are less sensitive to arsenic than humans, therefore, consumption of contaminated products can pose a health risk to humans (DWAF 1996).

2.3.3 Lead (Pb)

Pb is a highly toxic, non-essential metal of which Hippocrates noted the toxicity in 370 B.C. (Graeme and Pollack, 1998). Since 1904, various cases of Pb poisoning among children and adults have been reported and it was, therefore, banned from use as additive in paints as from 1977. Pb is still one of the most common elements used in the industrial sector in the production of storage batteries, ammunition, cable covering, ceramic glazes, casting metals and solders. Lead occurs as metallic lead, inorganic compounds and organometallic compounds where lead (II) is the stable form present in the aquatic environment. The major sources of Pb are deterioration of old paint, household dust, bare soil, air, drinking water, food and ceramics (DWAF, 1996).

In the form of salts, Pb is acutely toxic to aquatic invertebrates at concentrations above 0.1g/L and >40g/L for fresh-water organisms. For the same species, the 96-h LC_{50}s for fish vary between 1 and 27mg/L in soft water, and between 440 and 540mg/L in hard water. It is, however, the young life stages in fish that are more sensitive to Pb than are the mature or even the egg of fish. Spinal deformity and blackening of the caudal region are typical syndromes in fish with Pb poisoning. The toxicity of Pb varies depending on the availability and uptake of the Pb ion and some factors affecting toxicity are hardness (Maddock and Taylor, 1980), pH (Merlini and Pozzi, 1977) and salinity (Somero et al., 1977). The most common biochemical effect of Pb is the influence on delta amino levulinic acid dehydratase (delta ALAD) (Hodson et al., 1978; Johansson-Sjobeck and Larsson, 1979).

The aquatic ecosystem guideline (DWAF, 1996) for Pb is 0.5µg/L where the hardness should be adjusted for medium water (60-119mg $CaCO_3$/L). According to Heath and Claassen (1999) this value is unrealistically low for South African rivers. Therefore most of the data were compared to the guidelines compiled by Kempster et al. (1980) of <100µg/L for lead where no significant lead values were noted in fish species from fish collected from the five river catchments (Heath and Claassen, 1999) including water and sediment.

According to Goyer (1996), Pb can induce carcinogenesis, reproductive and developmental effects, nephropathies and neuropathies in rodents and humans. The first evidence of Pb as EDM has been reported by Telisman et al. (2000) who found that moderate exposure to Pb (blood

Pb<400µg/L) significantly reduced human semen quality without conclusive evidence of impairment of male reproductive endocrine function. Other endocrine systems affected by Pb included the gonadal steroids, adrenal steroids and the thyroid hormones. Pb can also disrupt reproductive neuroendocrine function in the Atlantic croaker (Khan and Thomas, 2000), where lead significantly inhibited the pituitary gonadotropin II (GTH II) response to stimulation by luteinizing hormone-releasing hormone analogue *in vivo* and caused reduced gonadal growth.

2.3.4 Mercury (Hg)

Hg is classified by the US EPA as hazardous because even slight exposure affects human health (Dallas and Day, 2004) and it may bioaccumulate in the body. Hg is naturally found in environment where human contributions steadily increased since the industrial revolution. Three important types that pose different health hazards are the pure element as the metal (liquid/vapour at room temperature), inorganic compounds such as mercuric chloride, iodide, sulphide and chloride, as well as organic mercurials such as ethylmercury, methylmercury, merbromin, merthiolate (Graeme and Pollack, 1998). The most toxic form of mercury is methyl mercury (MeHg), which is mainly produced by microscopic organisms in water and soil and it builds up in the tissues of fish. Hg enters the water and soil from natural deposits, disposal of wastes and volcanic activity.

The most common route for humans to be exposed to Hg is with the consumption of fish or shellfish contaminated with methyl mercury (Silbergeld and Devine 2000). Other sources of exposure include breathing vapours from spills, incinerators and industries that burn mercury-containing fuels and the release of Hg from dental work and medical treatments. Very young children are more sensitive than adults to Hg and the substance is also transferred to the developing fetus where accumulation may cause brain damage, mental retardation, incoordination, blindness, seizures and inability to speak (Agency for Toxic Substance Registry (ATSDR), 1999).

Heath and Claassen (1999) reported that Hg had the lowest levels detected of 12 metals measured in fish species in South Africa with concentrations generally lower than the levels of detection. These values were measured in the liver, gills and ovaries of selected fish species (n=12). The levels were also measured against the aquatic ecosystems guidelines compiled by Kempster et al. (1980) for mercury (<10µg/L).

Literature (WHO, 1993) indicated that all chemical forms of Hg during and even prior to gestation resulted in reproductive problems such as spontaneous abortion, stillbirths, congenital malformations, infertility, and disturbances in the menstrual cycle, inhibition of the ovulation and behavioural effects of the offspring. The endocrine effects of Hg on mammals have been studied

by monitoring the reproductive impairment in the Florida Panther. Research indicated that Pb contamination was the cause of death in a female panther, likely due to thyroid dysfunction (Facemire et al., 1995).

Few studies have investigated the effects of Hg on the endocrine system of fish. Kirubagaran and Joy (1989 and 1991) found induced morphological changes in the pituitary and thyroid gland as well as diminished blood cortisol levels in the catfish after chronic Hg exposure. Leblond and Hontela (1999) demonstrated that $HgCl_2$ had the lowest EC_{50} and LC_{50} and was the most toxic compared to others such as $CdCl_2$, $ZnCl_2$, CH_3HgCl_2 and *o,p'*-DDD. Acute exposures to sublethal concentrations of inorganic and organic mercury stimulate the pituitary-interrenal and the pituitary-thyroid axes (Bleau et al., 1996). This exposure includes increased plasma cortisol and plasma thyroxin (T_4) levels. Cortisol also influences the physiological fitness of fish by its effects on reproduction, growth and the immune function.

2.4 *In* vitro and *in vivo* bioassays

The environmental load of EDCs has reached critical levels at which human and wildlife is at risk (Colborn, 1994). *In vitro* assays are becoming increasingly attractive as screening tools because they are rapidly done and fairly easy to perform. They are usually amenable to high-throughput systems and they have the potential to reduce the number of animals needed for chemical testing. However, with the given diversity of endpoints a single assay is never suitable. A number of *in vitro* assays are useful for the initial screening of environmental samples for 'hot spot' identification and monitoring purposes.

In 1996, in response to public concern about EDCs, the American Congress passed amendments to the Food Quality Protection Act (FQPA) and the Safe Drinking Water Act (SDWA). These regulations require comprehensive screening for estrogenic and anti-estrogenic chemicals. The Endocrine Disruptor Screening and Testing Committee (EDSTAC) have made recommendations to the United States Environmental Protection Agency (US EPA) about which chemicals should be screened and what the process should include. The US EPA has indicated that at least 87 000 existing chemicals to be experimentally evaluated for their potential to disrupt activities in the estrogen, androgen and thyroid hormone systems (EDSTAC, 1998; Fang et al., 2000). Currently, both estrogen receptor (ER) and androgen receptor (AR) binding and transcriptional activation assays are being evaluated by the US EPA for inclusion in a Tier – 1 screening battery (T1S) to detect endocrine-active compounds, although particular assays have not been specified (Wilson et al., 2002). In South Africa there is increasing evidence that our aquatic systems are being polluted with EDCs. As a result there is a serious need to develop a battery of screening tests for not only anti- and estrogenic, but also anti – and androgenic effects relevant for local conditions.

A number of *in vitro* assays have been developed to screen substances for estrogenic and androgenic activity (Legler et al., 1999; Pons et al., 1990; Rogers and Denison, 2000; Wilson et al., 2002; Wilson et al., 2004; Zacharewski, 1997). Each assay is suited to a particular purpose; however many of these assays have drawbacks that limit their usefulness or are not freely available to the scientific community as a screening tool (Wilson et al., 2004). Competitive ligand binding assays assess the ability of a chemical to compete with the endogenous ligand (i.e., 17β-estradiol) for binding to the steroid receptor (i.e., estrogen receptor). Still they give no insight as to the chemical's ability to initiate or inhibit gene transcription as both estrogens and anti-estrogens bind ER similarly in a competitor binding assay (Wilson et al., 2004). Cell proliferation assays are limited by a lack of specificity, because mitogens other than estrogens can influence cell proliferation (Dickson and Lippman, 1995; Wilson et al., 2004). Yeast based assays are relatively simple to execute and can address transcription issues, but lack responsiveness to some estrogens and anti-estrogens. In addition permeability of compounds through the yeast cell wall differs from that of mammalian cell membranes (Gaido et al., 1997).

In the environment EDCs occur as complex mixtures with potencies ranging over several orders of magnitude (Giesy et al., 2002). Screening bioassays are useful techniques for the determination of receptor-mediated activities in environmental samples. International concern has led a number of countries to develop an *in vivo* and *in vitro* screening programme for EDCs. The Water Research Commission (WRC) is a member of the Global Water Research Coalition (GWRC, 2005) which has previously undertaken work on chemical analytical techniques with regards to EDCs. Currently the WRC is involved in a GWRC international project to determine which bioassays are effective test platforms and to compare chemical analysis with the bioassays. The first step is to look at the most relevant toolbox of EDC tests to detect estrogenic activity in environmental waters. This initiative addresses the need for multiple bioassays to evaluate the different modes of action. At the GWRC workshop held in Le Pecq, France in October 2005, a list of assay criteria were applied, this included, global applicability, sensitivity, reproducibility, robustness, ease of use or complexity of the assay, an easily transferable methodology, appropriate sample preparation and isolation protocol, relative environmental relevance and finally cost. Finally, five *in vitro* bioassays were suggested, namely, the recombinant yeast estrogen screen (YES), MELN reporter gene assay, Kbluc reporter gene assay, ER-CALUX reporter gene assay and the E-screen cell proliferation assay (GWRC, 2005). The following bioassays have been proposed for the battery of screening assays in South Africa (Aneck-Hahn et al., 2006).

2.4.1 The Recombinant Yeast Screen Assay (YES)

Yeast do not contain endogenous steroid receptors. However, mammalian steroid receptors introduced into yeast function as they do in mammalian cells as steroid-dependent transcriptional

activators (Gaido et al.,1998; Metzger et al. 1988; Purvis et al., 1991). As a result yeast are a useful tool for studying mammalian steroid receptor function in isolation of confounding factors found in mammalian cells. When a steroid responsive reporter gene is introduced into the yeast along with the steroid receptor, chemical interaction with that receptor can be determined by measuring the reporter gene product. A yeast based steroid receptor assay differs from a competitive binding assay in that it not only determines the ability of a chemical to bind to a receptor, but also to cause that receptor to dimerize and bind to the appropriate steroid responsive regions of the DNA to induce reporter gene activity. Other advantages of using yeast to study steroid receptor function include the ease of manipulation, rapid attainment of stable transformants, ability to process a large number of samples quickly and relatively inexpensively (Gaido et al., 1998).

2.4.2 The T47D-KBluc reporter gene assay

In comparison to other existing *in vitro* assays, reporter assays based on stably transfected cell lines provide the most specific, responsive, and relatively quick means to screen substances for estrogenic potential and anti-estrogenic activity (Legler et al., 1999). The 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. These cells were named the T47D-KBluc and were specifically developed for screening chemicals for estrogenic and anti-estrogenic activities. The ERE regulates the expression of a luciferase reporter gene. Stable transfection of this promoter-reporter construct into T47D cell resulted in a sensitive responsive clone. In principle, compounds enter the cell, estrogen receptor ligands bind to the ER, two ligand-bound receptors dimerize and bind coactivators, then the dimer 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 (Wilson et al., 2004). These cells are sensitive to potent estrogens, 17 β-estradiol, ethynyl estradiol and diethylstilbestrol and well characterised weaker environmental estrogens like genistein and 4- nonylphenol. ICI 182,780 is a potent anti-estrogen, and inhibits the effects of estrogen-dependent gene expression. When testing chemicals or environmental samples using the T47D-Kbluc cells, an estrogen was defined as a chemical that induced dose-dependent luciferase activity which could be specifically inhibited by the anti-estrogen ICI (Wilson et al., 2004).

2.4.3 The MDA-kb2 reporter gene assay

The MDA-kb2 assay was developed to screen androgen agonists and antagonists and to characterise its specificity and sensitivity to EDCs. The MDA-MB-453 breast cancer cell was

stably transformed with the MMTV.luciferase.neo reporter gene construct. Since both the glucocorticoid receptor (GR) and androgen receptor (AR) are present in the cells and both receptors act through the MMTV promoter, compounds that act through either AR or GR receptors activate the MMTV luciferase reporter. 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 androgenic activity of the test chemical. AR agonists such as dihydrotestosterone (DHT) and GR agonists such as dexamethasone (DEX) induce luciferase expression at appropriate concentrations (0.1-10nM DHT and 1- 1000nM DEX). To distinguish AR- from GR-mediated ligands, chemicals or environmental samples are assayed concurrently with the anti-androgen, hydroxyflutamide (OHF), which blocks the AR- but not GR- mediated responses (Wilson et al., 2002). When testing chemicals using the MDA-kb2 cells, an androgen was defined as a chemical that induced a dose-dependent luciferase activity, which could be specifically inhibited by the anti-androgen OHF. The positive control in this assay is DHT (agonist/androgen) and the negative control is the assay solvent ethanol (Wilson et al., 2002).

In the South African scenario this battery of assays will provide a platform to identify samples of interest and to provide basic information for further analysis and risk assessment in line with the GWRC.

In addition to the assays described above, there are a number of other *in vitro* assays that have been developed involving the use of a diverse range of human and animal tissues or primary cultures and cell free systems (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 1996; Holmes et al., 1998; Baker, 2001). However, most of these assays are not sufficiently well developed to be considered part of a testing strategy (Baker, 2001).

2.4.4 Catfish vitellogenin enzyme linked immunosorbent assay (cf-VTG-ELISA)

The use of a species specific developed vitellogenin (VTG) enzyme linked immunosorbent assay (ELISA) has become an accepted biomarker/tool in detecting the presence of endocrine disruption in different fish species (Johnsen et al., 1999; Fenske et al., 2001; Tolar et al., 2001; Hennies et al., 2003; Spano et al., 2004). VTG is a phospholipoglycoprotein produced in the liver of mature oviparous animals in response to the stimulation of estrogens. From the liver it is transported by the blood circulation to the ovaries and incorporated in the developing oocytes through receptor-mediated endocytosis. Once incorporated by the developing oocytes, VTG and its metabolites serve as a source of energy (Roubal et al., 1997).

Mature females usually produce VTG only during spawning. However, in both males and juveniles the responding genes are present, thereby enabling VTG expression when exposed to exogenous estrogens (Lomax et al., 1998). VTG expression in males and juveniles is, therefore, a good indicator of exposure to EDCs in the aquatic environment.

The sharptooth catfish, *Clarias gariepinus*, is an indigenous South African species (see 2.1) and widely used for environmental research. Intersex, the presence of male and female gonadal features in the same animal, has been reported in catfish from freshwater sources of South Africa (Barnhoorn et al., 2003). The implementation of a cf-VTG ELISA in combination with histopathological analyses of gonads should provide a useful tool to assess all water sources in South Africa, including most of Africa, for estrogenicity. The cf-VTG ELISA procedure is based on competition for the cf-VTG antibody between VTG-coated on wells of a 96-well Nunc Maxisorp microtitration plate (from AEC Amersham, South Africa) and free VTG in solution. The amount of antibody bound is measured in a subsequent step by an enzyme-labeled second antibody. The enzyme activity (measured colorimetrically) is inversely related to the concentration of VTG in the sample. The assay is calibrated using dilutions of cf-VTG standard as competitor (Tyler and Sumpter, 1990).

2.5 Bio-analysis of dioxin and dioxin-like PCBs

Polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) are not produced intentionally, but formed as by-products in industrial activities and all combustion processes (Fiedler et al., 1990). The general molecular structures are represented in Figure 2.1. Each of the generalised PCDD/PCDF-molecules can have chlorine atoms attached at the different numbered positions in a variety of combinations creating a possible 210 different molecules (75 PCDD- and 135 PCDF congeners). Only 17 of these molecules (Table 2.6) are significantly toxic to many laboratory animals and all have chlorine atoms in the 2,3,7 and 8 positions of the parent molecule (Fiedler, 2003).

Depending on the number of chlorine substituents and their position at the two rings (Figure 2.1), there are theoretically 209 congeners possible. From a toxicological point of view, 12 non-*ortho*- and mono-*ortho*-PCB-congeners (Table 2.5) are of special interest because these show toxicological properties that are similar to dioxins. These congeners are often called "dioxin-like" PCBs (Päpke and Fürst, 2003).

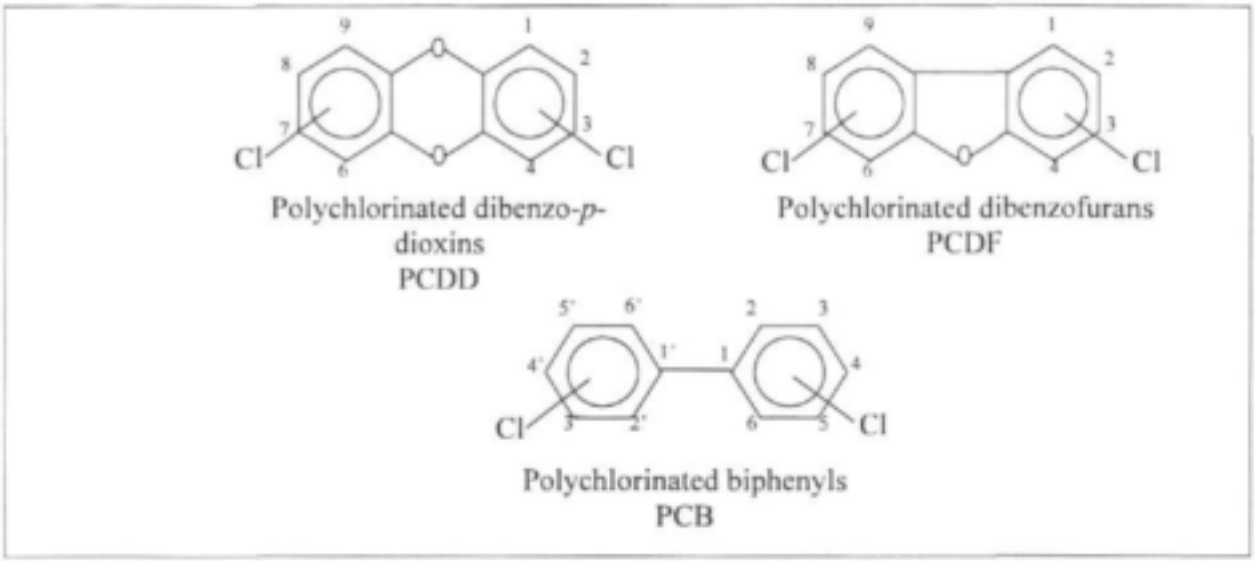

Fig. 2.1: Structural formula of polychlorinated dibenzo-*p*-dioxins (PCDD), polychlorinated dibenzofurans (PCDF) and polychlorinated biphenyls (PCBs).

PCDD/PCDF and PCB are multimedia pollutants, and once released into the environment, become distributed between environmental compartments. PCDD/PCDF and dioxin-like PCB are primarily bound to particulate and organic matter in soil and sediment. In biota, they are mainly concentrated in fatty tissues. Because of their chemical characteristics and very low solubility these compounds accumulate in most soil types, with very little water leaching and negligible degradation of the 2,3,7,8-substituted PCDD/PCDF-congeners. (This finding, however, has not been substantiated for South African conditions.)

They accumulate in aquatic fauna as a result of the ingestion of contaminated organic matter (Fiedler, 2003). The concentration of PCDD/PCDF and dioxin-like PCB in fish tissue is found to bio-magnify in the food web as a progressive ingestion of contaminated prey (EC, 1999).

The estimated half-lives of PCBs range from three weeks to two years in air and, with the exception of mono- and di-chlorobiphenyls, more than six years in aerobic soils and sediments. PCBs also have extremely long half-lives in adult fish: the half-life of PCB153 (the number refers to the IUPAC number of the compound) was more than 10 years in eels (Whiley et al., 2003).

Table 2.5: PCB, PCDD/PCDF-congeners regarded as the most toxic congeners (WHO, 1997).

PCB	PCDD	PCDF
3,3',4,4'-Cl_4B	2,3,7,8-Cl_4DD	2,3,7,8-Cl_4DF
3,4,4',5-Cl_4B	1,2,3,7,8-Cl_5DD	1,2,3,7,8-Cl_5DF
3,3',4,4',5-Cl_5B	1,2,3,4,7,8-Cl_6DD	2,3,4,7,8-Cl_5DF
3,3',4,4',5,5'-Cl_6B	1,2,3,6,7,8-Cl_6DD	1,2,3,4,7,8-Cl_6DF
2,3,3',4,4'-Cl_5B	1,2,3,7,8,9-Cl_6DD	1,2,3,6,7,8-Cl_6DF
2,3,4,4',5-Cl_5B	1,2,3,4,6,7,8- Cl_7DD	1,2,3,7,8,9-Cl_6DF
2,3',4,4',5-Cl_5B	Cl_8DD	2,3,4,6,7,8-Cl_6DF
2',3,4,4',5-Cl_5B		1,2,3,4,6,7,8-Cl_7DF
2,3,3',4,4',5-Cl_6B		1,2,3,4,7,8,9-Cl_7DF
2,3,3',4,4',5'-Cl_6B		Cl_8DF
2,3',4,4',5,5'-Cl_6B		
2,3,3',4,4',5,5'-Cl_7B		

PCB-mixtures, particularly those with lower chlorine contents (up to 48%) have weak estrogenic activity (Bulger and Kupfer, 1985). PCBs show weak binding affinity to estrogen receptors with a potency 10 000- to 100 000-fold less than estradiol (Nelson, 1974). PCBs may alter reproductive endocrine function directly by altering the function of neurotransmitters involved in the regulation of gonadotropin hormone secretion (Thomas, 1989). Enhanced metabolic clearance of steroids and a decline in steroid levels, due to induction of cytochrome P450, has been shown for AhR-active PCB. However, compensatory mechanisms of the hypothalamus-pituitary-gonadal axis may eventually overcome this clearance, so that reproduction is delayed but not prevented (Thomas, 1989).

Dioxin is not an anti-estrogen in the classical sense of blocking estrogen binding to the estrogen receptor. It may alter the metabolism of estrogen, potentially leading to a decrease in the active form of the hormone at target sites. It also leads to decreased numbers of estrogen receptors. Dioxin is also not an estrogen and does not bind to the estrogen receptor. It does not cause classical estrogenic responses, but some of the effect of dioxin does resemble those seen with potent synthetic estrogens such as DES (diethylstilbestrol). Estrogen appears to be necessary to allow the progression of TCDD-promoted endometriosis in mice (Birnbaum, 1995).

Developmental and reproductive toxicity, immunotoxicity, and neurotoxicity have been detected in rats, mice and non-human primates at levels in the animals which are less than 10 times higher than in the more highly exposed members of the general population. Many of these same endpoints have

been observed in children whose mothers are at the high end of the general population in terms of levels of dioxin and related chemicals (Schecter et al., 2006). The developmental effects are most likely subclinical on an individual basis, including respiratory infections, behavioural or cognitive difficulties, and altered breast or penile development at puberty (Den Hond et al., 2002.)

The concentrations of these pollutants in environmental matrices can be determined by chemical analyses and/or employing any number of bio-assays. Chemical analyses are done by GC and MS and the concentration for each congener determined very accurately. Quantitatively, chemical analysis is the best method to determine the concentrations of these environmental pollutants, but the clean-up procedures can be time consuming as well as costly (Hilscherova et al., 2000). Instrumentation analyses, however, do not account for interactions among the chemicals in complex mixtures and provide little information on their biological effects. Chemical analyses can also underestimate the potential risks posed by these chemicals (Behnisch et al., 2001; Hilscherova et al., 2000).

In vitro cell bio-assays offer a rapid, sensitive and relatively inexpensive solution to some of the limitations of instrumental analyses. They enable estimation of total biological activity of all compounds acting through the same mode of action in extracts of any environmental media. Bio-assays can also integrate possible interactions among chemicals. However, *in vitro* methods, while a useful adjunct to instrumental analyses, still do not substitute *in vivo* toxicological studies.

A recent development is the aryl-hydrocarbon-immunoassay method (Ah-I). This is a cell-free system that comprehensively analyses total toxicity potential contributed by dioxin and dioxin-like compounds. Analysis of toxicity is functionally performed by measuring their collective ability to bind to a cytosolic aryl-hydrocarbon receptor protein (AhR). This activated AhR-protein then binds an exogenous Arnt-protein to form an activated protein complex, which is able to bind an ELISA plate-bound oligonucleotide dioxin responsive element (DRE) and is then detected by an immuno-assay-based colour reaction. The Ah-I measures toxic dioxins and dioxin-like compounds, based on toxicity appearance mechanism without the need for live cell culture or radioactivity (Kobayashi et al., in press).

Another recently developed assay is the reporter gene assay. Different strains of tissue cultures such as the rainbow trout cell line RLT2.0, the rat hepatoma cell line H4IIE (Villeneuve et al., 1999), human 101L cells and mouse hepatoma H1L1 cells (Behnisch et al., 2001) are stably transfected with a luciferase reporter gene under control of dioxin-responsive elements (DREs). Binding of the AhR-ligand complex to the DREs results in an up-regulation of luciferase transcription (Figure 2.5.2), which upon addition of a substrate, luciferin, catalyses a light-producing reaction. The luminescent endpoint can be measured with a luminometer to provide a sensitive measure of AhR-mediated gene

expression potency (Villeneuve et al., 1999). This assay is commercially available as CALUX® (Chemically Activated Luciferase eXpression) (Koppen et al., 2001).

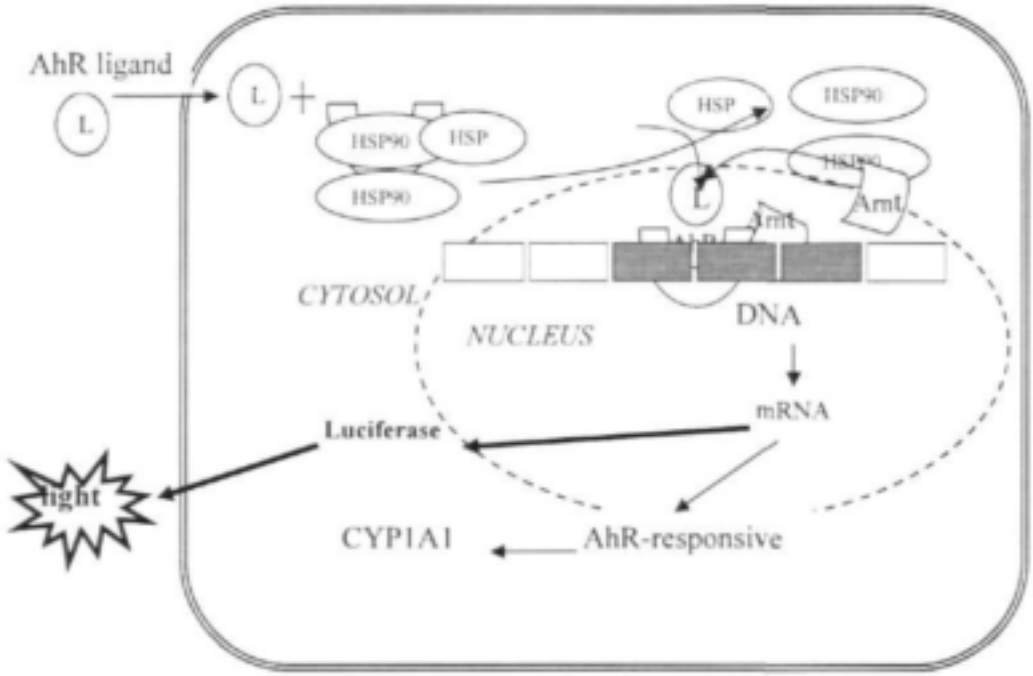

Fig. 2.2: The AhR response pathway. With the adoption for the bio-assay (parts in bold) HSP = heat shock protein; L = ligand; DRE = dioxin response element; Arnt = Ah receptor nuclear translocator. Adapted from Hilscherova et al. (2000).

2.6 Biosentinels

Several papers and reports stated that EDCs might be linked to reproductive, developmental and behavioural effects in humans and wildlife (Guillette et al., 1994; Bowerman et al., 1998; Colborn et al., 2000; Skakkebæk et al., 2001). Fish, alligators, frogs and rodents have been used to evaluate *in vivo* reproductive toxicity effects of EDCs in laboratory experiments (Guillette et al., 1994; de Jager, 1996; Nimrod and Benson, 1996).

2.6.1 Sharptooth catfish

The applicability of *in vivo* models enhances the ability to indicate the presence and physiological effects of EDCs in the aquatic environment (Kime, 1999). In general, fish provide a useful model for determining the effects of environmental aquatic pollutants on vertebrate reproductive function since they are most at risk from pollution (Kime, 1999). Numerous authors used fish as a model system to evaluate different types of pollution in the aquatic environment, such as metals and persistent organic pollutants (Heath, 1995; Kime, 1999; Heath and Claassen, 1999; van der Oost et al., 2003). Since rivers and estuaries are repositories for the enormous amounts of industrial and domestic waste containing thousands of natural and man made chemicals, the aquatic environment

is, therefore, an ideal medium to study the possible effects of EDC on wildlife populations (Jobling et al., 1998).

South Africa, a semi-arid developing country is lacking important arterial rivers and lakes and, therefore, requires extensive water conservation and control measures for its population of about 42,768,678 (est. growth 2003, 0.01%)(World Factbook, 2003). This country seems to be no exception to the rule and both the presence and possible effects of EDCs in South African water sources have been reported recently by De Jager et al. (2002) and Barnhoorn et al. (2003).

The sharptooth catfish, *Clarias gariepinus,* (Figure 2.3) an indigenous South African species, is considered to be one of the most important tropical catfish species for aquaculture (De Graaf and Janssen, 1996; Skelton, 1993; Skelton, 1995). Osteichthyes (bony fish) are part of the largest and most diverse group of vertebrates (Skelton, 1993; Burchett, 1996). *Clarias gariepinus* (Burchell, 1822) belongs to the order Siluriformes; suborder Siluroidei, and family Clariidae. It is distinguished from other Siluriforms by having a large armoured (bony) head with small eyes, a large terminal mouth and four pairs of barbels present. It has a long based dorsal (without spine) and anal fin, a stout serrated spine on the pectoral fins used for defense or "walking" overland, and a well developed suprabranchial organ for air breathing. No adipose fin is present.

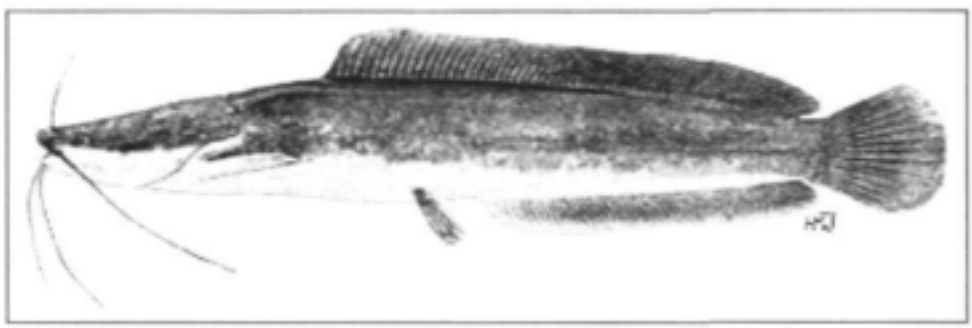

Fig. 2.3: A sketch representation of an African sharptooth catfish (Jubb,1967).

The colour varies from a sandy-yellow through gray to olive with dark greenish-brown markings, and a white belly (Bruton, 1988; Sketon, 1993; Skelton, 1995). The common name for *Clarias gariepinus* is the African sharptooth catfish (Skelton, 1993).

2.6.1.1 General biology

Sharptooth catfish comprise an ecologically diverse component of most river faunas throughout the world, preferring the slow- flowing reaches of rivers and also lakes. *C. gariepinus* is widely distributed throughout Africa, occurring in almost every aquatic habitat (Skelton, 1993). The species is an opportunistic omnivore capable of switching feeding modes depending on prey

availability (Teugels, 1986). It is a relatively hardy species, able to withstand harsh conditions (Bruton, 1988; Skelton, 1993), and does not easily succumb to disease (Teugels, 1986). Catfish are also typically very adaptable. The suprabranchial organ, and the catfish's ability to remain out of water for long periods of time and move overland during moist conditions, enables this species to survive in waters with low oxygen content, and to inhabit water bodies out of reach for most other fish (Bruton, 1988; Skelton, 1993; Teugels, 1986).

2.6.1.2 Reproduction and development

Sharptooth catfish spawn at night in summer, between September and March (Viljoen, 1999), with temperatures preferably above 22°C. This usually occurs after heavy rains, and runoff water has a strong stimulating effect on spawning (Van der Waal, 1974). Gonadal maturation starts a while before the rainy season (possibly in August), and is completed as the rainy season starts (September to February) (Viljoen, 1999). The gonadal maturation of adult *C. gariepinus* is summarized in Table 2.6.

Behaviour is an important factor in teleost reproduction, and migration and individual proximity are both important behavioural responses. *C. gariepinus* has limited migratory patterns, but may still compete in spawning runs. For external fertilization to occur, the proximity of two individuals of the opposite sex is crucial (Lagler et al., 1962), often preceded by aggressive postmigratory, and prenuptial displays. Spawning occurs in vegetative areas (Bruton, 1988; Skelton, 1993). Males and females swim together and the male starts swimming ahead, folding himself around the female (Van der Waal, 1974; Viljoen, 1999). The eggs are laid and the sperm released. Gas bubbles are released with the eggs, probably for egg dispersal, and the female also flicks her tail actively to mix and distribute sperm and eggs (Van der Waal, 1974; Viljoen, 1999). *Clarias* eggs are large adhesive disk which is extremely adhesive, insuring egg survival (Viljoen, 1999). Once released, the parents desert the eggs.

Egg development starts once the sperm penetrates the egg (impregnation) (Lagler et al., 1962), where after fertilization takes place. The fertilized egg undergoes different developmental changes (Table 2.7).

Table 2.6: The eight stages of gonadal maturation in adult *C. gariepinus* (adapted from Viljoen, 1999).

Stage	Description	Morphology
I	Immature virgin	Minute gonads under vertebral column. Testes and ovaries transparent elongate threads, apparently empty bags
II	Developing virgin	Sexual products have not yet begun to develop. Gonads very small, testes elongate transparent bags, ovaries transparent or translucent, red. Eggs invisible to naked eye, but visible under 10x magnification.
III	Developing	Eggs distinguishable to naked eye as white granules, developing into yellow spheres. Ovaries reddish-brown, becoming oval, testes changing from transparent to pale rose colour. Rapid increase of weight of gonads.
IV	Maturing	Testes enlarged, white; ovaries enlarged, opaque and orange, eggs large.
V	Maturity	Sexual products mature, gonads have achieved their maximum weight, but the sexual products are not extruded when light pressure is applied. Eggs round, opaque, yellow, present as discrete bodies. Ovary wall transparent. Testes swollen, white, sometimes pink with grey proximal edging. Testes or ovaries usually approximately equal in size. May remain at this stage for several months.
VI	Ripe	Fish on spawning run. Ovaries distend body cavity. Sexual products extruded by light pressure on the belly, weight gonads decreases rapidly from start of spawning to completion, gonad sacs collapse.
VII	Spent	Sexual products have been discharged, and remaining eggs are resorbed. Testes deflated grey-white sacs. Genital apertures inflamed and red. In females that didn't spawn, resorption begin with the ovary turgid, the ovary wall turns red, and a reddish-brown amorphous mass develops internally.
VIII	Recovering spent	Gonads very small, transparent or white sacs under vertebral column, eggs invisible to naked eye.

Table 2.7: Development of the fertilized egg until juvenile stage of *C. gariepinus* (TL – Total length), Viljoen, 1999).

Age	Mean TL (mm)	Morphology	Behaviour
0	1.6-1.9	Fertilized egg	Adheres to substrate by means of chorionic adhesive disk.
1 hr		Blastula visible as small red dot on pole opposite adhesive disk.	Adheres to substrate.
3.5 hrs		Meroblastic cleavage produces gastrula which is tightly confined by chorionic membrane.	Adheres to substrate.
9.2 hrs		Gastrulation completed by cellular overgrowth of yolk.	Adheres to substrate
16 hrs	3.6		Larva hatches
24-25 hrs		Head and pharyngeal regions project beyond yolk anteriorly and tail projects posteriorly. Two gill slits.	Tail begins to move, sometimes detaching yolk sac from substrate.
27 hrs	4.6	Larvae yello, yolk greenish orange, large olfactory sacs, notochord prominent, vertical fin fold and rudimentary intestine present.	Wriggle vigorously but attached by ventral sucker on vitelline membrane.
33 hrs		No visible external sense organs. Operculum present.	Larvae detached and swimming haphazardly, thigmotactic.
35 hrs	4.9	Yolk sac reduced, vascular network on branchiostegal membrane well defined.	Swim at 10-15mm/sec. When waterflow is reduced, activity decreases.
44 hrs	5.4	Mouth cavity and rudimentary eye formed, barbells form as buds.	Very active near substrate.
56 hrs	5.6	Pigmentation prominent on head.	Very active, swimming up to 200mm above the substrate in aquarium.
60 hrs	5.7	Opercula and mouth develop.	
66 hrs	6.2	Barbels longer and densely covered with sense organs, eye entire, mouth and intestine functional, mouth and perimeter of vertical fin fold lined with sense organs, yolk sac resorbed.	Begin feeding, mainly on water surface but also on substrate. When irrigation is stopped they form clumps with active movement of tails, possibly to produce a respiratory current. When inactive form tight groups.
80 hrs	6.3	Mouth assumes adult form.	Eats introduced zooplankton readily. Strong swimmers capable of evading pipette. Feed on water surface, substrate and sides of container.

Table 2.7 Continued

96 hrs	6.7	Head shield formed.	Intraspecific aggression, butting and conflict over food. Feed in midwater on *Pseudodiaptomus hessei* (Copepoda).
107 hrs	7.1	Vertical fin fold large, maxillary barbel reaches head length.	
120 hrs			Feed actively throughout 24-hour period, with irregular rest periods.
6 days	8.2	Caudal fin rays and pectoral fin bud appear. Barbels exceed head in length.	Feed on *Caridina nilotica* and dead catfish larvae, also grub in sand, expelling grains through opercular opening.
7 days	8.8	Pectoral fin forms.	First observation of aerial breathing.
8 days	9.2	Near adult body form.	Readily feed on small insects, crustaceans and bread.
9 days	9.5	Pectoral fin rays present.	Congregate at water surface when irrigation is stopped.
10 days	10.1	Dorsal fin rays form.	
11 days	12.1	External morphology closely resembles that of adult.	
14 days		Juvenile catfish	

The embryonic period is characterized by endogenous feeding, which ends with hatching. The newly hatched embryo is called a free-embryo, which becomes a larva once the yolk is absorbed and first oral feeding starts (Balon, 1990). The larva metamorphises into a juvenile, which eventually grows into a sexually reproductive adult. The sharptooth catfish has a relatively short developmental and embryonic period, possibly increasing the survival potential of the species, because these sensitive stages are not subjected to environmental stressors for extensive periods (Viljoen, 1999).

Males have distinct secondary sexual characteristics (Figure 2.4), which are absent in the females (de Graaf and Janssen, 1996).

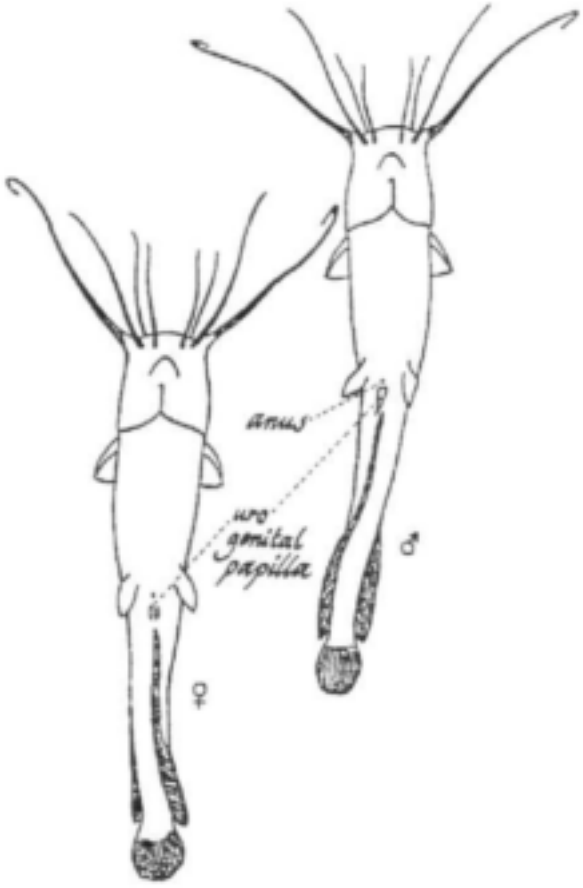

Fig. 2.4: Line diagram of the secondary sexual characteristics of male and female *C. gariepinus*.

The urogenital papilla (UGP) is a secondary sexual characteristic of catfish and like male nuptial colouration, the development is under hormonal control in fish (Kirby et al., 2002; Brion et al., 2004; Robinson et al., 2004). A number of these morphological characteristics are specific for

males, including breeding tubercles (occur in many cyprinid fish), fat pad (in mature male fathead minnows), and a modified anal fin (gonopodium in the mosquito fish and guppy) (Brion et al., 2004). These all develop as the male fish becomes sexually mature (Robinson et al., 2004). In medaka, however, the shape of the UGP is a female-specific sexual characteristic, probably under control of estrogens from the ovary (Brion et al., 2004). Subsequently, specimens can be sexed by examining the sexually dimorphic UGP (Kirby et al., 2002).

In principle, exposure of fish to EDC may influence the development and the morphology of the UGP. Robinson et al., (2003) concluded that secondary sexual characteristics of fish could be manipulated through exposure to pollutants. Exposure to estrogenic chemicals leads to feminization of these secondary sexual characteristics in adult male guppy and fathead minnow (Brion et al., 2004). Male fathead minnows exposed to estradiol showed atrophy of fat pads and nuptial breeding tubercles in a concentration-dependent manner (Robinson et al., 2003). In sand goby, the length of the UGP is estrogen-dependent (Robinson et al., 2004). The UGP is specifically important role during courtship and reproduction, and is used for sperm deposition and attachment. It seems possible, therefore, that malformation of the UGP may interfere with behaviour and compromise reproductive performance (Kirby et al., 2002). Likewise, the UGP appears to offer a useful endpoint for indicating exposure to estrogenic chemicals (Brion et al., 2004).

In river of the United Kingdom (UK), the occurrence of intersex (intersexuality, ovotestis, hermaphroditism) in wild roach (*Rutilus rutilus*; a cyprinid fish), characterized by the simultaneous presence of both male and female gonadal features in the same animal, is reported to be widespread (Jobling et al., 1998). Evidence indicates that the intersex condition has resulted from exposure to environmental estrogens and, therefore, that the condition is a consequence of the partial feminization of genetic male roach rather than of masculinisation of genetically female roach (Jobling et al., 1998). More recently, the intersex condition has been reported in other species of gonochoristic fish, including the gudgeon (*Gobio gobio*, U.K.) (Van Aerle et al., 2001) and the flounder (*Platichthys flesus*, UK) (Allen et al., 1999); *Pleuronectes yokohamae*, Japan (Hashimoto et al., 2000), suggesting that sexual disruption is not a species-specific phenomenon and is not restricted to U.K. waters alone. The exact causation of intersex is not known and currently under intense study. What is known, however, is that the populations of wild roach and gudgeon showing a high degree of intersex have been exposed to high concentrations of treated sewage effluents (Jobling et al., 1998). Exposure to estrogens (Hunter et al., 1983) or estrogen mimicking chemicals (Gimeno et al., 1996) during sexual maturation has been shown to induce sex reversal and/or intersex. Exposure during sexual maturation can inhibit gonadal growth and development (Jobling et al., 1996). Intersex has also been observed in the gonochoristic

sharptooth catfish (*Clarias gariepinus*) in freshwater sources of South Africa (Barnhoorn et al., 2003).

The steroid hormones have been frequently used as biomarkers of potential endocrine disruption. 11-ketotestosterone (11-KT) is the dominant androgen apart from testosterone in male teleost including Sharptooth catfish *C. gariepinus* (Cavaco et al., 2001). It was first isolated and recognized in the plasma of the sockeye salmon *Oncorhynchus nerka* during the 1960's (Idler et al., 1960). 11-KT is produced in liver of *C. gariepinus* by die conversion of 11-β-hydroxyandrostenedione (OHA) (Cavaco et al., 1997). Rising plasma 11-KT levels are coincident with the appearance of spermatids in *C. gariepinus* (Cavaco et al., 1997). A study by Chang and Chen (1990) indicated that sex steroids are not good detectors of possible endocrine disruption. Literature on fish 11-KT as an isolated biomarker of exposure is sparse. Folmar et al. (1996) pointed out that plasma 11-KT occurs in relationship with 17β-estradiol levels and the ratio is usually crucial during sexual differentiation in developing animals (Hileman, 1994). This ratio appears to be a more sensitive indicator of possible endocrine disruption in fish (Hileman, 1994; Folmar et al., 1996).

2.6.2 African clawed frog

During the last decade the loss of biodiversity has been a high-interest topic and the theme of many symposia and workshops. Amphibian declines have emerged as a key example of the global biodiversity crisis. Few other animal groups, if any, received as much attention as amphibians did over the past few years. According to Davidson et al. (2001) six primary hypotheses have been proposed to explain amphibian declines. Habitat destruction increased ultraviolet radiation, climate change and introduction of exotic predators, disease and agrochemicals.

In spite of the fact that every year many thousands of new chemicals are synthesized, there is an enormous lack of knowledge on their environmental properties such as distribution pathways in various compartments of bio-availability, toxicity, bio-degradation, etc. Pesticide use in some other countries has declined drastically over the past 20 years and when used correctly, they have enormous benefits in increasing crop yield (Zgajnar et al., 2001). On the other hand, there may be misuse and especially overuse of xenobiotics, like pesticides, resulting in an increasing concentration in surface and underground waters (Richardson, 1996).

Environmental chemicals affect endocrine function through effects on hormone biosynthesis, transport, activity and metabolism in animals (Van der Kraak et al., 1998). For example, the exposure of single fish species, White sucker (*Catostomus commersoni*) to bleached kraft pulp mill effluent, leads to significant reductions in plasma sex steroid hormone levels because of the

modulation of the endocrine function that causes interference with the biosynthesis or degradation of steroid hormones in the fish (McMaster et al., 1992).

The exact relationship between environmental factors and pesticide persistence in soil are not well quantified, because the rate at which pesticides degrade is influenced by several soil as well as weather factors. Soil is a complex medium in which chemical, physical, and biological factors such as pH, texture, organic matter, and micro-organisms are important variables. According to Graymore et al. (2001) there are five processes that determine the rate of pesticide degradation within soils namely hydrolysis, adsorption, volatilization, photo degradation, and microbial degradation. All of these factors may interact to determine the degradation rate as well as the specific compound-leaching rate and hence the persistence in a natural environment.

2.6.2.1 Effects in Amphibians

Living amphibians include three primary groups: frogs and toads (*Anura*), salamanders (*Caudata*), and caecilians (*Gymnophiona*), that are comprised of 4750 described species with perhaps another 500 extant species that have not been described (Sparling et al., 2000). Many amphibian species, especially frogs, complete their life cycle in temporary breeding sites or shallow ponds adjacent to agricultural fields that receive pesticide application. Furthermore, the timing of the application of these agrochemicals (spring and early summer) coincides with amphibian breeding and metamorphoses (Howe et al., 1998). Amphibians may be especially sensitive to agrochemicals or environmental contaminants because they have a permeable, exposed dermis (not covered by tough scales, hair or feathers), gills and eggs that readily absorb substances (not covered by hard or leathery shell) from the environment (Bantle et al., 1992). Amphibian larvae are primary consumers that feed on phytoplankton and periphyton, whereas adults act as higher-level carnivores, mainly feeding on insects and other invertebrates, which may expose them to the risks of biomagnifications of persistent environmental contaminants (Howe et al., 1998). Ouellet et al. (1997) described an increase frequency of deformity among frogs, *Rana clamitans* and *R. pipiens*, living in shallow ponds exposed to agricultural pesticide runoff. Of the 853 metamorphosing anurans examined in 14 agricultural habitats, 106 (12%) had severe degrees of ectromelia and ectrodactyly, compared to only two (0.7%) of 271 in the 12 control sites.

According to Foote (1964), the developmental basis for intersexuality in amphibians involves a sexual bi-potentiality of the gonocytes and gonaducts. Early amphibian tadpoles have differentiated gonads that consist of an outer cortex and an inner medulla. These two layers originate from the coelomic epithelium at the medial aspect of the mesonephric kidney in the developing embryo and support the primordial germ cells (George and Wilson, 1994). An

indifferent or bisexual state occurs before genetic factors induce sex-determining antigens, peptides and hormones that differentiate the cortex into ovarian tissue or medulla into testicular tissue (Noble, 1931).

A number of different environmental factors have been reported to influence sexual determination in amphibians. These factors include pH of aquatic medium, temperature, and the presence of chemicals that might pose an insult to the gonads (Reeder et al., 1998). The undifferentiated gonads of amphibians are highly sensitive to steroidal compounds, and treatment of males with estrogens during embryonic development can lead to sex reversal or the formation of an ovotestis (George and Wilson, 1994). A study conducted by Reeder et al. (1998), on cricket frogs (*Acris crepitans*), suggested that the overall prevalence of inter-sexuality from frogs collected between 1993 and 1995 was 2.6%. "*This may be consistent with the natural prevalence of intersexuality or may represent a prevalence altered by hormonally active environmental contaminants. Our 1994 data suggested a relationship between environmental contamination with atrazine and the prevalence of intersexuality in cricket frogs*" (Reeder et al., 1998).

Allran and Karasov (2001) found that there was no difference in hatchability of leopard frog (*R. pipiens*), wood frog (*R. sylvatica*), or American toad (*Bufo americanus*) embryos or mortality of 96-hour post-hatch larvae among atrazine treatments and controls. Atrazine also had no effect on swimming speed of *R. pipiens*. A study conducted by Birge et al. (1980) found that the 96-hour LC_{50} for atrazine on 4 day post-hatching *R. pipiens* larvae was 7.68mg/L. This is far less than was reported by Howe et al. (1998) on either early- (47.6mg/L) or late-stage (14.5mg/L) of *R. pipiens*. Howe et al. (1998) also observed abdominal edema in both *R. pipiens* and *B. americanus* larvae after 6 to 24 hour exposures to atrazine ranging from 2.8 to 23mg/L.

The gray tree frog (*Hyla versicolor*) metamorphosis, in pools exposed to atrazine at 200 and 2000µg/L by Diana et al. (2000) were 5% shorter and had a 10% lower body mass ($p<0.001$) than those in microcosms exposed to atrazine at 0 or 20µg/L. Larval period duration was 5% longer in the 2000µg/L group than in the 200µg/L group, but did not differ significantly among any other groups. This study does not contradict the assertion made by Solomon et al. (1996) that 20µg/L is the no-observed-adverse-effect-level (NOAEL) for atrazine in aquatic communities. By contrast, adverse effects were seen in the microcosms exposed to atrazine at 200 and 2000µg/L concentrations. Therefore, the NOAEL for atrazine in the experimental system used by Diana et al. (2000) was is between 20 and 200µg/L.

2.6.2.1 Effects in Xenopus laevis

One of the most widespread anuran species in the African sub-continent is the African Clawed Frog *Xenopus laevis*. It has been known to science for the past 200 years and was first described by Daudin in 1803 (Passmore and Carruthers, 1995). *Xenopus* belongs to a unique family of frogs, the Pipidae. The generic name *Xenopus* is derived from the Greek words "xenos", meaning strange or unusual, and "pous", for foot, while the specific species name *laevis* means smooth or slippery in Latin (Du Preez, 1996). *X. laevis* has a wide distribution area within the boundaries of South Africa, occurring from the Western Cape Province northwards, excluding the extreme north of the Northern Cape Province, northern KwaZulu-Natal and the Eastern Mpumalanga Provinces. It occupies any permanent body of water such as ponds (farm ponds), streams, dams, rivers and water holes (Weldon, 1999).

Tavera-Mendoza et al. (2002) conducted a study in which they exposed *X. laevis* tadpoles to atrazine at 21µg/L for 48-hours during gonadal differentiation (stage 56), which occurs during early metamorphosis, resulting in a decrease in testicular volume. The total testicular volume decreased from 0.026 ± 0.003mm^3 in controls to 0.01 ± 0.001mm^3 in atrazine exposed tadpoles; this represents a 57% decrease in testicular volume. Furthermore, testicular resorption in 70% of male tadpoles, as well as aplasia was observed in developing larvae exposed to atrazine. "*Atrazine may act as an endocrine disrupter at this sensitive stage in the developmental process and may subsequently significantly reduce the reproductive capacity of the organism (in this case X. laevis), for life – but no evidence was found of chromophores, indicating that the pituitary was actively secreting hormones*" (Tavera-Mendoza et al., 2002).

Hayes et al. (2002a) found that there was no effect on mortality, time to metamorphosis, length, or weight at metamorphosis at concentrations of up to 200µg/L atrazine. There also was a reduction in male metamorph laryngeal muscle size at concentrations >1µg/L as well as testicular abnormalities at a concentration as low as 0.1µg/L. The authors suggested that this may be a result of the disruption of steroidogenesis through an increase in the activity of aromatase ($P450_{ARO}$) during early larval development. Cross-sectional measurements of the laryngeal dilator muscle (Carr et al., 2003), revealed significant sex differences in both the reference and atrazine treatment groups, with males having a dilator muscle 20 to 25% larger than in females. The results of Carr et al. (2003) differ from those in the paper presented by Hayes et al. (2002a) with respect to laryngeal muscle size and gonadal abnormalities. The effects of atrazine on gonadal abnormalities observed at a concentration of 25µg/L in the study conducted by Carr et al. (2003) were only statistically significant, compared to the 0.1µg/L found by Hayes et al. (2002a). This is more than 250-fold greater than the smallest effective dose of atrazine reported to induced gonadal abnormalities in *X. laevis* when ethanol was used

as a vehicle for delivering atrazine in Holfretter's medium, rather than the usual Frog Embryo Teratogenesis Assay – Xenopus (FETAX) medium (Solomon et al., 2002).

Work of Tavera-Mendoza et al, (2002) and Hayes et al. (2002b) on *X. laevis* has been conducted under laboratory conditions. The question still remains as to what the situation would be when natural populations of *X. laevis* are exposed to pesticides. Due to the geographical distribution of natural *X. laevis* communities in the wild (endemic to Africa), South Africa is a suitable location in which to investigate the effect that pesticides have on *X. laevis* in its natural environment.

2.6.3 Freshwater snails

Various man-made agents like pesticides, industrial chemicals and some natural substances present in the environment, found in waste, ground and river water have been shown to affect the endocrine systems in birds, reptiles, fish and amphibians (Colborn et al., 1993; Jobling et al., 1998). However, until today only a few investigations have been done on the effects of EDCs on reproduction in exposed invertebrate aquatic species. Most attention has been given to fish (Arcand-Hoy and Benson, 1997). However, research on rats has shown that exposure to a range of synthetic EDCs during the pre-pubertal period are capable of influencing sexual maturity. Invertebrates, which are important primary consumers in limnic systems has received little attention.

The impact of chemical pollution on the reproductive success and population health of wildlife species is difficult to assess. This is because many factors such as habitat alterations, human interference and changes to natural food supplies can play an important role in determining population size. Therefore, it is difficult to determine to what extent, if any, EDCs may be contributing to observed effects on reproduction or population size in wildlife species. Some synthetic chemicals that have been shown to cause endocrine-related harmful effects in animals include (PCBs), 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane (DDT), other OC insecticides, TBT, pentachlorophenol, chlorinated dibenzo-p-dioxins (PCDD), and some estrogenically-active constituents from pulp mill effluents.

The hormonal regulation of biological functions is a common characteristic for all animal taxa, including the invertebrates. While the basic endocrine system to regulate biological processes has been conserved across all animal taxa (MacLachlan, 2001), some components of the endocrine system used in the various systematic groups have undergone significant evolutionary changes. These differences are especially true for invertebrates that exhibit a wide range of different chemical signalling systems, with some of them being unique to specific phyla. Other invertebrate

groups (e.g., prosobranch molluscs) seem to have at least partially comparable hormones to vertebrates, so that functional, vertebrate-type sex steroids are produced in these groups (DeFur et al., 1999; Lafont, 2000; Hines et al., 1992). However, sufficient knowledge regarding the role of these steroids in the endocrine system of most invertebrates is still lacking (Pinder et al., 1999).

The endocrine systems of invertebrates generally regulate the same processes (development, growth and reproduction) found in vertebrates. Because invertebrate species have developed many different life histories, it is evident that endocrine systems of invertebrates are much more diverse than those found in vertebrates (DeFur et al., 1999). Estrogens and EDCs can affect several vertebrate and non-reproductive functions, including the immune response. As it is virtually impossible to provide a complete overview of the various endocrine systems in invertebrates an overview will follow. More detail can be found in Lafont (2000), Hoffmann and Porchet (1984), Dorn (2000), Matthiessen and Gibbs (1998).

2.6.3.1 Invertebrate endocrine systems and their susceptibility to endocrine disrupting compounds

According to Janer et al. (2005) invertebrates represent 95% of all animal species. Just as in vertebrates, invertebrates are also susceptible to the action of EDCs (LeBlanc et al., 1999). The development of imposex in gastropods exposed to tributyltin is considered one of the clearest examples of EDC responses (WHO, 2002). Because invertebrates are represented by more than 30 different phyla, their endocrine systems are considerably more diverse than in vertebrates (comprising only the chordate phylum). In general, invertebrates use steroids, terpenoids, and peptide hormones, but the latter are the most common (Lafont, 2000; Matthiessen and Gibbs, 1998). Steroids are secreted in vertebrates from true glands, but the secretory structures in invertebrates are often neuronal in origin (neurosecretory organs or cells). Steroids, such as ecdysone and the vertebrate-type steroids, differ from both terpenoid and peptide hormones in their physical and chemical properties, solubility and resistance to degradation. Therefore, just as in vertebrates, it is likely that the endocrine systems in invertebrates can also be affected by EDCs.

Chemicals have been intentionally produced to disrupt the endocrine system of insect pests. These insect growth regulators (IGRs) were developed to disrupt the insect hormonal system, acting as ecdysone agonists, antagonists, or juvenile hormone analogues. Tributyltin (TBT) induced imposex and intersex in gastropods is regarded as one of the best examples of EDC activity in invertebrates (Matthiessen and Gibbs, 1998). The limited number of examples of endocrine disruption in invertebrates is due to the poorly understood hormonal systems, when compared with vertebrates. Endocrine changes following an exposure to certain compounds

may, therefore, be easily missed. Consequently, we can expect that far-reaching changes such as for TBT and its effects on prosobranch populations may not be unique within the invertebrates (Matthiessen and Gibbs, 1998). Studies on ED in invertebrates are important because invertebrates provide key species for ecological, and represent a large part of the global biodiversity.

2.6.3.2 Molluscs

Rittschof and McClellan-Green (2005) state that molluscs can be used to study endocrine disrupting compounds in invertebrates, as they are ubiquitous, have highly conserved control and regulatory pathways that are often homologous to vertebrate systems, and are extremely sensitive to anthropogenic inputs. The possibility to work with all the life history stages of molluscs, from cleaving eggs (Scheltema, 1967), to sexually mature adults (Blankenship et al., 1983), is a great advantage. The mostly sedentary lifestyle of most molluscs is also an advantage when investigating reproductive processes and morphological changes. Behavioural castration of some molluscs (Straw and Rittschof, 2004) and reproductive failure (Gibbs and Bryan, 1986; Gibbs et al., 1990; Gibbs et al., 1991) as a response to chemical pollution are reasons to study these invertebrates.

Steroid hormones apparently play an important role in the sexual development of molluscs (Lintelmann et al., 2003). In *Helix aspersa*, androstenedione metabolism produces several kinds of steroids, including testosterone, estrone, and estradiol-17 beta. These conversions indicate the action of several steroid conversion enzymes: dehydrogenases, reductase, and an aromatization system. It has also been shown that a secretion of neural factors from the pleural ganglia induces penis growth. Some gonadal activity, however, seems to be controlled by a gonadostimulin or mitogenic substance (Lintelmann et al., 2003). Biosynthetic pathways of steroid hormones are in many ways identical to those of other animal species. Thus, inhibition of the conversion of testosterone to estradiol catalyzed by P450-aromatase may affect the sexual development of snails as suggested for imposex caused by TBT. It is therefore important to carefully consider the various possible modes of action, before drawing a conclusion about the endocrine-disrupting mechanism of possible EDCs on molluses.

The responses of the freshwater snail *Marisa cornuarietis*, and two marine prosobranchs *(Nucella lapillus, Nassarius (Hinia) reticulatus)* to cyproterone acetate (CPA) and vinclozolin (VZ) were investigated by Tillmann et al. (2001). These known anti-androgens induced a number of biological responses in all three species. The lengths of the penis and of accessory male sex organs (such as the penis sheath and prostate) were significantly reduced. For *Marisa*, this was only found in sexually immature specimens and was reversible as the males

attained puberty. In the two marine species the sexually mature males responded to anti-androgens by showing a reduction of the male gonads, and an advancement of the sexual repose phase.

The effects of BPA, OcP and xeno-estrogens on freshwater and marine prosobranch species were investigated in another study, by exposing adult specimens of *Marisa cornuarietis* and *Nucella lapillus* to varying concentrations of BPA and OcP (Oehlmann et al., 2000). They found that the xeno-estrogens induced a complex syndrome of alterations in female *Marisa* referred to as "super-females" at the lowest concentrations. Affected specimens were characterised by additional female organs, enlarged accessory pallial sex glands, and gross malformations of the pallial oviduct section, resulting in an increased female mortality, as well as massive stimulation of oocyte and spawning mass production. Prosobranchs are therefore sensitive to endocrine disruption at environmentally relevant concentrations.

2.6.4 Small mammals

The evolutionary close association between man and small mammals that often live in close approximation of one another has received relatively little investigation. Small mammals should theoretically be excellent sentinel organisms, as they are endothermic, share many of the same physiology, parasites and pathogens, and come in contact with pollutants through similar routes of exposure as their human counterparts. Small mammals have indeed been used to investigate exposure to radioactivity (Rudge et al., 1993), heavy metals (Hunter et al., 1987; Shore, 1995), and chlorinated compounds (Batty et al., 1990). The well-known ability of mammals to tolerate, detoxify, or adapt to pollutants stress (Shore and Daubin, 1994), apply to both man and small mammals. This offers a unique possibility for investigation when these live in a close association. Only three studies relevant to the possible use of small animals as biosentinels could be found. A fourth study investigated the *F1* generation from wild caught mice. Some laboratory studies using rodents are included in this summary.

The population dynamics of wild small mammals are adversely affected by high dietary exposure to PCBs, because of the impact upon the size, maturation and structural integrity of the sex organs. White-footed deer mice *(Peromyscus leucopus)* living around a PCB-contaminated pond showed inhibited reproductive capability and dysfunction of the liver, spleen and adrenals, at total body concentrations of PCB average 2500µg/kg (Batty et al., 1990). Johnson et al. (1996) reported whole body concentrations of EPCB (Arochlor 1:1:1) and specific PCB congeners in three species of small mammals from a severely contaminated landfill site. These included the common shrew *(Sorex araneus)*, and two rodents species, a field vole (*Microtus agrestis)* and a wood mouse *(Apodemus sylvaticus)*. The interspecies differences with regard to PCB levels, suggested that the

food chain position occupied by a particular species is very influential upon the relative abundance of different PCB congeners in body tissue. Thus, a species in the upper trophic levels of a multi-faceted food chain (such as the shrew in this case) will be subjected to greater exposure to those congeners that are inducers of mixed- or phenobarbital-type, mammalian microsomal mixed-function oxidases-accepted indices of toxicity potential.

Boonstra and Bowman (2003) assessed whether PCBs adversely affected the population demography of the short-tailed shrew (*Blarina brevicauda*), living in their natural environment. *Blarina* were selected because they were expected to readily bioaccumulate PCBs from the soil via insects. Populations were intensively live trapped on 1-ha grids. There was no relationship between any demographic parameter and PCB soil concentrations. Population densities were high (usually exceeding 20/ha, and on two grids exceeded 60/ha in summer), survival was good, and sex ratios, reproduction rates, growth rates, and body mass were within the ranges reported in the literature. Thus, these shrew populations showed no detectable impact on their population demography from living on PCB-contaminated sites.

The study on the effect of BPA on the F1 generation of field voles is technically not a field study, but has relevance to the current project. A factor that confounds the extrapolation from laboratory to field studies may be differences in susceptibility to EDCs, possibly due to the inbred nature of the normal laboratory rats and mice. Nieminen et al. (2002) found that the F1 generation from wild caught field moles (*Microtus agrestis*) was more susceptible to BPA than established laboratory rodents, although this was done at relatively high dosage rates. BPA exposure increased the plasma testosterone concentrations at 250mg BPA/kg/day. The pooled plasma ghrelin levels increased and the leptin levels decreased after exposure to 50 or 250mgBPA/kg/day. The liver 7-ethoxyrufin-*o*-deethylase activity decreased slightly at all doses, as did the liver cytosolic glutathione *S*-transferase activity at 250mg BPA/kg/day.

Transgenerational effects of environmental toxins require either a chromosomal or epigenetic alteration in the germ line. Transient exposure of a gestating female rat during the period of gonadal sex determination to the endocrine disruptors VZL (an antiandrogenic compound) or methoxychlor (estrogenic) induced the adult phenotype in the F1 generation of decreased spermatogenic capacity (cell number and viability) and increased incidence of male infertility (Anway et al., 2005).

In utero and lactational exposure to an environmentally relevant organochlorine mixture adversely affected the reproductive system of male rats, perhaps via antiandrogenic effects during testis development, suggesting a possible reproductive health hazard for human and other species. The

organochlorine mixture was designed to resemble that found in Ringed Seal blubber from northern Quebec, which is part of the traditional Inuit diet (Idris Anas et al., 2005).

Han et al. (2004) showed that when male rats were treated with *p*-NP at 250mg/kg/day, the absolute and relative weight of the epididymis decreased dramatically, while the relative weight of the kidney and liver increased by 14 and 22%, respectively. In addition, the sperm density of the head of the epididymis and the testosterone level descended at 250mg/kg/day. Pathological changes were detected by microscopy and the number of apoptotic cells in testes increased with *p*-NP in a dose-dependent manner. In four months old male rats, different doses of OcP (1×10^{-5}, 1×10^{-7}, 1×10^{-9}M) caused an increase in tail abnormalities of epididymal sperm that could interfere with sperm motility, and the highest dose also decreased the sperm count. The findings raised the possibility that consumption of OcP in drinking water may adversely affect human male reproductive fertility (Blake et al., 2004).

In assessing the development of puberty in juvenile male rats after sub-acute exposure to BPA and *p*-NP, two groups of rats were administered orally with 100mg/kg body weight of either chemical and a third group a mixture of both. *p*-NP and BPA delayed the onset of puberty, caused testicular damage in all the treatment groups with spermatogenesis affected in most of the treated rats. Administration of *p*-NP and BPA in a mixture has caused less than additive effects (Tan et al., 2003).

De Jager et al. (1999a) showed that exposure of adult male rats to different concentrations of *p*-NP had an effect on the histology of the seminiferous tubules, signs of epididymal toxicity, impaired testicular mass and sperm count. In the groups with higher concentrations, spermatogenesis was affected in some animals. In another study done by De Jager et al. (1999b) the effect of maternal exposure of rats to *p*-NP was determined. An overall lower sperm count with increased *p*-NP concentrations was found which corresponded with the decreased testicular and epididymal masses. This emphasized the toxicity of *p*-NP on both testis and epididymis. The histological measurements of the seminiferous tubules were also smaller in the exposed groups. Lee et al. (1999) tested the testicular abnormalities in male rats after lactational exposure to *p*-NP. They found that lactational exposure of male rat pups decreased the size of their testes and male accessory glands, lack of differentiation of seminiferous tubules, lowering of sperm count and reduction in the percentage of motile sperm and modulation of a specific form of testicular proteinases.

Epidemiologic and experimental data suggest that consumption of diets that are rich in isoflavones (phytoestrogens) may decrease cancer risk in the breast, prostate, and other tissues. Rats received

dietary exposure to a mixture of soy-derived isoflavones containing 45% genistein, 23% diadzein, and 4% glycitein. Isoflavone exposure had no significant effects on spermatid count, sperm production, or sperm morphology in any group. The results of this study conducted in adult male rats differ from the significant alterations in reproductive parameters that have been reported in female rats receiving prenatal or juvenile exposure to isoflavones (Faqi et al., 2004).

In another study (Kilian, 2005) the interactive effects of a relevant environmental mixture of known EDCs on fertility parameters in Sprague-Dawley rats were determined. A modified protocol of the Organization for Economic Cooperation and Development (OECD) 415 one-generation test was used. Group A received cottonseed oil as control, and Groups B, C and D received deltamethrin (DM); DM and DDT; and a combination of DM, DDT, phytoestrogens, and *p*-NP, respectively. Estrogenicity appeared to increase with the combined exposure to the mixture of compounds, as a progressive decrease in seminiferous tubule diameter was observed. All experimental groups differed statistically significantly from the control group. Seminiferous epithelium thickness differed significantly amongst groups, with the exception of Groups C and D. These findings were mirrored by changes in the anogenital distance and sperm count, but to a lesser extent. The histology of the testes showed signs of apical sloughing and vacuolization. These findings emphasized that exposure to EDCs could be responsible for adverse reproductive effects (Kilian, 2005).

CHAPTER 3

THE URBAN NATURE RESERVE (UNR) AND OVERVIEW OF PREVIOUS PROJECTS

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3.1 Introduction

The Urban Nature Reserve (UNR) (Figure 3.1) covers a surface area of 3870 Ha in the city limits of the City of Tshwane (also known as Pretoria). Dam 1 and Dam 2 are situated in this reserve and they cover surface areas of 204.13 Ha and 19.47 Ha respectively. The Reserve is known as a Bakenveld area and as rocky highveld and grassland. Apart from the environmental importance, this reserve is one of the few recreational areas in close proximity of the metropoles of Johannesburg and Tshwane. The UNR was proclaimed as a game reserve in 1937 and opened to the public in 1994. The main attraction is the game viewing on the reserve where a variety of wildlife roams freely. Horse rides, hiking trails, angling and camping are also included in the activities Culture and recreation department, 1997).

The UNR forms an integral part of the catchment area, and the primary function of this Reserve is to act as an active wetland for the filtration of incoming water accumulating in Dam 2. The wetland stretches 8km in length and measures 400m in width. The wetland includes D1 until it reaches D2. Between D1 and D2 the wetland measures approximately 5km. The soil is mainly alluvial and the most abundant flora in this wetland is reeds (*Fragmites australis*). The UNR wetland is also classified as a peat land. Africa and South America have 1% of the worlds' peat lands, which gives this wetland a unique global importance. The number of wetlands around the world is diminishing due to anthropogenic activities. Industrial and mining, as well as farming activities are playing a major role in the pollution and possible destruction of the world's wetlands. Wetlands form an integral part of a river systems' ability to recover from pollution. This fact may play a major role in the future supply of water to the populations. These shallow water fed systems are central to the life cycle of many plants and animals; some of them endangered species (van Dyk, 2003). Wetlands are among the most biologically important and productive ecosystems on earth. Table 3.1 lists some of the important functions of wetlands, including the conservation concerns attached to the specific functions.

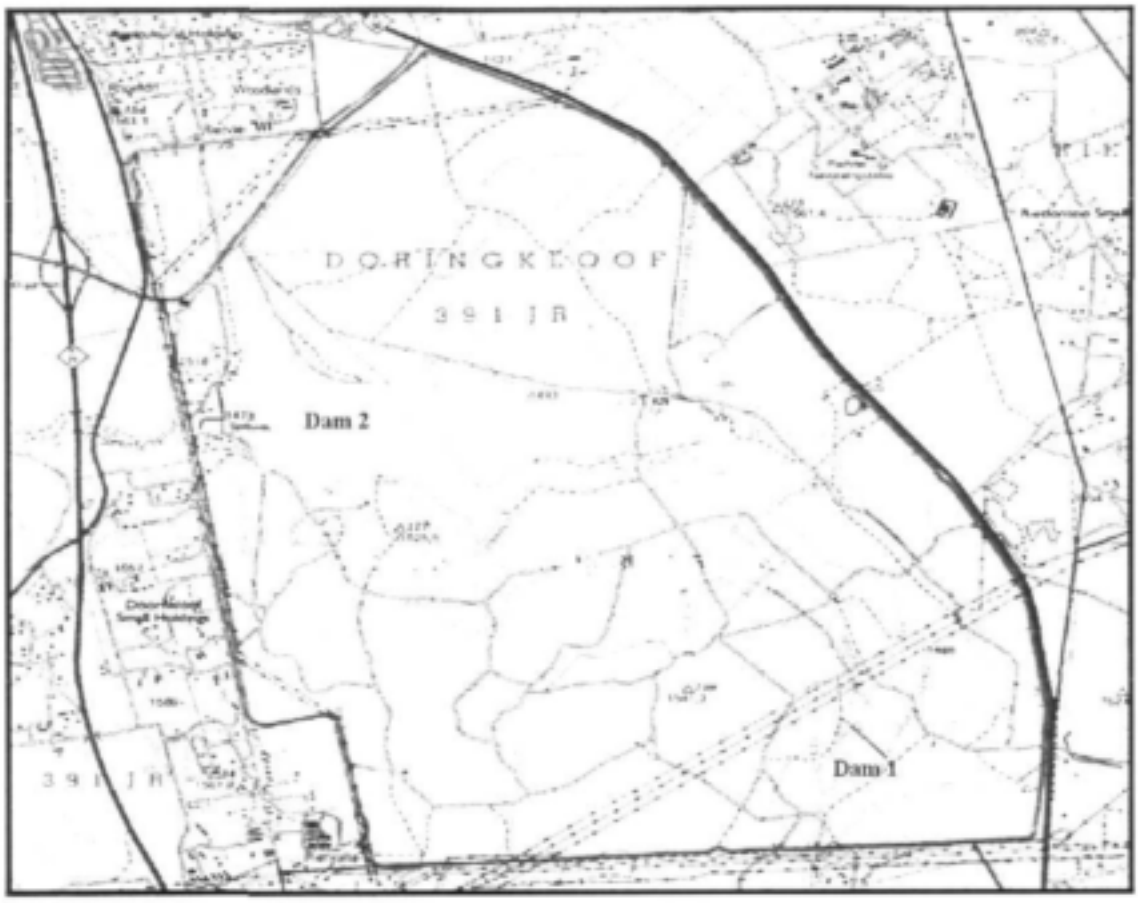

Fig. 3.1: Map of the Urban Nature Reserve.

Table 3.1: Wetland functions and conservation concerns (Kupchella and Hyland, 1993).

Wetland function	How wetlands perform function	Conservation concern
Flood storage	Some wetlands store and slowly release floodwaters.	Fill or dredging of wetlands reduces flood storage capacity.
Flood conveyance	Some wetlands (particularly those immediately adjacent to rivers and streams) serve as floodway areas by conveying flood from upstream to downstream points.	If flood flows are blocked by fills, dikes or other structures, increased flood heights and velocities result, causing damage to adjacent upstream and downstream areas.
Erosion control/wave barriers	Wetland vegetation, with massive roots and rhizome systems, binds and protects soils. Vegetation also acts as wave barriers.	Removal of vegetation increases erosion and reduces capacity to moderate wave intensity.
Sediment control	Wetland vegetation binds soil particles and retards the movement of sediment in slowly flowing water.	Destruction wetland topographic contours or vegetation decreases capacity to filter surface runoff and act as sediment traps. This increases water turbidity and siltation of downstream reservoirs, storm drains, and stream channels.
Pollution control	Wetlands act as settling ponds and remove nutrients and other pollutants by filtering and causing chemical breakdown of pollutants.	Destruction of wetland contours or vegetation decreases natural pollution control capability, resulting in lowered water quality of downstream lakes, streams, etc.
Fish and wildlife habitat	Wetlands provide water, food supply, and nesting and resting areas. Coastal wetlands contribute nutrients needed by fish and shellfish to nearby estuarine and marine waters.	Fills, dredging, damming and other alterations destroy and damage flora and fauna and decrease productivity. Dam construction is an impediment to fish movement.
Recreation	Wetlands provide scenery, wild areas, habitat, wildlife and water for recreational use.	Fill, dredging or other interference with wetlands causes loss of area for boating, swimming, bird watching, hunting and fishing.
Water supply (surface)	Some wetlands store floodwaters, reducing the timing and amount of surface runoff. They also filter pollutants. Some serve as sources of domestic water supply.	Fills or dredging cause accelerated runoff and increase pollution.
Aquifer recharge	Some wetlands store water and release it slowly to groundwater deposits. However, many other wetlands are discharge areas for a portion or all of the year.	Fills/drainage may destroy wetland aquifer recharge capability, thereby reducing base flows to streams and groundwater supplies for domestic, commercial, other uses.

3.2 Synoptic description of the geology and geohydrology in the Dam 2

3.2.1 Geology

Dam 2, with a full supply capacity of 12.2Mm^3, is underlain by indurated sedimentary strata (shale, siltstone and quartzite) of the Timeball Hill Formation and extrusive igneous rocks (lava) of the Hekpoort Formation (Figure 3.2). These formations lie at the base of the Pretoria Group assemblage of strata. Although the rivers draining into the dam (the Swartspruit from the south and an unnamed drainage from the southeast) rise on similar strata, they traverse dolomitic formations of the Chuniespoort Group in their lower reaches. The study area is also characterised by a variety of geological structures. The most striking of these are subvertical dolerite dyke intrusions. The importance of these structures is that they generally form a barrier to the lateral movement of groundwater. As such, they build compartments that subdivide especially the dolomitic strata into smaller, discretely interconnected geohydrological units. The numerous dolomitic springs in the area are an important derivative of these structures.

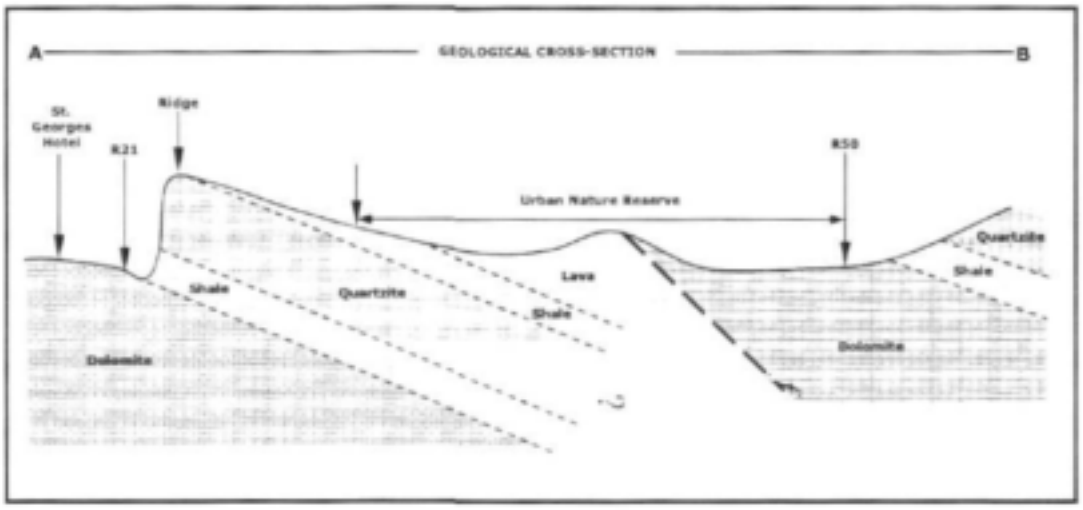

Fig. 3.2: Geological cross-section of Dam 2 area (CSIR communication).

3.2.2 Geohydrology

The dolomitic aquifer in the catchment contributes some 6.53Mm^3 (3.8%) to the current annual Tshwane Metropole water demand of some 173Mm^3. This testifies to the importance of groundwater as a source of potable water in the region. The contribution is derived from boreholes located in the UNR (126L/s = 3.97Mm^3/a) and the protected Grootfontein Spring (102L/s = 3.31Mm^3/a) to the southeast. The unprotected Elandsfontein Spring (46L/s), located to the south (upstream) of the Reserve at the head of a tributary of the Swartspruit, provides water for agricultural activities. The groundwater resource is under threat from various land use activities in the catchment (Figure 3.3). These include small holdings (located mainly to the west of the Reserve) that use septic tank systems for on-site sanitation purposes, agriculture (including

irrigation, tunnel and chicken farming) and industry (mainly brickworks) to the south, and residential estate development to the east. There is also a landfill site to the north-eastern side next to the border of the UNR.

Dam 2 has a catchment area of 479km^2 (Figure 3.2). This includes the Sesmylspruit and the Grootvlei Spruit. The stream that enters the system consists of effluent from the sewage treatment plants (STP) higher up in the catchment as well as inflow from industrial, agricultural and settlement areas before flowing into the UNR. The Grootvlei Spruit which is a 8km long wetland confluence with the wetland in the UNR after Dam 1. The Grootvlei Spruit flows through a urbanized area which include several industries and informal settlements before it enters the UNR.

Increased pressure of encroaching urban development, rural effluent, farming and industrial activities could have detrimental effects on this fragile ecosystem. Ecological assessment studies of the current and future environmental status of the UNR is of vital importance. Various research projects have been done in the UNR and the findings are summarised below.

3.3 Previous projects

The first study was conducted by Slabbert et al. (1998) for the WRC. Toxicity (mammalian cell colony froming inhibition, teratogenicity in *Xenopus laevis* and mutagenicity using the Ames *Samonella* test was detected in Dam 2 water. This project included the development of guidelines for toxicity bioassaying of drinking and environmental waters of South Africa.

3.3.1 Aneck-Hahn, 2002

Estrogenic activity in water samples in an Urban Nature Reserve

During this study, seven sites were identified in the UNR to standardise the recombinant yeast estrogen bioassay (YES) to measure estrogenic activity in water under local South African conditions. The results indicated five sites with estrogenic activity and two sites that showed no estrogenic activity.

Fig. 3.3: The UNR area and surrounding activities.

3.3.2 Barnhoorn et al., 2002

The occurrence of macro calcifications in the testis of Eland (Tragelaphus oryx) at the Urban Nature Reserve, South Africa

While collecting tissue samples from possible sentinel animals in a hunting programme for target analyses of specific endocrine disruptive chemicals, macroscopic focal white gritty areas were observed in testes of eland (*Tragelaphus oryx*). Testicular tissue samples were fixed in Bouin's solution for histopathology and fat tissue was frozen for analytical chemistry (n=17). Microscopically, sperm stasis, multiple intratubular dystrophic calcifications, interstitial chronic cell infiltrates and surrounding fibrosis were noted in all samples. Spermatogenesis was generally impaired. Full complement of spermatogenesis was observed only in focal areas and Sertoli cell vacuolization and sloughing of the seminiferous epithelium occurred. A few atypical germ cells were encountered and adenosis and adenoma of the rete testis, changes pathognomonic of chronic estrogenic exposure, were noted. Fat samples of 11 out of 17 animals (64.7%) contained the estrogenic chemical *p*-nonylphenol (*p*-NP; concentrations ranged from 35.0 to 214.0 (184.8 ± 24.6) µg/kg fat. No organochlorine chemicals or polychlorinated biphenyls were detected. These novel findings are the first indication of mammalian wildlife being affected by environmental pollution of endocrine disrupting chemicals in South Africa. The findings in eland are similar to

the testicular dysgenesis syndrome in humans attributed to developmental exposures to chemicals (Bornman et al., submitted).

3.3.3 Barnhoorn et al., 2004

Histological evidence of intersex in feral sharptooth catfish (Clarias gariepinus) from an estrogen polluted water source in Gauteng, South Africa

This study found the first histological evidence of intersex in a fish species inhabiting a South African water source. 100 Catfish, (*Clarias gariepinus),* were collected randomly from both the Dam 1 and Dam 2. Each fish was macroscopically evaluated, blood was drawn, and the gonads were macroscopically and histologically examined to verify intersex potentially related to endocrine disruption. Gonadal histology of several fish showed primary oocytes scattered between testicular tissues indicative of intersex. The results reported 20% of intersex in fish from both the dams. The GSI value for intersex fish were closer to the male GSI values, suggesting that the intersex fish were more likely from the feminisation of male catfish. Target chemical analyses of the water, sediment and serum samples tested positive for *p*-nonylphenol (*p*-NP). Water and sediment values at Dam 1 measured 6360µg/kg and 4.0µk/kg respectively while the *p*-NP level in sediment at Dam 2 was 113µg/kg. *p*-NP is commonly found in the effluent from sewage treatment plants and the presence of *p*-NP in water and sediment indicates estrogenic water pollution, which might have an effect on other wildlife and humans dependent on these sources.

3.3.4 Muteveri, 2004

Genetic and biomolecular responses of the sharptooth catfish (Clarias gariepinus) in the contaminated wetland system, South Africa

This study was done as part of a major project aimed at identifying and selecting suitable biomarkers to use in the UNR, South Africa. This study focused on the response of *Clarias gariepinus* (Burchell, 1822) to contaminants using three biomarkers: population genetic structure, DNA damage and metallothioneins (MTs). The main objectives of the study were to assess the potential of these biomarkers in *C. gariepinus* and to provide baseline data on the UNR. Eighteen enzyme loci (creatine kinase (Ck), esterase (Est), phosphoglucomutase (Pgm), glycerol-3-phosphate dehydrogenase (G3pdh-1 and -2), glucose-6-phosphatase isomerase (Gpi-A and -B), L-lactate dehydrogenase (Ldh-A and B), malate dehydrogenase (Mdh-1, -2 and -3), malate dehydrogenase (NADP+) (Mdhp), phosphogluconate dehydrogenase (Pgdh), isocitrate dehydrogenase (NADP+) (Idh); superoxide dismutase (SOD), and peptidase: substrate (leucyl tryrosine) (Pep-S1 and -2)) were screened for by horizontal starch gel electrophoresis. An agarose gel electrophoretic method was used for DNA damage determination and a spectrophotometric method for metallothionein. The population of the UNR (UNRP) showed higher genetic variability as shown by the average number of alleles per locus (A), proportion of polymorphic

loci (P) and expected average heterozygosity (HE) than the reference populations. Statistically significant deviations ($p < 0.05$) from Hardy-Weinberg proportions associated with heterozygote deficiencies occurred at Gpi-B, Idh, Pgdh, Ldh-A and Pep-S1 for UNRP. These deviations could have been a result of small sample size. There was high genetic differentiation between UNRP and the reference populations with FST values of 0.450 and 0.480 between UNRP and wild population (WP) and UNRP and Northern Cape population (NCP) respectively. A mean base-pair length of 6770.9 ± 10.68 (SE) bp was recorded in Dam 1 population (D1P) that of 6327.2 ± 17.69 (SE) bp was recorded in the Dam 2 population (R2P). The MDP had a higher proportion of DNA fragments with low base-pair lengths than R2P indicating a higher level of DNA damage. The level of DNA damage in D1 was higher than in the D2P. The mean MTs content was 3.63 and 0.643 (SE) nmol/g-1 wet weight in the D1P and 4.35 ± 0.580 (SE) nmol/g-1 wet weight in the D2P. There was no significant ($p = 0.40$) difference in the levels of MTs between the two dams. It was concluded that there were significant differences in genetic diversity between UNRP and the other populations in unpolluted sites, hence genetic diversity in *C. gariepinus* has the potential to be a biomarker of pollution.

3.3.5 Barson, 2004

Endoparasites of the sharptooth catfish, Clarias gariepinus (Burchell), from the Dam 2, Sesmyl Spruit system, South Africa

As part of a bigger aquatic health project in the Zoology Department, University of Johannesburg, aimed at finding suitable biomarkers for water quality monitoring in the system, this study was done to identify the major internal helminth parasites of the sharptooth catfish, *Clarias gariepinus*, that can be used in fish health assessment studies, and to determine their prevalence and intensity in the Dam 2. Fish were collected during one sampling survey and examined for endoparasites, also noting any ectoparasites that are recorded in routine fish health studies. Five species of helminths were identified: the adult cestodes, *Polyonchobothrium clarias* (intestine and stomach), *Proteocephalus glanduliger* (anterior intestine), the adult nematode *Procamallanus laevionchus* (stomach), larvae of the nematode *Contracaecum* sp. and many trematode metacercariae encysted in the muscles, of which only *Ornithodiplostomum* sp. was successfully excysted and identified. This trematode was recorded in South Africa for the first time, but could not be specifically identified because the reproductive system was still immature. Examination of piscivorous birds in the area or experimental infection of young birds is the only means by which the adult trematodes can be obtained. The adult cestodes and nematodes had specialised structures for attachment to the stomach and/or intestinal mucosa, adaptations associated with pathological effects in the host. *Polyonchobothrium clarias* had a crown of 26-30 hooks on its rostellum, and this number differs from those of specimens described from catfish in other African countries. Scanning electron microscopy showed that the rostellum of the *P. clarias* specimens from Dam 2

was different from that of specimens from other localities in South Africa. *Proteocephalus glanduliger* in *C. gariepinus* from Dam 2 differed in strobila size and size of glandular organ from specimens described by Janicki (Egypt) and Mashego (South Africa), the present specimens being much longer but with smaller glandular organs. *Procamallanus laevionchus* is a common parasite of catfish from many African countries, including South Africa, and scanning electron microscopy showed some form of transverse markings and presence of papillae-like structures at the posterior end of female specimens, an observation which was not described in previous studies. Larval *Contracaecum* are also common in *C. gariepinus* and other fish species, and adults have been identified in several species of fish-eating birds from South Africa. The sample size of fish collected in this survey was too low for a full health assessment index (HAI) study to be undertaken. *Polyonchobothrium clarias* and *Contracaecum*, however, were highly prevalent in the host species, and *Contracaecum* and *Ornithodiplostomum* occurred at high intensity (up to 44 and 140 respectively). Endoparasites of *C. gariepinus* can therefore be used in the fish HAI as a bioindicator of water quality. Only two ectoparasitic species were found on *C. gariepinus*: *Argulus japonicus* (skin and fins) and *Lamproglena clariae* (gills). Most water quality variables from the dam were within the target limits recommended by the Department of Water Affairs and Forestry (DWAF), but the levels of inorganic nitrogen (nitrate and ammonia) and phosphorus (orthophosphate) exceeded the limit. If uncontrolled, these may lead to eutrophication of the dam.

3.3.6 Mlambo, 2004

Active biomonitoring (ABM) of the UNR Wetland System using antioxidant enzymes, non-enzymatic antioxidants and histopathology as biomarkers

A suite of biomarkers of oxidative stress and histopathology were investigated in the fish *Oreochromis mossambicus* and the mollusc *Melanoides tuberculata*. The organisms were bred under laboratory conditions. They were deployed during the high-flow and low-flow periods, in cages at three sites down the flow gradient of the UNR wetland system, to determine spatial and temporal variations in biomarker responses and general water chemistry in the system. The oxidative stress biomarkers analyzed were catalase (CAT), superoxide dismutase (SOD), peroxidase (GPx), as well as levels of reduced glutathione (GSH) and lipid peroxidation as malondialdehyde (MDA). There was evidence of presence of chemicals inducing oxidative stress in the organisms as indicated by the high levels of MDA, GSH and GPx. Induction of CAT and SOD was not substantial due to possible inhibitory factors. This study established that organism transplantation is a feasible strategy for biomonitoring. Overall, no distinct variations were observed in the spatial and temporal comparisons in all the biomarker responses. The findings of this investigation also provide a basis for further investigation into the application of these biomarkers in ecological risk assessment.

3.3.7 Mbizi, 2004

The use of genotoxic and stress proteins in the active biomonitoring of the UNR system, South Africa

In this study, DNA damage, HSP 70 expression, lactate dehydrogenase and alkaline phosphatase were evaluated for their usefulness and applicability in the active biomonitoring of the UNR system using *Melanoides tuberculata* and *Oreochromis mossambicus* as test organisms. Levels of DNA strand breakage were measured in the exposed test organisms and the references that were kept in the laboratory under unpolluted environment. Different levels of DNA damage were recorded in *M. tuberculata* that were exposed during high-flow and those from the low-flow exposure period. There was no difference in DNA between the control and high-flow exposure *M. tuberculata* but the low-flow exposure duration had significantly higher DNA damage for all the three sites when compared to the high-flow and the controls. There was no difference in the amount of DNA damage in both exposed and control *O. mossambicus*. Fish samples were only available for the high-flow four weeks exposure because of the test organisms dying during the low-flow exposure. Heat shock protein 70 expression was determined in *O. mossambious* only and significant differences in expression between sites were demonstrated with highest expression at site 1 decreasing down stream at sites 3 and 5. The activity of LDH in *M. tuberculata* was not different in both control and exposed fish at all the sites. There was no difference in the activity of LDH between the high-flow and low-flow exposure groups in *M. tuberculata*. In *O. mossambicus*, the activity of LDH was significantly inhibited at site 1 with the effects of the contaminants decreasing downstream at sites 3 and 5 where LDH activity did not differ from the controls. There was no alkaline phosphatase activity in both exposed and control *M. tuberculata* but in *O. mossambicus* alkaline phosphatase was significantly inhibited at the three sites although inhibition did not differ from site to site. This suggests that LDH and alkaline phosphate are not sensitive biomarkers in *M. tuberculata* in the UNR system but are sensitive in *O. mossambicus*. DNA damage was shown to be a sensitive biomarker using *M. tuberculata* but it was difficult to assess this in *O. mossambicus* since fish were only available during the 4 weeks high-flow exposure, which did not show any differences from the control. The lack of DNA damage in *O. mossambicus* may be a result of low concentration of contaminants during high-flow or this biomarker is not sensitive in fish.

3.3.8 Moolman, 2004

The use of selected freshwater gastropods as biomonitors to assess water quality

In the ABM study, *M. tuberculata* bioaccumulated the metals, zinc (Zn), iron (Fe) and manganese (Mn). The gastropod did reveal variations in biomarker responses of which CEA was the most sensitive response. The gastropod species used in the laboratory studies revealed interspecies variation in metal uptake and depuration as well as the resultant metal bioaccumulation and

biomarker responses. Although, the bioaccumulation and biomarker measurements often varied between species and between metals, similar trends were found between the test species. Bioavailable Zn and Cd concentrations were bioaccumulated and all the biomarker responses were affected. From the results obtained in both the field and laboratory exposures, it can be concluded that *M. tuberculata* can be regarded as a good representative of the selected freshwater gastropods. Thus, this gastropod can be used as a suitable biomonitor in order to assess water quality. To validate the use of *M. tuberculata* as an effective bioindicator, it should be used in several future biomonitoring studies.

3.3.9 Vos, 2005

The identification of freshwater crabs on the Urban Nature Reserve and the influence of water quality on these aquatic invertebrates

Most studies have so far focussed on the freshwater crab *Potamonautes warreni* and to some extend *Potamonautes perlatus*. *Potamonautes warreni* has been advocated as a species with potential use in bio-monitoring. *Potamonautes unispinus* can possibly fulfil the same role as bio-indicator of the freshwater system on the Urban Nature Reserve. In this study the identification of the freshwater crabs was necessary since no previous studies have been done. Two different freshwater crab species were identified on the UNR, one was *Potamonautes unispinus* and the second (*Species B*) still unidentified to date. Selected physico-chemical characteristics of the water on the UNR as well as the levels of metals accumulated by the freshwater crab, *Potamonautes unispinus* were measured. The results of this study shows that metal levels in *Potamonautes unispinus* are indicative of the environmental levels of metals to which these crabs are exposed. Higher metal levels as compared to the habitat were found in the organisms at most localities. These organisms can therefore be seen as useful bio-accumulative indicators.

CHAPTER 4

ANALYTICAL CHEMISTRY OF WATER AND SEDIMENT

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4.1 Organic chemical analyses

4.1.1 Introduction

A target chemical analysis is one of the methods to detect the presence of possible EDCs in various matrices. However, some of the major limitations are applicable methodology, limited availability of equipment and expertise to measure sufficiently low detection limits for specific chemicals and the general high cost to perform these assays in South Africa.

4.1.2 Materials and Methods

4.1.2.1 Sample sites and sampling

Surface water ± 45cm deep was collected in 1-litre glass bottles, pre-washed with chromatography grade ethanol. Sediment samples were collected on the same day at approximately 1m below the water surface into pre-washed clean wide mouth glass flasks. The opening of the sampling bottles was covered with foil and sealed before stored at 4°C in the laboratory, prior to sample preparation for chemical analyses.

Sampling was done at both the UNR and two possible sites that were suggested as possible 'control' sites.

The following sites were selected at the UNR

(GPS = Global Positioning System)(amsl = above mean sea level)

- Dam 1 (D1) (GPS: S25°54.469′; E28°18.449′; 1494amsl)
- Channel (GPS: S25°53.048′; E28°17.991′; 1481amsl)
- Wetland (Vlei): GPS: S25°52.876′; E28°17.730′; 1476amsl)
- Dam 2 (D2)(GPS: S25°53.231′; E28°17.085′; 1482amsl)

4.1.2.2 Sampling sites in "control" water sources

4.1.2.2a Lapalala

Water and sediment samples were collected from the Palala River, which is the main water source entering the area known as the Lapalala Wilderness Game Reserve (LWGR). This nature reserve is in the Waterberg area of Limpopo Province and is one of the last

remaining true wilderness areas in South Africa (Africa Explorer, 2003; van Dyk, 2003). The "control" site was chosen within the Palala River as it enters the reserve.

4.1.2.2b Suikerbosrand Nature Reserve

Suikerbosrand Nature Reserve (SBR) is a highveld reserve approximately 50 km south of Johannesburg and 25km west of the town of Heidelberg. The reserve covers an area of 13,337 hectares and the major habitat is grassland. There are two main types of grassland within the reserve, montane (above 1800m) and a non-montane savanna type. Broad-leaved woodland is present in the valleys, *Acacia* savanna and *Protea* veld (which gives the reserve its name) make up the rest of the reserve. The fast disappearing Bankenveld grassland also occurs here, making this one of the Highveld's most valuable reserves. Mammalian species include mountain reedbuck, common duiker and steenbok, grey duiker, baboon, oribi, eland, blesbok, springbok, red hartebeest, black wildebeest, kudu and zebra. The area has a relatively low runoff, high evaporation and periodic drying out of the catchment with long periods of no rainfall. The stream at Suikerbosrand is a seasonal stream and only flowing after storm events.

Water, sediment and fat samples from seven eland culled in the hunting programme at SBR were analyzed for target chemical residues. All the eland had macroscopic calcifications similar to those of the UNR (Barnhoorn et al., 2002.).

4.1.2.2 Determination of organochlorine pesticides (OCs), PCBs and alkylphenols (APs) in water

For target chemical analysis, the compounds selected were the OC (Table 4.1), PCB153 as representative of PCBs (Spano et al., 2005) and the alkylphenols, nonylphenol (*p*-NP) and octylphenol (OcP). Technical nonylphenol (t-NP) was used as chemical standard for the p-NP analysis.

Table 4.1: Organochlorine pesticides analysed.

Alpha-BHC, gamma-BHC (lindane), heptachlor, aldrin, dieldrin, beta-BHC, delta-BHC , heptachlor epoxide, endosulfan I, endosulfan II, endosulfan sulfate, alpha-chlordane, gamma-chlordane, o,*p*'- and *p,p*'-DDT, -DDD and -DDE, endrin, endrin aldehyde, endrin ketone, methoxychlor

In the analytical laboratory water samples were filtered through a glass fibre filter before analysis commenced. The samples were analysed at the Residue Laboratory of the Agriculture Research Commission (ARC) at Onderstepoort Veterinary Institute, using standard methods

RNON 055 and GCMS 003. OcP, *p*-NP and OCs were extracted from water samples using solid phase extraction. Clean up was performed on a C18 cartridge and the analytes eluted with hexane-diethyl ether. Quantification was accomplished via fortified calibration curve. The APs were detected using fluorescence detection at a detection limit of 0.5µg/L. OCs were detected using GC coupled to a quadrupole MS detector and a detection limit of 0.5µg/L.

4.1.2.3 Determination of OCs and APs in sediments

The samples were analysed using standard methods RNON 059 and GCMS 010. OCs was extracted from sediment samples with dichloromethane-acetone (1:1, v/v) using soxhlet extraction procedure. Sample clean up was performed on a C18 cartridge and the analytes eluted with hexane-petroleum ether (1:1, v/v). OcP and *p*-NP were extracted from sediment with Tween 80, sample clean up performed on a C18 cartridge and the analyte(s) eluted with acetone. Quantification was accomplished via fortified calibration curve. APs were detected using fluorescence detection and a detection limit of 0.05mg/kg. OCs is detected using GC coupled to a quadrupole MS detector at a detection limit of 0.05mg/kg.

4.1.2.4 Phthalates determination of water and sediments samples

These analyses were performed according to Fatoki and Awofolu, (2003b) at the University of Venda, Thohoyandou.

4.1.2.4a Instrument

Perkin Elmer Clarus 500 Gas Chromatograph, with FID detector and Capillary column (Col- Elite 5-30 m 0.25µm – 0.25mm) supplied by Perkin Elmer SA (Pty.) Ltd., Cresta, Johannesburg, South Africa.

4.1.2.4b Optimizing GC Condition

GC conditions like oven/inlet temperatures, carrier gas flow, and detector temperature were optimized as follows: oven (initial temperature, 180°C, ramp rate of 12 minutes (min.), final temperature, 280°C with 2 and 7 min. hold time respectively); injector temperature, 180°C; carrier gas set point of 2.00ml/min. These conditions gave an analysis time of 17.33 minutes.

4.1.2.4c Retention Times

The phthalate esters determined are dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP). Butyl benzoate (BUBE) was used as internal standard. The esters were obtained from SUPELCO and Fluka AG. The stock solution (1000mg/L) for each ester was prepared in 20ml volumetric flask and

diluted as appropriate. Using the optimized GC conditions the following retention times were obtained - DMP, 2.252 ± 0.018; DEP, 3.027 ± 0.025; DBP, 5.727 ± 0.032; and DEHP, 8.984 ± 0.046.

4.1.2.4d Response Factors

Using a mixture of the phthalates and internal standard (n-butyl benzoate) at 100mg/L concentration with ten replicate injections (1μL), the response factors was calculated.

4.1.2.4e Detection Limits

The method detection limit (MDL) was determined following the method described by Miller and Miller, (1984). The values obtained were as follows (ng/L): DMP, 20.5; DEP, 10.5; DBP, 3.8; DEHP, 1.1 respectively.

4.1.2.4f Recovery Studies

Pre-extracted sediments and water samples were respectively spiked in triplicates with 1ml, 10mg/L mixtures of the phthalates. The samples were extracted with dichloromethane (DCM): 3 × 20mL DCM for the water samples and 120mL DCM for the Soxhlet extraction of the spiked sediment samples. The extracts were left to dry at ambient temperature, reconstituted with 2ml DCM and cleaned in a short silica gel (Kieselgel 60, 230-400 mesh) column, conditioned with hexane and eluted with benzene/ethyl acetate mixture (95:5). The percentage recoveries are as shown in Table 4.2, respectively for spiked water and sediment samples.

4.1.2.4g Sample Preparation

Water: 500mL water sample was salted with 50g NaCl in a 2L separatory funnel. The sample was then extracted with 3 × 20mL DCM. The combined extracts were left to stand until dried at room temperature. The dried extract was reconstituted with 2mL DCM and cleaned in a packed silica gel column as described earlier.

Sediment: 10g air dried and sieved (0.45μm, pore size) sediment samples were extracted with DCM for 10 hours in a Soxhlet extractor. The extract was left to dry on standing at room temperature. A silica gel column was packed with hexane and with a 0.5 – 1.0mL top layer of anhydrous sodium sulphate. The phthalates residue was run through the column and then eluted successfully with hexane and 20mL benzene/ethyl acetate mixture (95:5) as with the water samples. The eluates were left to dry at room temperature.

4.1.2.4h GC Analysis

The dried cleaned extracts were reconstituted with 0.5mL butyl benzoate (internal standard) solution, transferred into the GC sample vials for the GC analysis. The response factors obtained from the peak areas were then used to calculate the concentrations of the DMP, DEP, DBP, and DEHP contained in the water and sediment samples following the method described by Fatoki and Awofolu, (2003b).

Table 4.2: Percentage recoveries of phthalates from spiked pre-extracted water and sediment samples.

	Average % water	Average % sediment
Dimethyl phthalate (DMP)	82.13 ± 1.32	89.08± 0.51
Diethyl phthalate (DEP)	89.72 ± 0.52	89.95 ± 0.34
Dibutyl phthalate (DBP)	86.47 ± 0.39	88.72 ± 0.55
Diethyl hexyl phthalate (DEHP)	90.70 ± 0.32	89.04 ± 0.48

4.1.3 Results

4.1.3.1 Water and sediment sampling

Every two months pH, temperature, Oxygen (% and mg/L), conductivity and Total dissolved salts (TDS) were recorded. The physico-chemical parameters measured are shown in table 4.3 were indicative of stable conditions. The pH averaged around the 7.5 mark, except the continuous higher values in D2.

The results of the sites and target analytes detected are summarized in Table 4.4-7.

The target analysis performed at LWGR detected residues and no fish samples were analysed. Table 4.8 summarizes the results of the analyses performed at the possible "control" site SBR. The water samples contained residues of *p*-NP and OcP, but nothing was detected in sediment. The fat samples contained lindane in one sample, but both *o,p'*-DDT and –DDE, as well as *p,p'*-DDD in most animals in high concentrations. All seven eland had similar findings on testicular histopathology as previously described in the UNR eland microcalcifications (Barnhoorn et al., 2002).

Table 4.3: The physico-chemical water quality values measured at each locality during nine surveys (TDS, Total dissolved salts; N/A = Not available).

Locality	Date	pH	Conductivity µS/m	TDS µg/L	Temperature °C	Oxygen %	Oxygen mg/L
Dam 1	Sep 2004	9	662	N/A	31.9	143.2	10.17
Channel	Sep 2004	N/A	N/A	N/A	N/A	N/A	N/A
Vlei	Sep 2004	N/A	N/A	N/A	N/A	N/A	N/A
Dam 2	Sep 2004	9	473	N/A	25.9	96.4	7.28
Dam 1	Nov 2004	7.4	695	348	21.5	70.1	5.97
Channel	Nov 2004	7.7	673	330	22.8	75.5	6.85
Vlei	Nov 2004	7.7	616	309	18.9	50.8	4.62
Dam 2	Nov 2004	9.6	574	288	26.3	138.3	11.09
Dam 1	Jan 2005	7.19	N/A	N/A	22.4	45.6	4.12
Channel	Jan 2005	N/A	N/A	N/A	N/A	N/A	N/A
Vlei	Jan 2005	N/A	N/A	N/A	N/A	N/A	N/A
Dam 2	Jan 2005	8.93	N/A	N/A	26.1	89.9	6.97
Dam 1	Mar 2005	7.46	558	279	18.8	N/A	N/A
Channel	Mar 2005	7.48	494	248	17.8	N/A	N/A
Vlei	Mar 2005	7.46	493	248	16	N/A	N/A
Dam 2	Mar 2005	N/A	N/A	N/A	N/A	N/A	N/A
Dam 1	May 2005	7.34	730	367	12	N/A	N/A
Channel	May 2005	7.48	720	353	12.5	N/A	N/A
Vlei	May 2005	6.9	654	321	7.6	N/A	N/A
Dam 2	May 2005	8.72	490	249	17.1	N/A	N/A
Dam 1	Jul 2005	7.49	798	395	17	92.3	8.74
Channel	Jul 2005	7.25	750	383	13	56.8	5.73
Vlei	Jul 2005	7.06	657	321	13	58.1	5.79
Dam 2	Jul 2005	8.4	548	277	16	123.2	11.62
No data available for 2005/09/24							
Dam 1	Nov 2005	7.54	773	387	20.8	59.6	5.33
Channel	Nov 2005	7.6	655	327	20.6	35.4	3.21
Vlei	Nov 2005	7.4	621	311	19.9	55.2	5.04
Dam 2	Nov 2005	10.19	559	278	23.9	134	11.46
Dam 1	Jan 2006	7.45	504	253	20.2	48.4	4.6
Channel	Jan 2006	7.52	499	244	24.7	51.3	4.12
Vlei	Jan 2006	7.44	528	265	21.1	54.5	4.83
Dam 2	Jan 2006	9.57	534	266	22.8	55.6	4.79

Table 4.4: Chemicals detected in water samples at the UNR (All the open spaces indicated not detected).

WATER	α-BHC	Lindane	Heptachlor epoxide	Endrin	Methoxychlor	*p,p'*-DDT	*p,p'*-DDD	*p,p'*-DDE	*p*-NP	OcP	DMP	DEP	DBP	DEHP
	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L	mg/L	mg/L
Sep 2004 D1														
Vlei						3.2		0.002						
Dam 2														
Nov 2004 D1						0.597		0.021				3.16	8.33	0.35
Channel						0.247						3.19	4,78	0.86
Vlei						0.625						3.19	4.05	0.51
Dam 2						2.272						3.23	5.7	0.33
Jan 2005 D1												3.33	5.12	0.58
Channel		14.905										3.21	3,84	0.42
Vlei		0.645				1.057						3.26	4.07	0.37
Dam 2		14.042										3.18	3.93	0.46
Mar 2005 D1												3.16	5.48	0.79
Channel		2.934										3.63	4.97	2.78
Vlei		4.918										3.59	4.2	0.62
Dam 2												3.77	5.29	1.33
May 2005 D1	15.351	0.582												
Channel		6.596							0.600					
Vlei						0.642								
Dam 2		1.178												
Jul 2005 D1									50.050					
Channel									6.480					
Vlei									0.930					
Dam 2									0.920					
Oct 2005 D1									3.730					
Channel														
Vlei														
Dam 2														
Dec 2005 D1			0.680				1.100		9.290	6.560				
Vlei				0.650	1.140		1.100		3.660	2.260				
Dam 2				0.570			1.100							
Jan 2006 D1							0.900		3.040	2.560				
Channel						0.860			1.040					
Vlei														
Dam 2						0.880								

Table 4.5: Chemicals detected in sediment samples at the UNR (All the open spaces indicated not detected.)

SEDIMENT	*p,p'*-DDT	*p,p'*-DDD	*p*-NP	OcP	DMP	DEP	DBP
	mg/kg	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)		
Sep 2004 D1							
Channel		6.429					
Vlei							
Dam 2							
Nov 2004 D1		9.751				0.16	0.23
Channel		1.1812				0.16	0.26
Vlei						0.16	0.21
Dam 2						0.16	0.23
Jan 2005 D1						0.18	0.26
Channel			0.117			0.32	6.5
Vlei						0.27	0.34
Dam 2							
Mar 2005 D1			1.132			0.18	0.3
Channel						0.17	0.4
Vlei						0.16	0.71
Dam 2							
May 2005 D1			0.635				
Channel			0.229				
Vlei			0.080				
Dam 2							
Jul 2005 D1			1.089				
Channel							
Vlei							
Dam 2							
Oct 2005 D1							
Channel							
Vlei			0.229				
Dam 2			0.182				
Dec 2005 D1		0.067	0.171				
Channel	0.065	0.086	0.251				
Vlei			0.051				
Dam 2							
Jan 2006 D1			0.251				
Channel			0.051				
Vlei		0.080					
Dam 2		0.081	0.096				

Table 4.6: Chemicals detected in water summarized per site at the UNR (All the open spaces indicated not detected).

WATER	α-BHC	Lindane	Endrin	Endrin Aldehyde	Methoxychlor	*p,p'*-DDT	*p,p'*-DDD	*p,p'*-DDE	*p*-NP	OcP	DMP	DEP	DBP	DEHP
	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L	mg/L	mg/L
D1 Sep 2004														
D1 Nov 2004						0.60		0.02				3.16	8.33	0.35
D1 Jan 2005												3.33	5.12	0.58
D1 Mar 2005												3.16	5.48	0.79
D1 May 2005	15.35	0.58												
D1 Jul 2005									50.05					
D1 Oct 2005									3.73					
D1 Dec 2005							1.10		9.29	6.56				
D1 Jan 2006							0.90		3.04	2.56				
Channel Sep 2004						0.25						3.19	4.78	0.86
Channel Nov 2004		14.91										3.21	3.84	0.42
Channel Jan 2005		14.91										3.21	3.84	0.42
Channel Mar 2005		2.93										3.63	4.97	2.78
Channel May 2005		6.60							0.60					
Channel Jul 2005									0.93					
Channel Oct 2005														
Channel Dec 2005				0.65	1.14	1.10			3.66	2.66				
Channel Jan 2006						0.86			1.04					
Vlei Nov 2004		4.92										3.59	4.20	0.62
Vlei Jan 2005		0.65				1.06						3.26	4.07	0.37
Vlei Mar 2005		4.92										3.59	4.20	0.62
Vlei May 2005						0.64								
Vlei Jul 2005									0.93					
Vlei Oct 2005														
Vlei Dec 2005			0.65		1.14		1.10		3.66	2.26				
Vlei Jan 2006														
D2 Sep 2004	N/S													
D2 Nov 2004						2.27						3.23	5.70	0.33
D2 Jan 2005		14.04										3.18	3.93	0.46
D2 Mar 2005												3.77	5.29	1.33
D2 May 2005		1.18												
D2 Jul 2005									0.92					
D2 Oct 2005														
D2 Dec 2005			0.57				1.10							
D2 Jan 2006						0.88								

Table 4.7: Chemicals detected in sediment detected per site at the UNR (All the open spaces indicated not detected).

SEDIMENT	*p,p'*-DDT	*p,p'*-DDD	*p*-NP	OcP	DMP	DEP	DBP	DEHP
	mg/kg	mg/kg	mg/kg	mg/kg	µg/g	µg/g	µg/g	µg/g
D1 Sep 2004								
D1 Nov 2004		9.751				0.16	0.23	0.03
D1 Jan 2005						0.2	0.3	0.1
D1 Mar 2005			1.1			0.2	0.3	0.6
D1 May 2005			0.6					
D1 Jul 2005			1.1					
D1 Oct 2005								
D1 Dec 2005		0.1	0.2					
D1 Jan 2006			0.3					
Channel Sep 2004		6.4						
Channel Nov 2004		1.1812				0.16	0.26	0.03
Channel Jan 2005			0.1			0.3	6.5	0.6
Channel Mar 2005						0.2	0.4	0.0
Channel May 2005			0.2					
Channel Jul 2005								
Channel Oct 2005								
Channel Dec 2005	0.1	0.1	0.3					
Channel Jan 2006			0.1					
Vlei Sep 2004								
Vlei Nov 2004						0.2	0.2	0.0
Vlei Jan 2005						0.3	0.3	0.2
Vlei Mar 2005						0.2	0.7	
Vlei May 2005			0.1					
Vlei Jul 2005								
Vlei Oct 2005			0.2					
Vlei Dec 2005			0.1					
Vlei Jan 2006		0.1						
D2 Sep 2004								
D2 Nov 2004						0.2	0.2	0.0
D2 Jan 2005								
D2 Mar 2005								
D2 May 2005								
D2 Jul 2005								
D2 Oct 2005			0.2					
D2 Dec 2005								
D2 Jan 2006		0.1	0.1					

Table 4.8: Chemical residues in water, sediment and eland fat samples from SBR.

	Lindane	*o,p'*-DDE	*p,p'*-DDE	*p,p'*-DDD	*o,p'*-DDT	*p,p'*-DDT	PCB153	*p*-NP	OcP
	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg
Water								2.208	1.916
Sediment									
Eland fat (n=7)	<10.000	27.66±9.93	<10.000*	79.48±30.39	114.87±31.05	127.32±49.89	91.87±115.65	137.84±77.88	50.21±30.91

(Average ± SD); * present in all samples, but below detection limit

4.1.4 Discussion

The detection of chemical residues in samples of LWGR and SBR disqualified both as possible "control" sites. Of specific concern was the presence of several EDCs including lindane, DDT and metabolites, PCB153 and the alkylphenols. Lindane was only present in one animal and the concentration was low, but it raises the question where the agrochemical was coming from since the sampling site was high up in the mountains. The presence of DDT and PCB153 probably reflects the impact of air pollution and precipitation of these well without the areas where they have been produced or used. Similarly, the industrial pollutants OcP and *p*-NP were not supposed to be detectable at SBR, but the possible route/source is not clear. However, the corresponding high levels in the eland confirmed the reliability of the water results screened for those. It also emphasized the importance of wildlife as biomarkers to detect chemical pollution and bio-accumulation in the food chain.

The *p*-NP levels in eland from SBR (137.8 $\pm$ 77.9 µg/kg fat) were slightly lower than previously found at the UNR (178.2 ± 60.8 µg/kg) (Bornman et al., submitted). The SBR eland also contained residues of DDT and metabolites, as well as PCB153. Several samples from both groups of eland contained analytes at concentrations below the detection limits of the assays (10µg/kg). The presence of adenomatous lesions of the rete testis in the eland was similar to diethylstilbestrol-induced rete adenocarcinoma in laboratory animals (Newbold et al., 1985; Newbold et al., 2000) and was, therefore, indicative of possible chronic estrogenic exposure.

In the 35 water samples analysed from the UNR residues of α-BHC, lindane, endrin, heptachlor epoxide, methoxychlor, *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE, *p*-NP, OcP, DMP, DEP, DBP and DEHP were detected. These samples were collected every two months over a two year period at the specific sites. Lindane was the most prevalent chemical in 11 (31.4%) samples followed by *p,p'*-DDT in 9 (25,7%) samples. The phthalate esters, however, were determined during a high- and low-flow period and except for DMP, all the samples (100%) contained phthalate residues. Therefore, in the water samples a mixture of agrochemical pesticides and industrial pollutants were present. This supported the initial view that D2 is as an area where contamination from various sources occurs (Burger, 2005).

The values of lindane varied between 0.58 and 14.92ug/L and several samples exceeded the toxic range. The temporary oral adult daily intake (ADI) recommended by the WHO is 1ppb (ug/L) bw/day (FAO, 1998) and the oral reference dose (RFD) is 0.3ug/L bw/day (US EPA, 1998). The recommendations for drinking water are 0.2ppb (USA EPA, 1998), 0.1mg/L Netherlands (ATSDR, 2002). The MRL recommended by the WHO is 0.003ppm. The possible impact of these high values in water, are addressed in Chapter 11: Health risk assessment.

In sediment samples, none of the abovementioned agrochemicals were present, but 4,4'-DDD occured in 7/35 (20%) samples. *p*-NP was the most prevalent chemical in 14 (40%) samples and although DMP was present in one sample, the other phthalate ester residues found in sediment were similar to water. The "normal" phthalate concentration in unpolluted water is in the µg to undetectable range and the values found in this study indicated pollution of the water systems with phthalate ester plasticizers. Freshwater studies have revealed detectable concentrations of phthalates in Italian (Guidotti and Cremisini, 1997) and Malaysian (Tan, 1995) water and sediment samples, and in river water samples taken in the UK (Fatki and Vernon, 1990), Nigeria (Fatoki and Ogunfowokan, 1993) and USA (Sheldon and Hites, 1978). DEHP levels of up to 628mg/kg dry weight were once reported in River Ronnebyan, Sweden (Thuren, 1986). Higher concentrations are frequently reported in samples from less developed countries, a fact that has been attributed to lack of legislation and/or facilities to treat industrial effluents (Guidotti and Cremisini, 1997; Fatoki and Ogunfowokan, 1993). Values found here are comparable to reported for those rivers, but less than those reported for the Eastern Cape water systems (Fatoki and Noma, 2002) and the levels found in some sediments were comparable to those reported for the River Ronnebyan (Sweden) (Thuren, 1986).

Lindane, the gamma-HCH (γ-HCH) isomer of hexachlorocyclohexane (HCH) or benzene hexachloride (BHC) is used as insecticide on fruit, vegetables, forest crops, animals and – premises. The use of HCH is restricted in South Africa (ATSDR, 2003). Lindane is also an EDC and has endocrine effects specifically on the reproductive system. Estrogenic activity in rodents was found with disruption of the estrous cycles, reduced testicular, pituitary and uterine weights (Raizada et al., 1980) and vitellogenin induction in male fish (Okoumassoun et al., 2002). However, it may also be an anti-estrogen (Chadwick et al., 1988). No agonistic or antagonistic activity was found on the androgen receptor (Schrader and Cooke, 2000).

The water samples collected from the all the sites had more or less a similar frequency of positivity for compounds detected (D1 21, Channel 23, Vlei 22, D2 16). The sediment in D1 and the Channel seemed to be particularly contaminated (16 positive each), followed by the Vlei (12) and D2 (6). Dam 1 receives water through a stream higher up in the catchment and serves as a sludge dam, supplementing the water supply of the bigger Dam 2. Since the Channel is man-made and ensures the constant flow of water parallel with the wetland to D2, it is not surprising that the chemical contamination is rather similar. Lindane at high levels was present in the Channel and D2 and to a lesser extent the Vlei, but not D1 between Nov 2004 and May 2005. This may imply that contamination occurred directly into the Channel and D2 from an unknown source and via an unknown route, and not from the water in D1.

4.2 Metal analysis

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4.2.1 Introduction

Cadmium (Cd), Arsenic (As), Lead (Pb) and Mercury (Hg) have been identified as the four endocrine disrupting metals (EDM) globally and have been analysed.

4.2.2 Material and methods

Surface water and sediment samples were collected in 250ml plastic jars for metal content evaluation with an ICP-MS (Inductively Coupled Plasma – Mass Spectrometry). The jars were left in a 2% hydrochloric acid solution for 12 hours and then rinsed with distilled water prior to sample collection. Samples were kept at 4°C in the laboratory, before sample preparation for metal analysis. Samples were collected on the same dates and at the same localities as samples for other contaminant analyses. Sediment samples were prepared according to methods described by Bervoets and Blust (2003), whilst water samples were filtered and acidified. Waterlab, Pretoria, conducted the analyses.

Water and sediment samples were analysed in duplicate for 70 different elements, including the four known endocrine disrupting metals (EDMs) Cd, As, Pb, Hg (Table 4.9). Concentration levels were read in parts per billion (ppb or µg/L) for water and sediment and were recalculated to mg/kg for sediment. Two different extractions were done for the sediment (Bervoets and Blust, 2003). The first was an easy reducible extraction, where elements that are bio-available to aquatic organisms and can affect them, are extracted. The second was a total extraction, were all the elements that are bound to the sediment are extracted, because not all elements are bio-available due to speciation.

4.2.3 Results

The average values for all the elements were calculated for both water and sediment, in each of the four localities and graphically shown in Figures 4.1-4. These were used for comparative purposes of the EDMs between the four chosen localities. Other elements above the target quality ranges were also identified.

Table 4.9: Information about the four EDMs as well as their quality range and effect values (DWAF, 1996).

Metal	Introduction	Occurrence	TWQR (µg/L)	CEV (µg/L)	AEV (µg/L)
Cadmium	-highly toxic to aquatic life -potentially hazardous (USEPA) -toxic and relatively accessible	-natural weathering processes -industrial industry -associated with Zn, Pb and Cu sulphide	From ≤ 0.15 to ≤ 0.40 *	0.3-0.8*	3-13*
Arsenic	-very toxic and carcinogenic -relatively available (USEPA)	-limited extent in nature -high concentrations due to industrial pollution	≤ 10	20	130
Lead	-potentially hazardous (USEPA) -toxic and relatively accessible	-weathering of sulphide ores -associated with suspended sediments	From ≤ 0.2 to ≤ 1.2*	0.5-2.4*	4-16*
Mercury	-concentration in environment usually low -very toxic -bio-accumulates	-high concentrations due to industrial pollution -strong affinity for sediments and solids	≤ 0.04	0.08	1.7

TWQR = Target Water Quality Range;* depends on water hardness (mg $CaCO_3/L$); CEV = chronic effect value; µg/L = ppbAEV = acute effect value

Cd levels were detected in water samples at all four the sites. Both D1 and Vlei samples had levels, on average, above the target water quality range (TWQR) of 0.216µg/L and 0.154µg/L respectively. Channel and D2 sample levels were just below the TWQR. Levels above 0.3µg/L (above chronic effect value (CEV)) were also detected at all four sites at some point. As levels were all below 10µg/L at all four the sites, during the entire sampling period. The highest level for As was detected in a December 2005 sample taken in D2 , at 5µg/L. Dam 2 had the highest concentrations of As of the four sites, on average. All the levels for Pb in all the samples collected were above 15µg/L, and higher than the acute effect value (AEV) for Pb (DWAF, 1996). Both D1 and the Vlei had higher Pb levels than the other two sites, on average, at 20.446µg/L and 20.144 µg/L respectively. It is, therefore, possible that the plants in the wetland area between D1 and D2 could have removed some amounts of both Cd and Pb from the water. Mercury was only detected in a D1 sample collected during the first field collection (Sep 2004), and was above the AEV (0.323µg/L average between the duplicates).

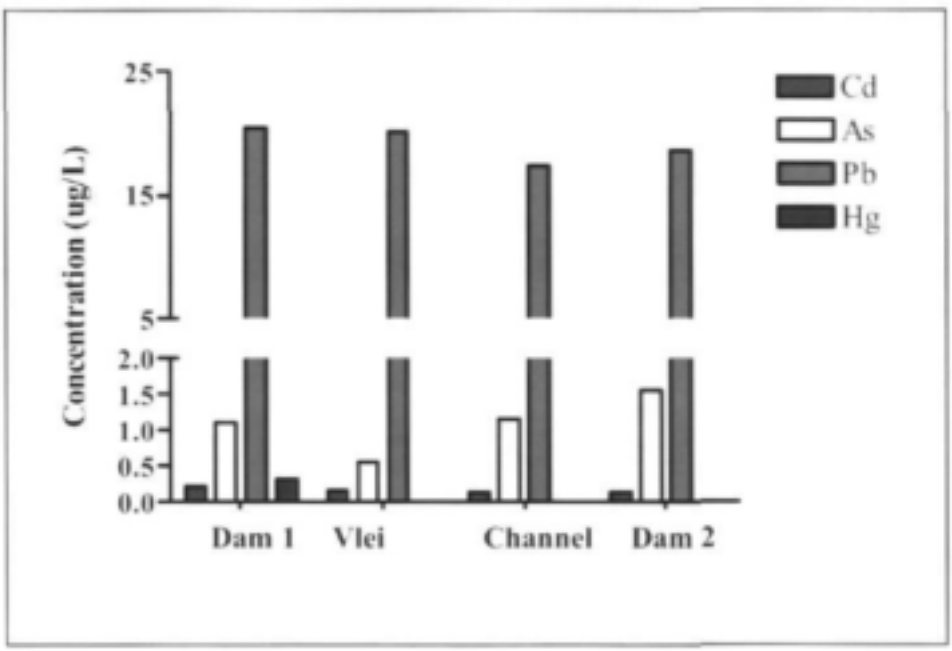

Fig. 4.1: Average Cd, As, Pb and Hg levels in water samples collected at the four different sites.

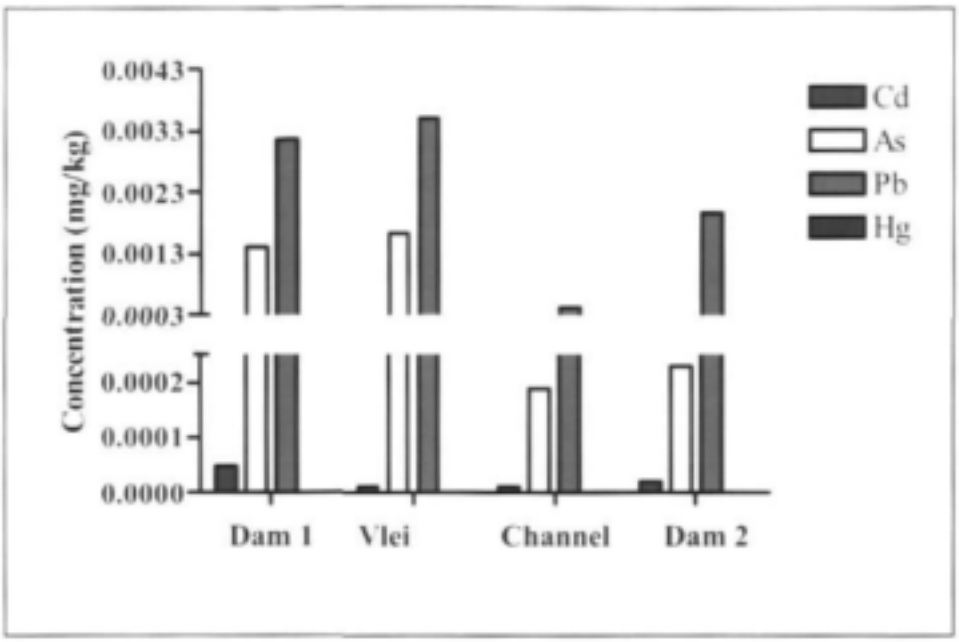

Fig. 4.2: Average easily reducible Cd, As, Pb and Hg levels in sediment samples.

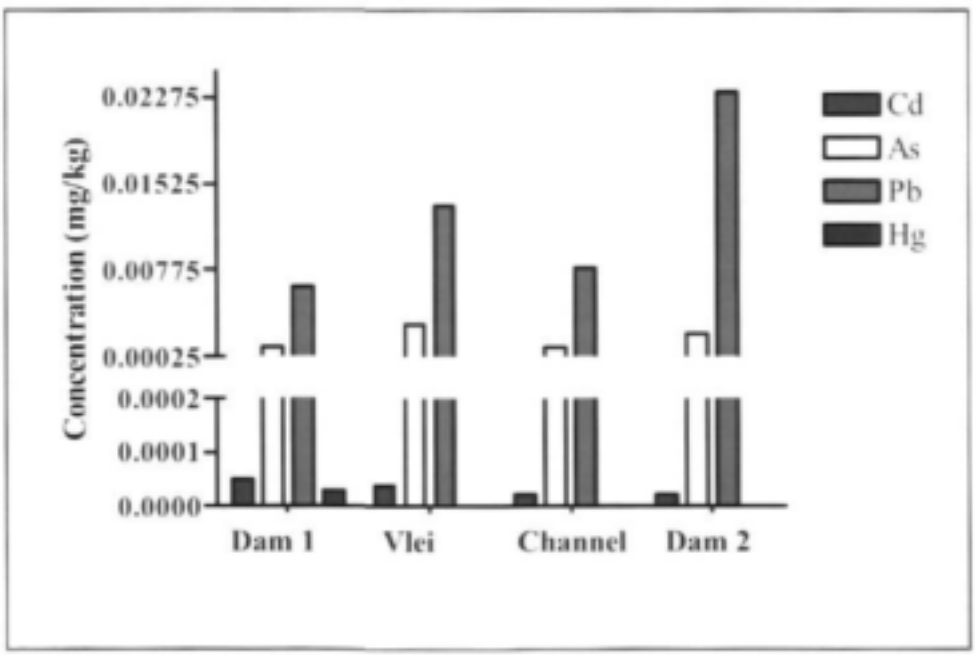

Fig. 4.3: Average total Cd, As, Pb and Hg in sediment samples collected at the different sites.

4.2.4 Discussion and conclusion

When comparing the easy reducible elements to the total available, it is clear that the sediment tends to hold on to large concentrations of elements. Only a smaller amount is bio-available to the organisms, and the availability depends on the pH. The water pH also affects the amount of an element that will either be attached to the sediment or in the aqueous mix.

Low levels of Cd were detected in sediment in both the easy reducible and the total extractions. Cd concentrations in the water were high and it is possible that the sediment released Cd into the water, due to the pH (DWAF, 1996). The Cd levels detected were on average very similar, but D1 was slightly higher than the other three sites. As concentrations were 0.0010 (D1), 0.0031 (Vlei), 0.0011 (Channel) and 0.0022mg/kg (D2) for the total extractions, but the channel and D2 easy reducible extractions indicated that less As was bio-available than in the other two sites. The levels of As were higher in sediment than in water. Pb levels were the highest of the EDMs in both the water and the sediment. The highest total Pb on average was detected in D2 at 0.02328mg/kg, whilst the lowest was in D1 at 0.00630mg/kg. The opposite was noted for the bio-availability of Pb with 0.00198mg/kg available in D2 and a higher level detected in D1 (0.00317mg/kg). The lowest levels of Pb were detected in the Channel, for both the total and easy reducible extractions. Hg levels were only detected in the total extractions done on samples of D1 during September and November 2004, with an average of 0.00003mg/kg between the duplicate extracts. There was no Hg detected in the easy reducible extractions, which could possibly indicate that the small amount of Hg present was not bio-available. Hg levels in the water samples were also almost non-existent, possibly indicating that there was either no Hg present in the system during the majority of the sampling period.

4.2.4.1 Other problematic metals/elements

Of the 70 elements scanned for, some were present at very high concentrations in the water system (majority above TWQR and even the AEV). Table 4.10 summarizes the total water quality ranges, chronic effect values and acute effect values for seven metals that could be problematic based on aquatic system guidelines (DWAF, 1996). As seen in the table, concentrations on average as detected in water samples of the four sites, are all above the TWQR and the majority are above the AEV. To what extent these metals might affect the health of organisms alone or in combination with the EDMs, is not clear.

Table 4.10: Other metal concentrations detected in water samples collected at the four sites, compared to aquatic system guidelines (DWAF, 1996)

Metal	Aquatic systems TWQR (µg/L)	CEV (µg/L)	AEV (µg/L)	Detected levels (µg/L)
Aluminium	5 to 10	10 to 20	100 to 150	All averages for all sites above CEV. Dam 2 under AEV.
Chromium	Cr(6) = 0.7 Cr(3) = 0.12	Cr(6) = 14 Cr(3) = 24	Cr(6) = 200 Cr(3) = 340	Average for D1 above CEV. Other sites above TWQR.
Manganese	0.180	370	1300	All averages for all sites above TWQR, but under CEV.
Iron	Shouldn't vary >10% of background dissolved Fe	N/A	N/A	D1 = 478.o13; Vlei = 163.034; Channel = 37.759; D2 = 0
Copper	From 0.3 to 1.4 *	From 0.53 to 2.8	From 1.6 to 12	Average for D1 above AEV. Other 3 sites falls in the CEV.
Selenium	0.2	5	30	Average for D1 and D2 above CEV. Other two sites below CEV, but above TWQR.
Zinc	2	3.6	36	Averages for all sites above AEV at over 300µg/L.

* depends on hardness of water

CHAPTER 5

IN VITRO BIOASSAYS FOR ESTROGENIC ACTIVITY

5.1 Recombinant cell bioassay (*saccharomyces cerevisiae* (yes) assay)

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5.1.1 Introduction

Yeast does not contain endogenous steroid receptors. However, mammalian steroid receptors introduced into yeast function as they do in mammalian cells as steroid-dependent transcriptional activators (Metzger et al., 1988; Purvis et al., 1991; Gaido et al., 1998). As a result yeast are a useful tool for studying mammalian steroid receptor function in isolation of confounding factors found in mammalian cells. When a steroid responsive reporter gene is introduced into the yeast along with the steroid receptor, chemical interaction with that receptor can be determined by measuring the reporter gene product. A yeast based steroid receptor assay differs from a competitive binding assay in that it not only determines the ability of a chemical to bind to a receptor, but also to cause that receptor to dimerize and bind to the appropriate steroid responsive regions of the DNA to induce reporter gene activity. Other advantages of using yeast to study steroid receptor function include the ease of manipulation, rapid attainment of stable transformants, ability to process a large number of samples quickly and relatively inexpensively (Gaido et al., 1998).

The recombinant cell bioassay utilises the yeast *Saccharomyces cerevisiae* transformed with plasmids encoding the human estrogen receptor and an estrogen responsive promoter fused to the structural gene for β-galactosidase (Klein et al., 1994) (Figure 5.1). This assay detects 100 fold lower estradiol levels than the most sensitive radioimmunoassay available. The yeast assay is sensitive enough to consistently detect an increase in β-galactosidase production with 17 β-estradiol at concentrations above 1×10^{-11} M (Beresford et al., 2000). This compares with the detection limit of 3×10^{-11} M 17 β-estradiol reported for the MCF-7 cell proliferation assay (Sonnenschein et al., 1995). A limitation to the yeast-based assay is that the permeability of the cells to some substances may generate false negative results and some strain specific effects have been reported (Gray et al., 1997). The yeast estrogenicity assay of Routledge and Sumpter (1996) has certain advantages over other yeast-based assays, in that the colour can be monitored over a period of time.

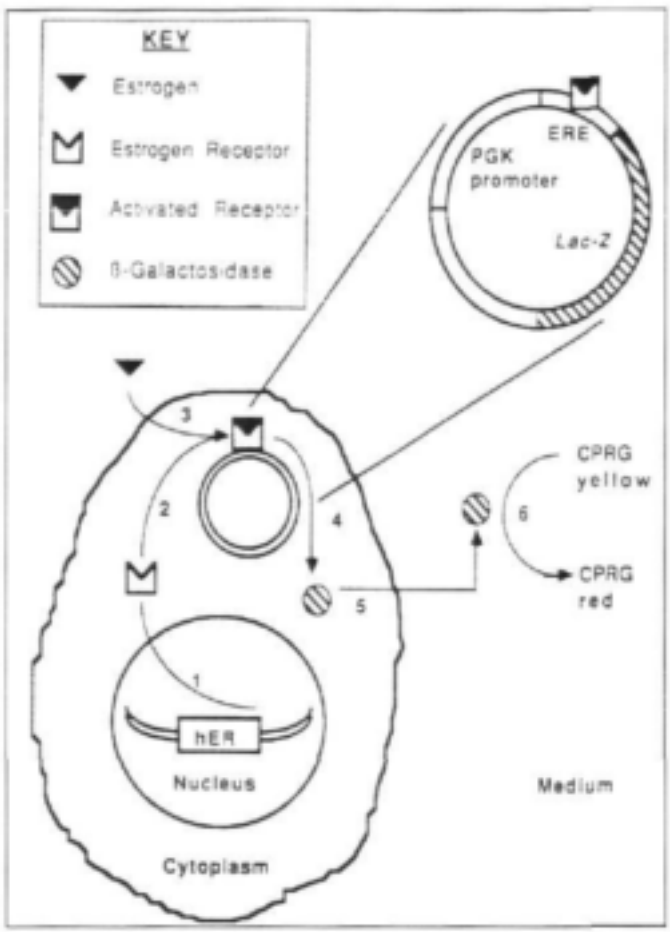

Fig. 5.1: A schematic representation of the estrogen-inducible expression system in the yeast. The human estrogen receptor gene is integrated into the main genome and is expressed (1) in a form capable of binding to estrogen response elements (ERE) within a hybrid promoter on the expression plasmid (2). Activation of the receptor (3), by binding of ligand, causes expression of the reporter gene Lac-Z (4) which produces the enzyme β-galactosidase. This enzyme is secreted into the medium (5) and metabolises the chromogenic substrate CPRG (normally yellow) into a red product (6), which can be measured by absorbance (Routledge and Sumpter, 1996).

Among various bioassays developed to elucidate estrogenic activities of individual chemicals and environmental water samples (Flouriot et al., 1993; Feldman and Krishnan, 1995; Jobling et al., 1995; Pawlowski et al., 2000a,b), the yeast estrogen screen (YES) received particular attention as a rapid and comparatively simple screening assay in laboratory and field experiments (Soto et al., 1995; Routledge and Sumpter, 1996; Harries et al., 1997; Johansson, 1999; Garcia-Reyero et al., 2001; Murk et al., 2002; Pawlowski et al., 2004).

5.1.2 Materials and Methods

A recombinant yeast strain was developed in the Genetics Department at Glaxo Laboratories in the UK for use in a test to identify compounds that can interact with the human estrogen receptor-α (hER). The yeast was obtained from Prof JP Sumpter's laboratory, in the Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex, UK. The assay was performed according to the method described by Routledge and Sumpter (1996).

5.1.2.1 Preparation of assay components

5.1.2.1a Minimal Medium (pH 7.1)

Minimal medium was prepared by adding

13.6g	KH_2PO_4	(Cat. No. P-0662, Sigma)
1.98g	$(NH_4)_2SO_4$	(Cat. No. R 0350/500g, NT Laboratory Supplies)
4.2g	KOH pellets	(Cat. No. 504 44 00, Saarchem)
0.2g	$MgSO_4$	(Cat. No. 291184P, BDH)
1mL	$Fe_2(SO_4)_3$ solution (40mg/50mL water)	(Cat. No. F-1135, Sigma)
50mg	L-leucine	(Cat. No. 371213W, BDH)
50mg	L-histidine	(Cat. No. 372214E, BDH)
50mg	Adenine	(Cat. No. 1.00838, Merck)
20mg	L-arginine-HCL	(Cat.No. 1.01543, Merck)
20mg	L-methionine	(Cat. No. 371315E, BDH
30mg	L-tyrosine	(Cat. No. 371562R, BDH)
30mg	L-isoleucine	(Cat. No. 371236G, BDH)
30mg	L-lysine-HCL	(Cat. No. 371293P, BDH)
25mg	L-phenylalanine	(Cat. No. 1.07256, Merck)
100mg	L-glutamic acid	(Cat. No. 371024T, BDH)
150mg	L-valine	(Cat. No. 37160, BDH)
375mg	L-serine	(Cat. No. 371465R, BDH)
1L	Double distilled water	

Aliquots of 45mL were dispensed into 150ml glass flasks, sterilised at 121°C for 10 minutes and stored at room temperature.

5.1.2.1b Vitamin solution

8 mg	Thiamine	(Cat. No. 440055N, BDH)
8mg	Pyroxidine	(Cat. No. 449865Q, BDH)

8mg	Pantothenic acid	(Cat. No. 111 993, Merck)
40mg	Inositol	(Cat. No. 380443M, BDH)
20mL	Biotin solution (2mg/100mL water)	(Cat. No. 44011 4H, BDH)
180mL	Double distilled water	

The solution was then filtered through 0.2µm pore size Whatman PURADISC filters (Cat. No. 6780-2502), and 10mL aliquots were stored at 4°C in sterile glass bottles.

5.1.2.1c Glucose solution

A 20% weight/volume solution of D-glucose (Cat. No. ART.8337, Merck) was sterilised in 20mL aliquots at 121°C for 10 minutes and stored at 4°C.

5.1.2.1d L-aspartic acid solution

A stock solution of 4mg/mL L-aspartic acid (Cat. No. 370225W, BDH) was sterilised in 20ml aliquots at 121°C for 10 minutes and stored at 4°C.

5.1.2.1e L-threonine solution

A stock solution of 24mg/mL L-threonine (Cat. No. 371505Y, BDH) was sterilised in 5mL aliquots 121°C for 10 minutes and stored at 4°C.

5.1.2.1f Copper (II) sulphate solution

A 20mM Copper (II) sulphate (Cat. No. 278504G) solution was prepared (31.92mg/100mL) and filter sterilised through 0.2µm pore size Whatman, PURADISC filters (Cat. No. 6780-2502) into sterile glass bottles and stored at 4°C.

5.1.2.1g Chlorophenol red-β-D-galactopyranoside (CPRG)

A 10mg/L stock solution of CPRG (Cat. No. 884 308, Boehringer Mannheim, Roche Diagnostics) and filter sterilised through 0.2µm pore size Whatman, PURADISC filters (Cat. No. 6780-2502) into sterile glass bottles and stored at 4°C.

5.1.2.1h Growth medium

Growth medium was prepared by adding the following:

45mL	Minimal medium
5mL	Glucose solution
1.25mL	L-Aspartic acid solution

0.5mL	Vitamin solution
0.4mL	L-Threonine solution
125µL	Copper (II) sulphate solution

5.1.2.1i 17 β-estradiol stock solution

A 54.58µg/l stock solution of 17 β-estradiol (Cat. No. E8875, Sigma) was prepared in ethanol (Cat. No. 27,0741, Sigma-Aldrich) in a sterile glass bottle and stored at -20°C.

5.1.2.2 Assay Procedure

5.1.2.2a Assay medium

Growth medium as described above was prepared for the assay. The growth medium was then inoculated with 125µL of the 10x concentrated yeast stock and incubated at 28°C in a rotating water bath at 150-155upm until turbid. A new flask of growth medium was then prepared and 0.5mL CPRG was added to the medium, this medium was then inoculated with 0.5mL of the 24-hour yeast culture.

5.1.2.2b Sample procedure

The assay was carried out in a type II laminar flow air cabinet, to minimise aerosol formation. Serial dilutions were made of the 26 water sample extracts and controls, in 96 well microtiter plates (Cat. No. 95029780, Labsystems). 100µL of the solvent, ethanol (Cat. No. 27,0741, Sigma-Aldrich) was placed in wells 2-12 on the plate. 200µL of the sample extract was placed into the first well and this was serially diluted (100µL) across the plate, using a Finpipette multichannel pipette (AEC Amersham). 10µL Aliquots were then transferred to a 96 well, optically flat bottom microplate (Cat. No. 95029780, Labsystems). This was allowed to evaporate to dryness on the assay plate.

Aliquots (200µL) of the assay medium containing the yeast and chromogenic substrate (CPRG) were then dispensed into each sample well using a multichannel Finnpipette. Each plate contained at least one row of blanks (assay medium and solvent ethanol) and a standard curve for 17β-estradiol (Cat. No. E8875, Sigma) ranging from $1x10^{-8}$M to $4.8x10^{-12}$M (2.274µg/L to 1.3ng/l) which was extended to a concentration of $1.19x10^{-15}$M ($3.24x10^{-13}$g/L). The plates were sealed with parafilm (Cat. No. P7793, Sigma) and placed in a naturally ventilated incubator (Heraeus, B290) at 32°C for 3 to 6 days.

After 3 days incubation the colour development of the medium was checked for 3 days (day 3 to 5) at an absorbance (abs) of 540nm for colour change and 620nm for turbidity of

the yeast culture. The absorbance was measured on a Titertek Multiskan MCC/340 plate reader to obtain data with the best contrast. After incubation the control wells appeared light orange in colour, due to background expression of β-galactosidase and turbid due to the growth of the yeast. Positive wells were indicated by a deep red colour accompanied by yeast growth. Clear wells, containing no growth indicated lysis of the cells and colour varied. All experiments were performed in quadruplicate. The following equation was applied to correct for turbidity:

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

The 17 β-estradiol standard curve was fitted (sigmoïdal function, variable slope) using Graphpad Prism (version 2.01), which calculated the minimum, maximum, slope, EC50 value and 95% confidence limits. The detection limit of the yeast assay was calculated as absorbance elicited by the solvent control (blank) plus three times the standard deviation. The estradiol equivalents (EE) of the water samples were interpolated from the estradiol standard curve and corrected with the appropriate dilution factor for each sample on day 4. Any curve that failed to reach the maximum response obtained with 17 β-estradiol will be referred to as a submaximal response.

5.1.3 Results

5.1.3.1 Water

In order to facilitate interpretation of the results for each sample site has been summarised into table format indicating the type of response and the EE-EC_{50} and maximum estradiol equivalents (EE-Max) (ng/L) as extrapolated from the 17 β-estradiol curve for that experiment. The results are tabulated in Table 5.1-4 and graphically shown in Figures 5.2a-5.5b.

5.1.3.1a Sample site: Dam 1 (D1)

Table 5.1: Estrogenic activity in D1 expressed as the type of response and estradiol equivalents (ng/L).

Date sampled	Type of response			YES	EE–EC_{50}	EE-Max
	Toxic	Submaximal	Maximal	Result*	(ng/L)	(ng/L)
January 2005	X			2		
March 2005	X			1		
May 2005	X			2		
July 2005	X			1		
October 2005	X			1		
December 2005		X		3	0.68	1.24

*0 = Below detection limit; 1 = One point above detection limit; 2 = Two points above detection limit; 3 = Three or more points above detection limit (positive for estrogenic activity)

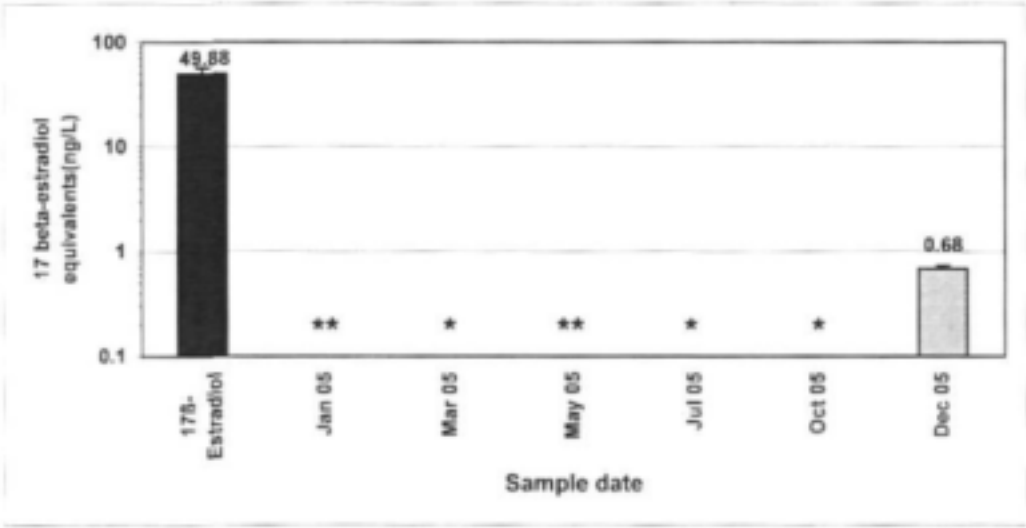

Fig. 5.2a: D1 EC50 values expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit.

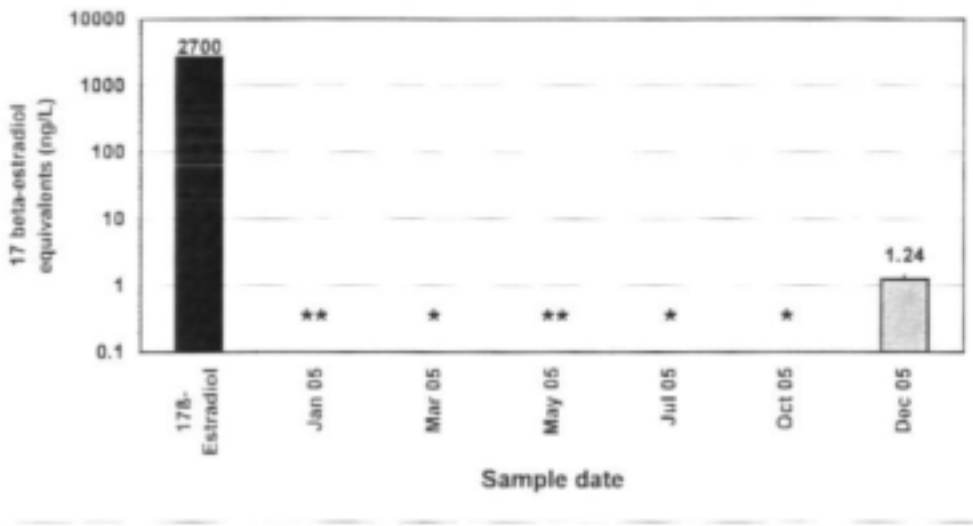

Fig. 5.2b: D1 maximum estrogenic activity expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit.

5.1.3.1b Sample site: Vlei

Table 5.2: Estrogenic activity in the Vlei expressed as the type of response and estradiol equivalents (ng/L).

Date sampled	Type of response			YES Result*	EE–EC_{50} (ng/L)	EE-Max (ng/L)
	Toxic	Submaximal	Maximal			
January 2005	X	X		3	< 0.03	<0.03
March 2005	X			1		
May 2005				1		
July 2005				1		
October 2005		X		3	0.78	1.92
December 2005	X			0		
January 2006	X			0		

*0 = Below detection limit; 1 = One point above detection limit; 2 = Two points above detection limit; 3 = Three or more points above detection limit (positive for estrogenic activity)

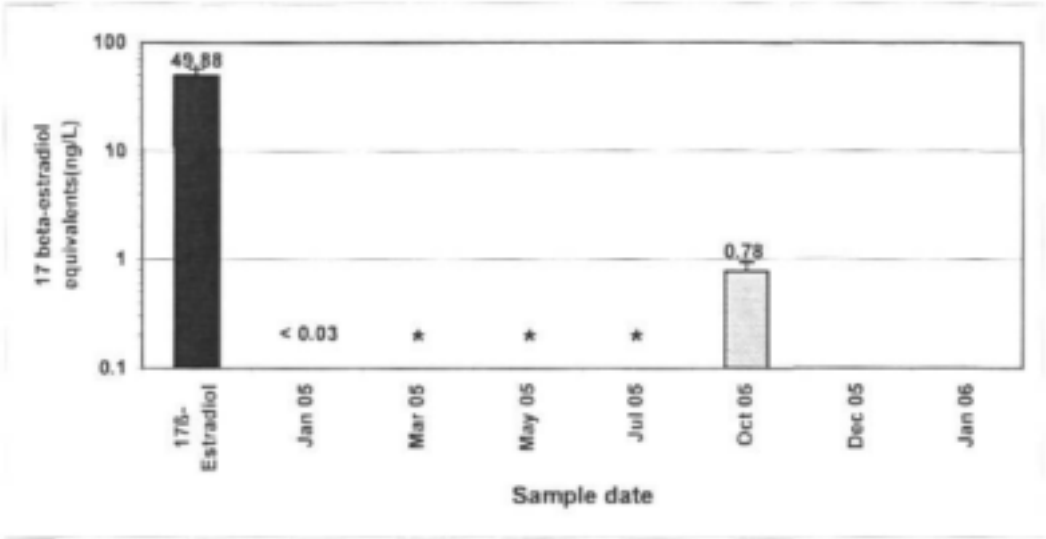

Fig. 5.3a: Vlei EC50 values expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit.

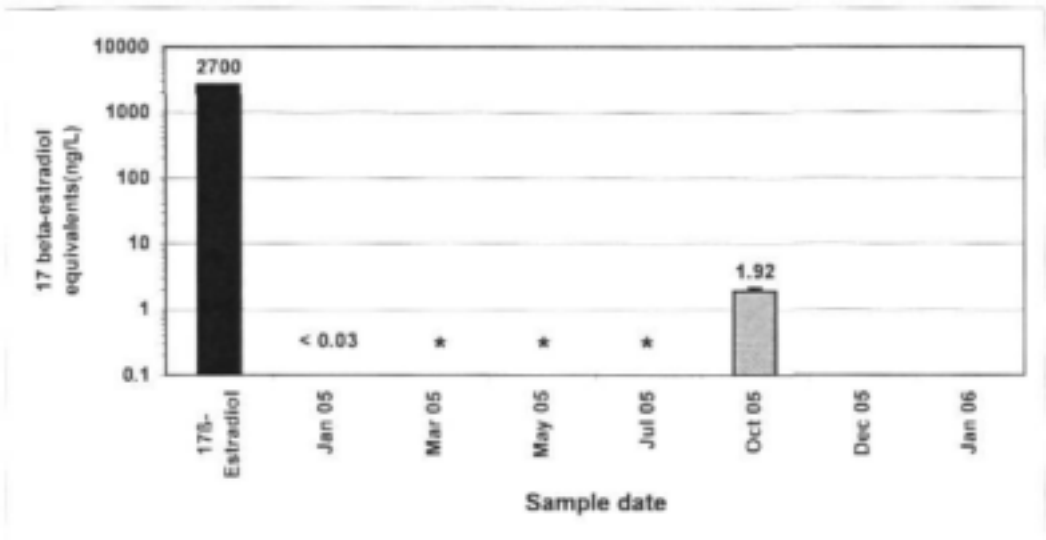

Fig. 5.3b: Vlei maximum estrogenic activity expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit.

5.1.3.1c Sample site: Channel

Table 5.3: Estrogenic activity in the Channel expressed as the type of response and estradiol equivalents (ng/L).

Date sampled	Type of response			YES Result*	EE–EC_{50} (ng/L)	EE-Max (ng/L)
	Toxic	Submaximal	Maximal			
January 2005	X			2		
March 2005	X	X		3	< 0.03	0.16
May 2005	X			0		
July 2005	X	X		3	0.25	0.44
August 2005	X			0		
October 2005	X			1		
January 2006		X		3	0.43	0.74

*0 = Below detection limit; 1 = One point above detection limit; 2 = Two points above detection limit; 3 = Three or more points above detection limit (positive for estrogenic activity)

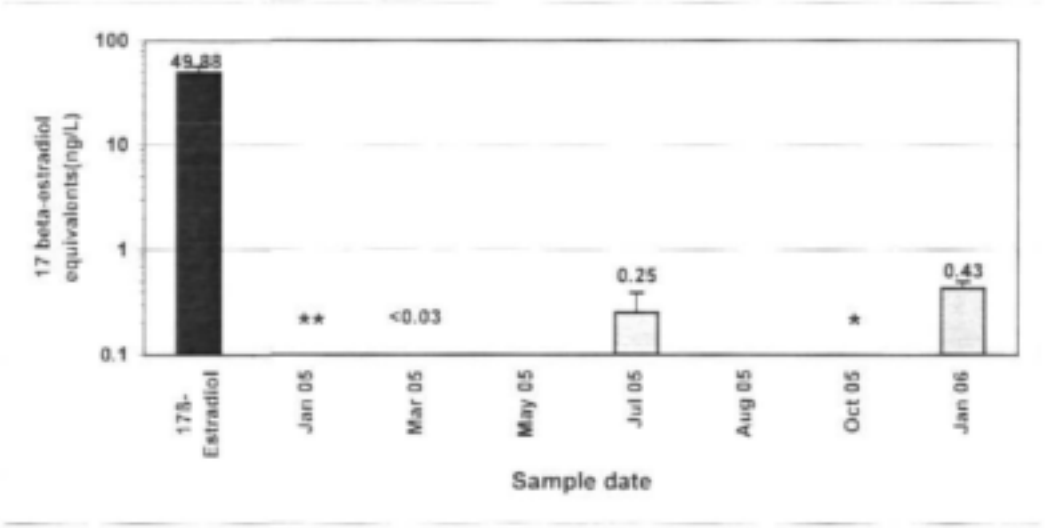

Fig. 5.4a: Channel EC50 values expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit.

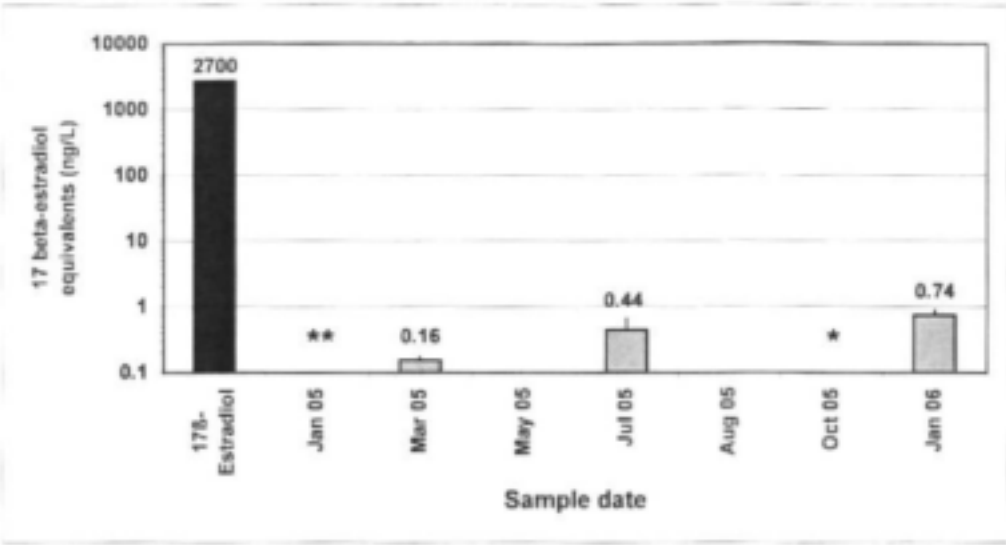

Fig. 5.4b: Channel maximum estrogenic activity expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit.

5.1.3.1d Sample site: Dam 2 (D2)

Table 5.4: Estrogenic activity in D2 expressed as the type of response and estradiol equivalents (ng/L).

Date sampled	Type of response			YES Result*	EE–EC_{50} (ng/L)	EE-Max (ng/L)
	Toxic	Submaximal	Maximal			
January 2005	X			0		
May 2005	X	X		3	<0.03	0.16
July 2005				1		
October 2005		X		3	0.52	1.02
December 2005	X			0		
January 2006		X		3	0.36	0.65

*0 = Below detection limit; 1 = One point above detection limit; 2 = Two points above detection limit; 3 = Three or more points above detection limit (positive for estrogenic activity)

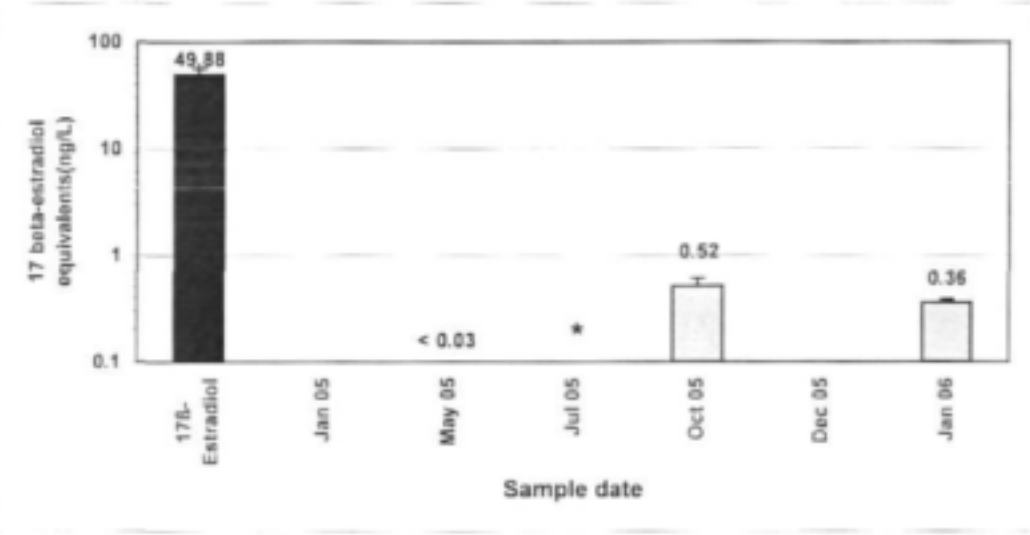

Fig. 5.5a: D2 EC50 values expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit.

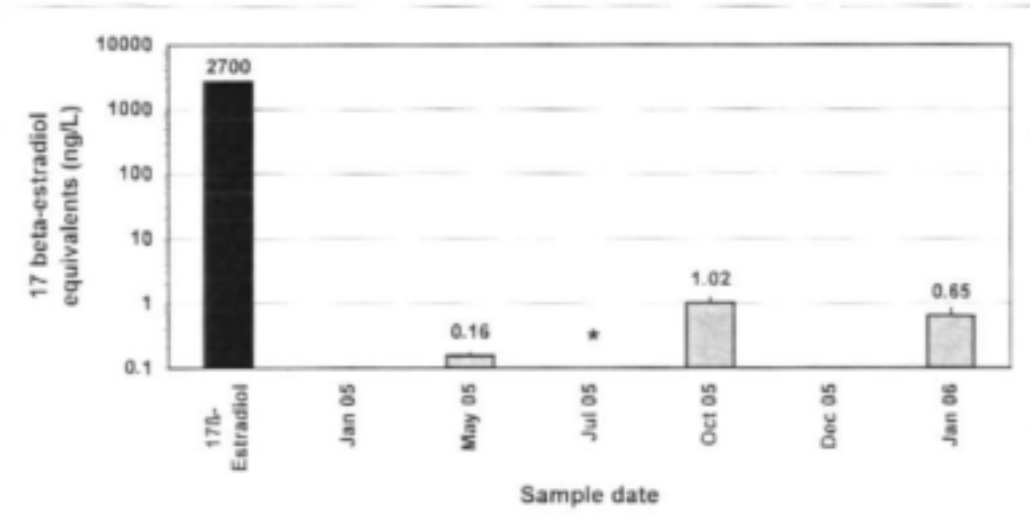

Fig. 5.5b: D2 maximum estrogenic activity expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit.

Of the four sampling sites three of the sites had no points above the detection limit of the assay, with D1 being the exception. Table 5.5 shows the sample sites and dates that were below the detection limit of the assay. The frequency of sampling indicates the percentage of the samples at a particular site that were below the detection limit (<dl) in Table 5.5.

Table 5.5: Summary of the frequency of sampling of the sites that were below the detection limit of the assay.

Sample site	Date sampled	Frequency of sampling (%)*
Vlei	December 2005	29
	January 2006	
Channel	May 2005	29
	August 2005	
Dam 2	January 2005	33
	December 2005	

% Frequency is calculated as follows:

Number of samples < dl / Total samples per site x 100

All four sites had samples that were positive for estrogenic activity. None of the samples were able to reach the maximum response obtained with 17-β-estradiol. D2 had the highest frequency of positive samples (50%) (Table 5.6). Although the Vlei and D1 had lower % estrogenic positive frequencies they had the highest maximum activities 1.24 and 1.92ng/L EE, respectively.

Table 5.6: Summary of the frequency of sampling of the sites that had estrogenic activity.

Sample site	Date sampled	Frequency of sampling (%)*
Dam 1	December 2005	17
Vlei	January 2005	29
	October 2005	
Channel	March 2005	43
	July 2005	
	January 2006	
Dam 2	May 2005	50
	October 2005	
	January 2006	

*% Frequency is calculated as follows:

Number of samples with estrogenic activity / Total samples per site x 100

Four of the nine samples that were positive for estrogenic activity, were also cytotoxic at higher concentrations. The Channel had the highest sample frequency at 29% (Table 5.7).

Cytotoxicity was found in 18 of the 26 samples, in other words 69% of the samples were cytotoxic. The Channel and D1 had the highest frequency of cytotoxicity (86% and 83%) respectively (Table 5.8). This was followed by the Vlei (57%) and D2 (50%).

Table 5.7: Summary of the frequency of sampling of the sites that had a cytotoxic response and estrogenic activity.

Sample site	**Date sampled**	**Frequency of sampling (%)***
Vlei	January 2005	14
Channel	March 2005	29
	July 2005	
Dam 2	May 2005	17

*% Frequency is calculated as follows:

Number of samples with cytotoxicity and estrogenic activity / Total samples per site x 100

5.1.4 Discussion

It is clear from the results that there is evidence of estrogenic activity in water sources from the UNR. It is important to note that although nine of the samples were below the assay detection limit, they are not necessarily negative for estrogenic activity. Likewise, samples with less than three points above the detection limit are not negative, but cannot be quantified for estrogenic equivalents in this assay.

All the sites had samples that were positive for estrogenic activity. The submaximal estrogenic response of these samples could be attributed to the complexity of the sample mixture. As some endocrine disrupting chemicals such as the hydroxylated PCBs may have antiestrogenic activity and this could inhibit the response (Moore et al., 1997). Of concern is the large number of samples that exhibited cytotoxicity. There was a number of samples that had a combined reaction of cytotoxicity and estrogenic activity, in these samples the estrogenic activity is almost certainly underestimated. This can be seen in samples from D1 that exhibited cytotoxicity and estrogenic activity two points above the detection limit of the assay. In those samples that exhibited cytotoxicity the estrogenic activity could be masked at these concentrations and therefore give a false negative result, as the estrogenic response may lie in the toxic range.

Table 5.8: Summary of the frequency of sampling of the sites that had a cytotoxic response.

Sample site	Date sampled	Frequency of sampling (%)*
Dam 1	January 2005	83
	March 2005	
	May 2005	
	July 2005	
	October 2005	
Vlei	January 2005	57
	March 2005	
	December 2005	
	January 2006	
Channel	January 2005	86
	May 2005	
	March 2005	
	July 2005	
	August 2005	
	October 2005	
Dam 2	January 2005	50
	May 2005	
	December 2005	

* % Frequency is calculated as follows:

Number of samples with cytotoxicity / Total samples per site x 100

In pilot study assessing the estrogenic activity in water in the Urban Nature Reserve using the YES a number of sites were positive for estrogenic activity (Aneck-Hahn, 2002). The EC_{50} - EEs ranged between 0.31-2.1ng/L and none of these samples exhibited cytotoxicity. In this study five of the nine samples that had estrogenic activity had EC_{50} – EEs ranging from 0.36-0.78ng/L which falls within the range of Aneck-Hahn's study. These values also corresponded with a study done by Matsui et al. (2000) in Japan. The concentration of estrogenic substances present in the sewage treatment effluents ranged from 5ng/l up to 15ng/L E_2–equivalents and lake water 1ng/L. Using the E-screen to determine estrogenic activity in water samples in Korea, Oh et al. (2000) found the total estrogenic activity to between 0.5pg/L and 7.4ng/L. Another study found E_2–equivalents levels higher than 10ng/l in Flemish rivers (Witters et al., 2001). A study on the estrogen like

potential of Taihu water, estimated with two luciferase reporter gene assays reported, E_2-equivalents in the range of 2.2 to 12.1ng/L (Shen et al., 2001). It is clear however from the large number of samples with cytotoxicity and the possible masking of estrogenic activity it appears that the water quality within the UNR has deteriorated.

These results highlight the need to include the proposed estrogenic (T47D-kBluc) and androgenic (MDA-kb2) reporter gene assays as they are more sensitive, have a lower detection limit and can distinguish between anti-estrogenic and anti-androgenic activity, compared to the yeast screen assay (estrogenic activity only).

Wetlands are among the most biologically important and productive ecosystems on earth. Estrogenic pollution does not only threaten the ecological environment but also the reproductive ability of freshwater fish and aquatic life in general. It is therefore important to develop early warning procedures to prevent disturbances in the normal reproductive cycles of aquatic organisms that would threaten their survival. With the increasing anthropogenic activities of humans, it has also now become important that further research is done on the removal of EDC from water system. A comprehensive understanding on the water quality of the UNR needs to be established.

5.1.5 Conclusions

All the sites had samples that were positive for estrogenic activity. The submaximal estrogenic response could be attributed to the complexity of the sample mixture. Of concern is the large number of samples that exhibited cytotoxicity alone or in combination with estrogenic activity, since the estrogenic activity is almost certainly underestimated.

5.2 Reporter gene assays

5.2.1 Introduction

In comparison to other existing in vitro assays, reporter gene assays based on stably transfected cell lines provide the **most specific, responsive, and relatively quick** means to screen substances for potential estrogenic and antiestrogenic activity (Legler et al., 1999). Recently, a few such cell lines have been developed (Legler et al., 1999; Pons et al., 1990). These cell lines are sensitive and responsive to estrogens but are not freely available.

5.2.2 T47D-Kbluc assay

US EPA developed an estrogen-dependent stable cell line by stably transfecting an estrogen-responsive luciferase reporter gene construct into T47D human breast adencarcinoma cells expressing endogenous estrogen receptors alpha and beta.

T47D cells express a relatively high number of endogenous ER, reportedly 67.6±6.2 mol/mg cytosolic protein (Watanabe et al., 1990). Western blot analysis indicated that these cells contain both the alpha and beta isoforms of the ER protein with very slightly higher levels of beta than alpha (Power and Thompson, 2003). The reporter gene construct consists of three estrogen response elements (ERE) upstream from a 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 principle, 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. When testing chemicals using the T47D-Kbluc cells, an estrogen is defined as a chemical that induced dose dependent luciferase activity which could be specifically inhibited by the antiestrogen ICI (Wilson et al., 2004).

T47D human breast cancer cells, which contain both endogenous ERα and ERß, were transfected with an ERE luciferase reporter gene construct. It provides an *in vitro* system that can be used to evaluate the ability of chemicals to modulate the activity of estrogen-dependent gene transcription. This cell line has the potential to be used both for screening chemicals and as an aid in defining mechanism of action of chemicals with estrogenic and antiestrogenic activity. This is valuable for a first-pass type in vitro assay, as a ligand for either receptor could drive the luciferase reporter gene thereby eliminating the need for a separate assay for Erα and Erß.

Advantages of this assay are that it is relatively rapid (2 days), eliminates the need for transfection and can be conducted in 96 well plates and consistent results are produced.

5.2.3 MDA-kb2 assay

The US EPA proposed that *in vitro* assays for both estrogen receptor (ER) - and androgen receptor (AR) – mediated reactions be included in a Tier-I screening battery to detect hormonally active chemicals.

The US EPA then developed a stable cell line used to identify compounds that bind AR and, unlike standard receptor binding assays, are able to discriminate androgen agonists from antagonists thereby aiding in defining the mechanisms of action.

The breast cancer cell line, MDA-MB-453 was stably transformed with the MMTV.luciferase.neo reporter gene construct. Both the glucocorticoid (GR) and AR are present in the MDA-MB-453. To distinguish between AR from GR mediated ligands, chemicals assayed concurrently with the anti-androgen, hydroxyflutamide (OHF) which blocks the AR but not GR mediated responses. The cells are relatively easy to culture and maintain and are stable for more than 80 passages (Wilson et al., 2002).

Advantages of this assay are that it is relatively rapid (2 days), eliminates the need for transfection and can be conducted in 96 well plates and consistent results are produced. These make it an ideal assay for screening.

5.2.4 Comments

Both the above mentioned cell lines were ordered through Highveld Biological in June 2005. After a long delay at ATCC the cell lines were delivered on 5 April 2006. While attempting to grow up stock cultures the cells were found to be contaminated. It can be said with certainty that the contamination was not from the Andrology laboratory as up until this point there had been no contamination in any of the other cell lines in that laboratory or subsequently. A new batch of cells has to be ordered. As soon as the cell lines arrive in the country stock cultures will be prepared and the method will be validated in the laboratory. In order to get a result from a mammalian cell line 10 of the water extracts used in the YES assay were sent to Bio Detection Systems in Amsterdam

5.3 ER-Calux reporter gene assay

5.3.1 Introduction

The estrogen receptor (ER)-mediated chemical activated luciferase gene expression (ER-CALUX) assay (Legler et al., 1999) uses T47-D human breast adenocarcinoma cells expressing endogenous ER α and β, which are stably transfected with an estrogen responsive luciferase reporter gene. Exposure to xenoestrogens results in transactivation of ER and consequent induction of the luciferase gene, which is easily assayed by lysing cells and adding the substrate luciferin and measuring light output (Legler et al., 2002). The amount of luciferase produced is proportional to the extent of receptor binding and it is quantitated by reaction with luciferin to produce a light signal, which is detected by a luminometer (CALUX®).

5.3.2 Results

Estrogenic activity was detected in all 10 samples sent for analysis. The EEs ranged from 0.32-16.0 ng/L. The following table compares the results of the YES assay and the ER-Calux assay.

Table 5.9: Comparison of the YES assay and the ER-Calux assay results.

Sample site	Sample date	ER-Calux EE (ng/L)	YES EE (ng/L)
Dam 1	January 2005	0.33	Toxic (2 points)
	October 2005	2.9	Toxic (1 point)
	December 2005	16.0	1.24
Vlei	January 2005	0.32	Toxic (3 points ; < 0.03ng/L)
	October 2005	1.3	1.92
Channel	January 2005	3.5	Toxic (2 points above)
	October 2005	3.0	Toxic (1 point above)
	December 2005	2.0	0.74
Dam 2	January 2005	1.1	Toxic
	October 2005	1.1	1.02

5.3.3 Discussion

These results suggest that estrogenic contamination is widespread within the Urban Nature Reserve. The levels of estrogenic activity found in these samples lie in the same range (0-10ng/L EEQs) as those reported in agricultural surface waters in Israel (Shore et al., 1995, 2004) and North America (Soto et al., 2004). Matthiessen et al. (2006) found estrogenic activity in UK streams ranging from 0-26.5ng/L (Mean = 2.0ng/L) EEQs. More importantly the implication from published information on fish studies (Metcalfe et al., 2001; Seki et al., 2005; Young et al., 2002) is that average long-term EEQ concentrations in excess of 1ng/L, if bioavailable, are likely to cause ovotestis and other estrogen-induced intersexual abnormalities (eg. Vitellogenin induction) in some fish (Matthiessen et al. 2006). Eight of the ten samples tested were above this level; this suggests that the aquatic biota, especially the fish may be at risk of endocrine disruption.

When comparing the results from the two different assays it is clear that there is a need for more than one screening bioassay. With a suitable choice of assays the results can complement each other and give a clearer assessment on the estrogenic activity in the environmental samples. Although some of the samples had a toxic response in the YES, the ER-Calux was able to assess the estrogenic activity. This confirms the hypothesis that the toxic response in the YES could well be underestimating estrogenic activity or giving a false negative. In some cases the YES EEQs were similar to those of the ER-Calux (Vlei: Oct '05 and Dam 2: Oct '05) and in others (Dam 1: Dec '05 and Channel: Dec '05) were very different. This difference could be due to the impermeability of the yeast membrane to some substances and therefore decreasing the estrogenic activity.

5.34 Conclusion

There is clearly estrogenic pollution in the UNR and according to the ER-Calux results, Dam 1 and the Channel have the highest estrogenic activity. The EEQs throughout the sampling period at these sites was not always consistent, the levels of estrogenic activity remained higher than that of the other sites during the same sampling period. The EEQs exceeded the Predicted-No-Effect-Concentration (1ng/L) for 17β-estradiol in water in all cases for the Channel and two out of three for D1. This activity was probably sufficient to cause reproductive damage to fish. Despite the limited sample numbers evaluated in the ER-Calux assay, it cannot be concluded that the UNR is safe from estrogenic pollution. Further research and continuous monitoring using a relevant battery of assays is essential to establish the extent, sources and ecological consequences of estrogenic contamination in the aquatic system.

5.4 Catfish vitellogenin (cf-VTG) in male fish

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5.4.1 Introduction

Various laboratory and domestic animal species can be used to address specific aspects of the possible effects *in vivo* of (endocrine disrupting chemicals) EDCs. These should be particularly useful for studies of delayed effects (i.e. where exposure to the chemicals(s) occurs in fetal/neonatal life and the reproductive consequences are manifest in adult life and for studies of the effects of chronic low level exposure to EDCs).

The EDSTAC believes inclusion of *in vivo* methods in Tier 1 can help reduce false negatives in the absence of knowledge of absorption, distribution, metabolism, and excretion. *In vivo* assays are often apical (that is, while they incorporate endocrine-specific endpoints, disruption of a number of hormone regulation/delivery mechanisms can be evaluated in the same assay). Therefore, they are less specific, but more comprehensive, than *in vitro* assays. *In vivo* assays can be made more specific if accompanied by target organ/cell dosimetry of biologically active metabolites. *In vitro* data are enhanced if the actual concentration of the chemicals in the media is determined, to account for metabolism, stability, and solubility, and to determine whether these concentrations compare to those that can be achieved *in vivo* (EDSTAC, 1998).

In association with the intersex condition the use of a species specific developed vitellogenin (VTG) enzyme-linked immunosorbent assay (ELISA) has become an accepted biomarker/tool in detecting the presence of endocrine disruption in different fish species (Johnsen et al., 1999; Fenske et al., 2001; van Aerle et al., 2001; Tolar et al., 2001; Hennies et al., 2003; Spano et al.,

2004). VTG is a phospholipoglycoprotein produced in the liver of mature oviparous animals in response to the stimulation of estrogens. From the liver it is transported by the blood circulation to the ovaries and incorporated in the developing oocytes through receptor-mediated endocytosis. Once incorporated by the developing oocytes, VTG and its metabolites serve as a source of energy (Roubal et al., 1997). Mature females usually produce VTG only during spawning but males and juveniles have the responding genes enabling VTG expression when exposed to exogenous estrogens (Lomax et al., 1998). VTG expression by males and juveniles in plasma as a result of xenoestrogens exposure is, therefore, a good indicator of endocrine disruption in the aquatic environment.

5.4.2 Materials and methods

Plasma catfish vitellogenin (cf-VTG) was analyzed using an enzyme linked immunosorbent assay (ELISA) (Figure 5.2.1) developed by Barnhoorn et al. (unpublished) specifically for *Clarias gariepinus*. Plasma cf-VTG was defrosted overnight on ice at 4°C before use (Van Aerle, 2002). Ninety-six well microtiter plates (Nunc) were pre-coated with 100µL cf-VTG (100ng cf-VTG/mL) in coating buffer (0.05M sodium bicarbonate, pH 9.6). A serial dilution of standards with known VTG concentrations was made in separate uncoated Titertek microtiter plates. Apart from the standards the plasma samples with unknown VTG concentrations were also diluted in blocking buffer (1% bovine serum albumin in washing buffer [0.01M sodium phosphate; 0.9% NaCl and 0.05% Tween 20; pH 7.4]) and together with the standards, pre-incubated with the primary antibody (PAS 7441).

The unknown samples were diluted between 1:10 and 1:1,000,000 depending on the estimated concentration of VTG in the plasma sample (Van Aerle, 2002). Both the coated plates and the standard/sample plates were incubated overnight at 4°C. The next day the coated plates were washed three times in washing buffer and the unbound sites blocked using blocking buffer and together with the samples/standards containing plates incubated for 30' at 37°C. The VTG coated plates were washed three times and to establish competition for the antibodies between the VTG coated in the wells and the VTG in sample solution. 100µL/well of pre-incubated standard/samples were transferred into the coated plates and incubated for a further 60' at 37°C. The plate was then incubated for two hours with the secondary antibody (goat-ant-rabbit; 1:2000). Then substrate buffer (horse-radish peroxidase) was added to the wells and left at room temperature in the dark enabling the substrate buffer to change from colourless to yellow. The enzyme reaction was stopped after 10' using 3N H_2SO_4, left to stabilize for 10' and the optical density (OD) of each well in the plate were read in a multi well plate reader at 490nm. Non-specific (NSB) and maximum binding (Bo) were also measured respectively in four wells. For NSB measurement the wells were not coated with VTG but all the steps were included as if a

normal ELISA. Maximum binding was measured in wells where there was only assay buffer with no competition for the coated VTG and the antibodies.

5.4.3 Results

After development and the first assessments of the ELISA, the control males were all diluting parallel to the standard and all fitted nicely on top of each other as shown in figure 5.6. This meant that whatever VTG they have is constant. The female however, never reached the top of the curve. The results indicated the need to do a Western Blot to again verify the specificity of the antibody produced against catfish VTG.

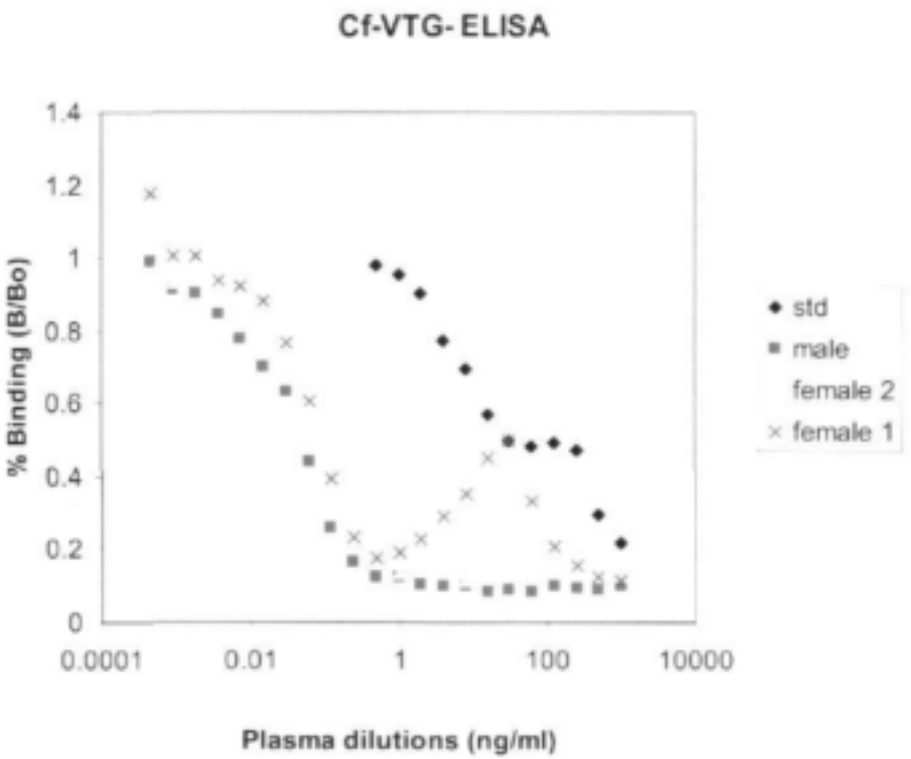

Fig. 5.6: Troubleshooting the Cf-VTG-ELISA. Binding curves of serial dilutions of plasma samples from mature females and a mature unexposed male (control) with the catfish vitellogenin (c-VTG) standard curve. The dilution of the mature unexposed male should have been represented on the upper x-axis. This procedure was repeated with numerous males but the results shown here are the average results from all the experiments. This indicates that all the males are estrogenised or the ELISA is not specific for catfish VTG.

5.4.4 Discussion and conclusion

These screening assays determine contamination by pollutants that act through specific modes of action. In the South African scenario this battery of assays allows an approach to EDC pollution that is efficient and cost effective. It provides a platform to identify samples of interest and to provide basic information for further analysis and risk assessment. There is yet no final outcome on the cf-VTG-ELISA.

CHAPTER 6

BIO-ANALYSIS OF DIOXIN AND DIOXIN-LIKE PCBS

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6.1 Introduction

The rat hepatoma cell line H4IIE (Villeneuve et al., 1999) is a reporter gene assay available in South Africa as a bio-assay to assess dioxin and dioxin-like PCBs.

6.2 Materials and methods

6.2.1 Choice of environmental matrix to be analysed

Aquatic sediments serve as a sink for a number of contaminants and provide an integrative measure of exposure to the aquatic ecosystem. Sediment may contain mixtures of biologically active compounds acting through different mechanisms. Bottom-dwelling animals are directly exposed to these chemicals and through them these pollutants may enter the aquatic food chains. In addition, contaminants in sediments can directly affect micro- and meiobenthic communities (Hilscherova et al., 2002).

6.2.2 Chemical extraction

Composite sediment samples were collected from D1, channel, vlei and D2 at two-monthly intervals and stored in pre-cleaned glass vials. The samples were protected from UV degradation and stored and transported at 4 °C. All apparatus and instruments, with which the samples came into contact, were washed with phosphate free soap, rinsed with double deionised water and thrice with high grade acetone and hexane. The acetone removed any polar organic compounds and hexane the non-polar organic compounds in order to exclude external contamination of the samples.

The samples were freeze-dried and manually grinded in a pre-cleaned mortar and pestle before being sieved (0.5mm mesh size) to obtain homogenous samples. 40g of each sample was mixed with the same volume of anhydrous sodium sulphate (to remove any trace of water) before loaded onto a Soxhlet extraction apparatus for a minimum period of 16 hours. 400mL HPLC grade hexane was used as the solvent (US EPA, 1996). After allowing the extract to cool, it was concentrated to 20mL in a rotary evaporator. 10mL was further concentrated under a gentle stream of high purity nitrogen gas to 1mL. This will be referred to as the raw extract. The remaining 10mL of extract was treated with concentrated sulphuric acid to remove any traces of polycyclic aromatic hydrocarbons (PAHs), which are known ligands of the Ah-receptor

(Hilscherova et al., 2001). Therefore, the raw extracts would still contain PAHs, PCDD/Fs and PCBs, whereas the acid treated extracts, should contain only PCDD/Fs and PCBs, if present. An equal volume of sulphuric acid was added to the extract and mixed gently, venting often, while the phases were allowed to separate. The acid phase was discarded and the process repeated until the acid phase was clear. The extract was subsequently concentrated to 1mL under nitrogen stream. Both the raw and acid treated extracts were stored at 4°C in amber gas chromatography vials until the bio-assay commenced.

6.2.3 Bio-assay

The H4IIE-*luc* cells were grown at 37°C in an incubator: 5/95% CO_2/air, >90% humidity in Dulbecco's Modified Eagle's medium (with sodium bicarbonate, but without phenol red) supplemented with 10% fetal bovine serum (Whyte et al., 2004). For the assay, cells were plated into 96-well micro plates (optical bottoms with opaque white walls) at a concentration of 50 000 cells/well on day one (adapted from Whyte and Tillitt, 2004). Endogenous hormones in the fetal bovine serum and growth medium were removed using dextran coated charcoal. Cells were only transferred to the inner 60 wells and the outer wells were filled with phosphate buffered saline to create a micro-climate of homogenous humidity across the plates.

After an incubation period of 24 hours, the cells were exposed to either the raw or acid treated extracts, the solvent control hexane or the standard: 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (TCDD). To determine a dose-response relationship, cells were exposed to six different concentrations of the extracts (2.5µl of a specific dilution/well) (Masunaga et al., 2002). Every alternating plate was dosed with a series of TCDD ranging from 0.5 - 120pg TCDD/well. After 72 hours of exposure, the luciferase activity was measured. Before measurement, confluent cells were examined microscopically to check for possible cytotoxicity or microbial/fungal contamination. Culture medium was removed, the cells were washed with phosphate buffered saline, and incubated for 10 min with LucLite™ (Perkin Elmer, Johannesburg) reagent at 37°C. Luciferase activity was measured as luminescence with a microplate-scanning luminometer (Microplate Fluorescence Reader FLX 800, Bio-Tek Instruments) (Hilscherova et al, 2001).

6.2.4 Data analysis

The response of the cells was registered as relative light units (Hilscherova et al., 2002). The response from the cells exposed to the solvent control was subtracted from the responses of the extracts or TCDD responses. Sample responses were converted to a percentage of the maximum response observed for the TCDD standard ($TCDD_{max}$) and plotted as a function of log µl sample. Regression equations were derived for the linear portion of each dose-response curve. 2,3,7,8-TCDD equivalents (TCDD-EQs), based on bio-assay results were then calculated from the amount

of sample producing a response equivalent to 20% of the maximal response (EC_{20}) produced by the standard (TCDD) (Hilscherova et al., 2001). The EC_{20} was selected because of the low concentrations present in the samples.

6.3 Results

Over the entire period of investigation, 54 samples were exposed in the bio-assay. All of them, except one (Table 6.1), showed no response, thereby implying that the cells could not detect any measurable quantities of Ah-ligands in the extracts. However, many of the undiluted extracts were cytotoxic, which could be masking positive responses from the cells. The minimum detectable quantity (MDQ) quantified with H4IIE was 0.2pg TEQ/g sediment (dry weight). The highest $\%TCDD_{max}$ measured was 24.4% in the channel July 2005.

Table 6.1: Summary of responses prepared from the sites at different intervals.

	Dam 1		**Channel**		**Vlei**		**Dam 2**	
Sampling dates	Raw extract	Acid treated	Raw extract	Acid treated	Raw extract	Acid treated	Raw extract	Acid treated
Sep 04	x	x	BDL	BDL	x	x	X	x
Nov 04	x	x	BDL	BDL	x	x	X	x
Jan 05	x	x	BDL	BDL	x	x	-	-
Mar 05	x	x	BDL	BDL	x	x	-	-
May 05	x	x	BDL	BDL	x	x	BDL	BDL
Jul 05	x	x	BDL	BDL	24.4%	BDL	-	-
Oct 05	x	x	BDL	BDL	BDL	BDL	X	x
Jan 06	-	-	-	-	x	x	X	x

-: no samples; x: cytotoxicity, BDL: below detection limit of the assay

6.4 Discussion

The cytotoxicity, which is so evident in Table 5.1, may be ascribed to sulphur in the sediment (Hilcherova et al., 2000). The first dilutions were either slightly cytotoxic or not at all and if there were measurable quantities of dioxin-like chemicals present in the extract, but masked by the cytotoxicity in the undiluted extract, their presence would have been evident in the first dilutions, which they were not. A good example to illustrate this condition is the sample collected at Vlei during May 2005 (Figure 6.1). The extract elicited a very low response (3.5%) from the cells. The first dilution however showed some response (10.6%), but still lower than the detection limit of the method. The shape of the graph is typical that of cytotoxicity at high concentrations of the sample. Cytotoxicity is usually detected by microscopic inspection of the 96 well microplate before the

commencement of the assay, and was duly noted. A graph such as the one in Figure 6.1, confirms what has been seen during the inspection of the plate.

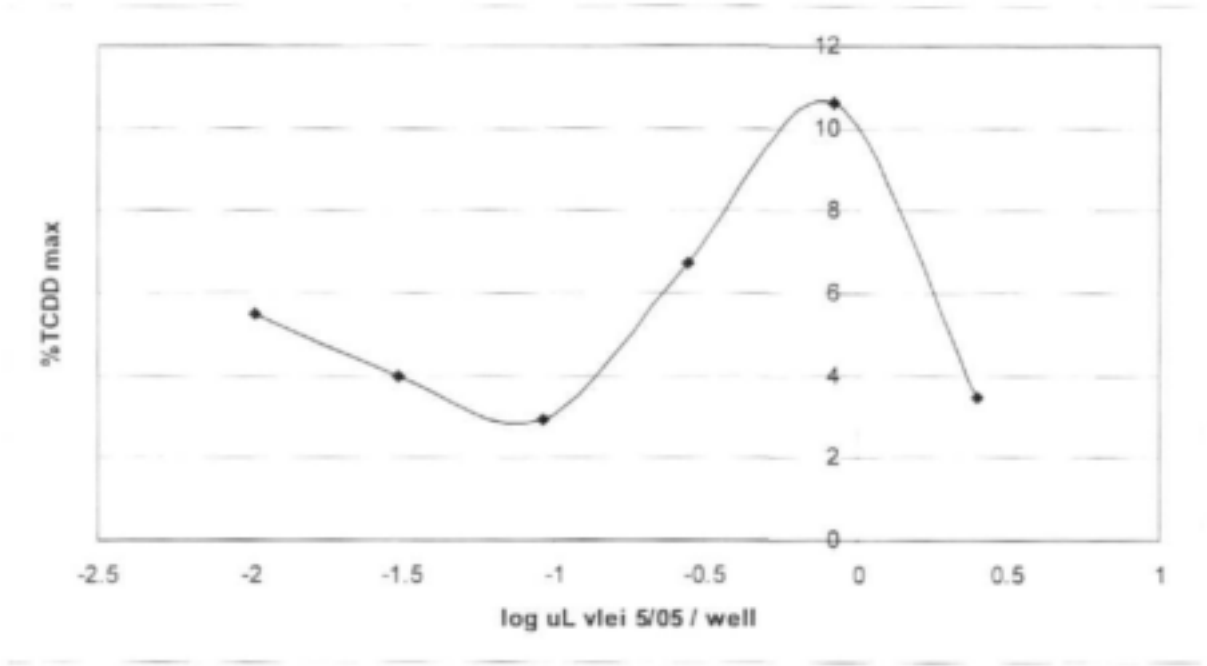

Fig. 6.1: The %TCDD$_{max}$ of the Vlei sample collected in May 2005.

The only extract that showed some positive response was the raw extract of Vlei collected in July 2005 (Table 6.1). A TCDD$_{max}$ of 24.4 % (Figure 6.2) was detected for the raw extract. Unfortunately the responses of the dilutions were below the detection limit of the method and no valid regression equation of the straight line could be calculated.

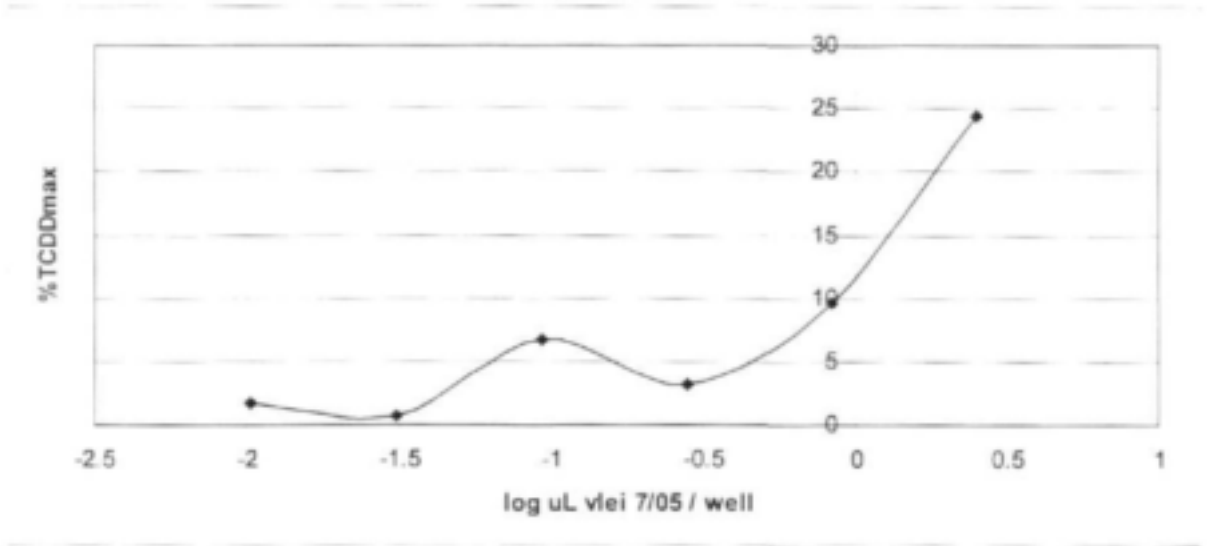

Fig. 6.2: The %TCDD$_{max}$ of the raw Vlei sample collected in July 2005.

The same sample that was treated with sulphuric acid showed no response at all. The cells probably responded to the PAH's present in the raw extract. Certain PAHs may act as agonists or antagonists for the oestrogen receptor, producing "feminising" of "masculinising" effects (Walker 2001). Aromatic hydrocarbons often consist of several fused benzene rings. As with PCDD/Fs and the planar PCBs, the largest release of PAHs is due to the incomplete combustion of organic compounds during the course of industrial processes and other human activities. Important sources include the combustion of coal, crude oil and natural gas for both industrial and domestic purposes, the use of such materials in industrial processes (e.g. the smelting of iron ore), the operation of the internal combustion engine and the combustion of refuse (Walker, 2001).

Because of the fusion of adjacent rings, PAHs tend to have rigid planar structures and as with PCDD/Fs and PCBs, they are of low water solubility and marked lipophilicity, and have high K_{ow} values. Because they have no functional groups, they are chemically rather unreactive. They can however, be oxidised both in the natural environment and biochemically. Photodecomposition can occur in air and sunlight to yield oxidative products. Nitrogen oxides and nitric acid can convert PAHs into nitro derivatives, and sulphuric acids can produce sulphanilic and sulphonic acids (Walker, 2001).

6.5 Conclusion

Although PCDD/Fs and planar PCBs are known endocrine disruptors, congeners of these groups of POPs do not seem to have been present in the sediment of the sites that were under investigation. In the event of cytotoxicity, the presence of dioxin-like chemicals was masked. However, the first dilutions of all of these samples were not cytotoxic and they too did not show any response at all, implying that there were no dioxin-like compounds present in the sediment.

The only sample that showed any indication of eliciting a response from the cells, was one that still contained the PAHs (Vlei July 2005). This response too was very limited. If endocrine disruptive tendencies were to be present in the biota that was collected at the same time, it would not be likely because of the presence of dioxin-like compounds.

CHAPTER 7

SENTINEL SPECIES AS BIOMARKERS OF EDC EXPOSURE

Various species were evaluated as possible biosentinels of environmental pollution.

7.1 The sharptooth catfish (*clarias gariepinus*)

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Department of Urology, University of Pretoria[1]; Department of Zoology, University of Johannesburg[2].

7.1.1 Introduction

This fish species was selected because a lot of scientific information is available on the species, including research data collected at the Urban Nature Reserve (UNR).

7.1.2 Material and Methods

7.1.2.1 Sampling of *Clarias gariepinus*

Ten male sharptooth catfish were each collected during 2 low-flow (September 2004 and 2005) and 2 high-flow periods (January 2005 and 2006) from Dam 1 (D1) and Dam 2 (D2) dams situated in the UNR (n=80). The mean mass and length of each fish was recorded. Blood was taken from the caudal aorta, using ice-cold vacutainers with EDTA additive and Aprotinin (protease inhibitor) as well as in vacutainers without additive. The samples were kept on ice until centrifuged at 1300 *g* for 15minutes (min.) using a Mistral 3000i centrifuge. Plasma and serum samples were then stored at –20°C for vitellogenin (refer to Chapter 5.2) and 11-ketotestosterone (11-KT) analyses respectively.

Fish were sacrificed and the external sex determined by evaluating the sexual papilla and then grouped into Male (M) or Intersex (I) (Barnhoorn et al., 2004). After laparotomy, the gonads were inspected macroscopically and the fish again allocated to male/intersex.

Gonads were removed, weighed and part of each gonad fixed in Bouins solution. The length and width of the papilla were measured, then removed and placed in 10% buffered neutral formalin (BNF) (Culling, 1974). After dehydration in graded ethanol and embedding in paraffin wax, sections (5 µm) were cut and stained with hematoxylin and eosin. The slides were histologically examined (Fig. 7.1) using light microscopy and a range of magnifications (20 × to 100 ×) (Barnhoorn et al., 2004).

The gonado-somatic index (GSI) was calculated according to the equation: gonadal weight/(body weight-gonadal weight) × 100 (van Aerle et al., 2001). The urogenital papilla

length index (UGPLI) was also calculated and it represents the UGP length as a percentage of the total body length (Kirby et al., 2002) (Table 7.3).

7.1.2.2 Determination of selected EDCs in fish fat

Available abdominal fat of each fish was removed, wrapped in foil, placed in plastic bags and kept at –20°C until target analyses was done (Table 7.5). The samples were analysed using standard methods in the Residue Laboratory, Onderstepoort, RNON: 057 and GCMS: 008. The alkylphenols (APs) octylphenol (OcP) and *p*-nonylphenol (*p*-NP) were extracted from fat samples with acetonitrile and sample clean up is performed on a florisil cartridge followed by a further clean up on a C18 cartridge. Analytes were eluted with methanol from C18 and quantification is accomplished via fortified calibration curve. The alkylphenols were detected using fluorescence detection at a quantification limit of 0.05mg/kg. OCs in fat samples (Chapter 4.2.2) was extracted and clean up was performed on a C18 cartridge followed by florisil solid phase extraction (SPE). The analytes were eluted with petroleum ether–diethyl ether. Aldrin was used as an internal standard and quantification was done via fortified calibration curve. The organochhlorine pesticides (OCs) were detected using gaschromatography (GC) coupled to a quadrupole mass spectrometry (MS) detector at a detection limit of 0.010 mg/kg.

7.1.2.3 Catfish 11-KT analysis

The androgen 11-KT was measured using a steroid radioimmunoassay (RIA) as previously describe by Schulz et al. (1996) at the laboratory of Dr RW Schülz, University of Utrecht, The Netherlands (Table 7.6)

7.1.2.4 Water and sediment sampling

Water and sediment samples were also collected and analyzed. For results, refer to Chapter 4, Table 4.3.

7.1.2.5 Statistical calculations

For the purpose of this study differences in selected groups of samples were calculated using the Microsoft software package Excell.

7.1.3 Results

7.1.3.1 Sex determination

Fish were sexed according to the external genital papilla, macroscopically after laparotomy and microscopically. The findings were summarized in table 7.1 and the findings and suggested a multisystem approach, which includes the use of papilla, laparotomy and histology

in comparison to do sex determination in sharptooth catfish. Table 7.2 summarizes the number of fish with one or more features of intersex (UGP, macro- or microscopic) during the different seasons and the individual dams. There was no significant difference between the low- and high-flow seasons (18 vs 20), but more fish with intersex features were collected from D1 (24 (63.2%) vs 14 (36.8%)). 28.9% of sharptooth catfish from D1 and D2 were intersex fish (Table 7.2).

Table 7.1: Comparison of external phenotypic sexual characteristic findings (papilla) and at laparotomy.

Locality	**Dam 1**			**Dam 2**		
Sex allocation	External (papilla)	Macroscopic (laparotomy)	Microscopic (histology)	External (papilla)	Macroscopic (laparotomy)	Microscopic (histology)
Male	**36**	**37**	**34**	**35**	**40**	**35**
Uncertain	**7**	**3**	**0**	**3**	**1**	**0**
Intersex	**9**	**12**	**18**	**7**	**4**	**10**
Total	**52**	**52**	**52**	**45**	**45**	**45**

Table 7.2: Total of intersex features in fish collected in the study.

	Low-flow season			**High-flow season**		
	First	**Second**	**Total**	**First**	**Second**	**Total**
Dam 1	7	3	**10**	4	10	**14**
Dam 2	1	7	**8**	1	5	**6**
Total			**18**			**20**

7.1.3.2 Gonadal development

The mean (± SD) GSI of male fish from D2 and D1 ranged between 0.104 ± 0.07 and 0.5 ± 0.32 respectively (Table 7.3). There were no significant differences between of the fish collected at D2 or D1. The GSI in the intersex fish from D2 were not significantly different from the GSI of males in D2 and D1.

7.1.3.3 Gonadal histology

The histology of the male testes of catfish from the 1D and D2 showed normal testicular development (Fig. 7.1a,b). The tubules contained spermatids, with no indication of primary oocytes. Histological evaluation of the male gonadal slides indicated intersex in several of the fish. Intersex was observed as primary oocytes scattered between the testicular tissue (Fig. 7.1c,d). Although some of the testes seemed morphological normal, histological valuations identified the presence of primary oocytes scattered among the testicular tissue (Fig. 7.1d).

Table 7.3: Mean- weight ± SD, length ± SD, GSI ± SD of sexed fish from both dams and the mean GSI ± SD of all intersex fish (SD, standard deviation).

Survey	Mean weight ± SD (g)	Mean length ± SD (cm)	Mean GSI ± SD (%)	Mean Intersex GSI ± SD
D1 (Sep 2004) low	3421 ± 1981	70 ± 13	0.195 ± 0.100	
D1 (Jan 2005) high	2562 ± 1092	72 ± 7	0.500 ± 0.320	D1; n = 20
D1 (Sep 2005) low	4000 ± 2603	77 ± 11	0.560 ± 0.207	0.349 ± 0.049
D1 (Jan 2006) high	2566 ± 1020	71 ± 9	0.300 ± 0.190	
D2 (Sep 2004) low	3836 ± 1942	74 ± 13	0.104 ± 0.070	
D2 (Jan 2005) high	3490 ± 1518	76 ± 8	0.327 ± 0.070	D2; n= 9
D2 (Sep 2005) low	2283 ± 1291	62 ± 8	0.170 ± 0.163	0.281 ± 0.074
D2 (Jan 2006) high	4100 ± 1599	76 ± 12	0.661 ± 0.990	

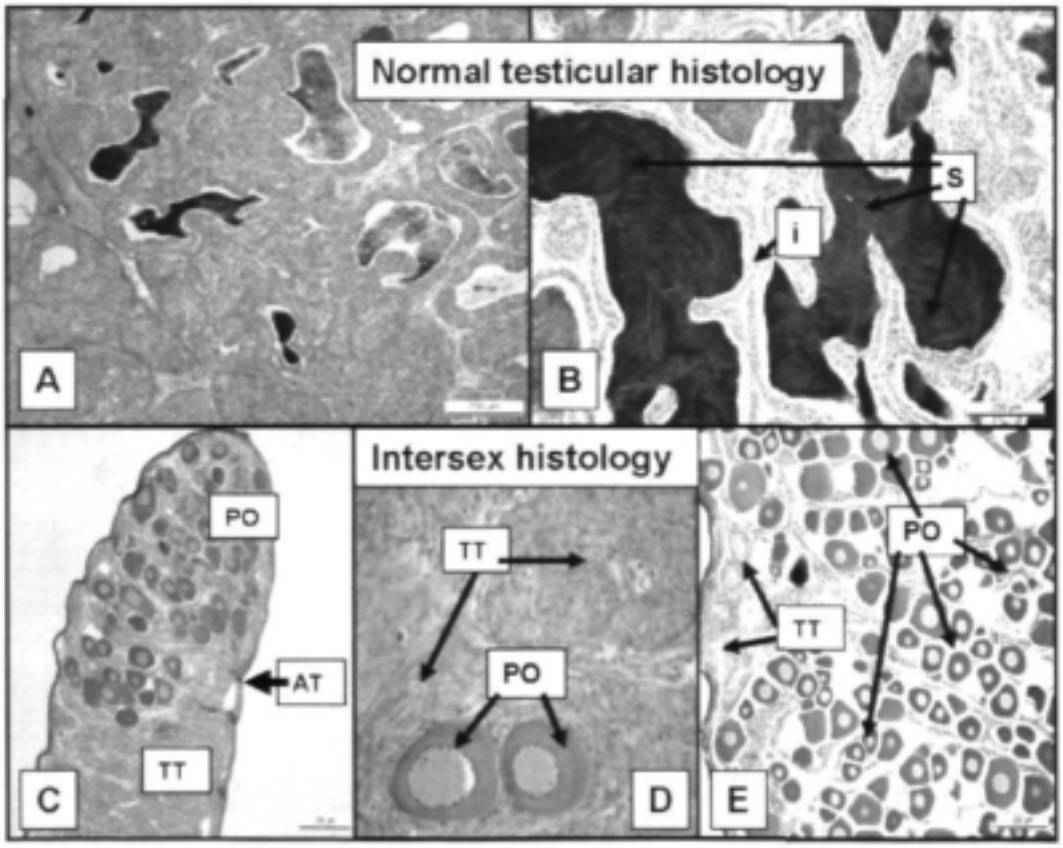

Fig. 7.1: Transverse section through the normal male (A & B) and intersex testes (C, D & E) of mature and maturing catfish. Showing normal testicular organization as well as intersex gonads with primary oocytes (PO) scattered through the testicular tissue (TT) [S, spermatids; i, interstitial tissue; scale bar 200µm, 10× magnification; scale bar 50µm, 100× magnification].

7.1.3.4 Urogenital papilla length index (UGPLI)

The mean (± SD) UGPI of the male fish from D2 and D1 ranged between 1.830 ± 0.5 and 2.481 ± 0.357 respectively (Table 7.4), which was not significant. The UGPLI in the intersex fish from D2 were not significantly different from the UGPLI of males in D2 and D1.

Table 7.4: Mean weight ± SD, length ± SD, UGPLI ± SD of sexed fish from both dams and the mean UGPLI ± SD of all intersex fish (SD, standard deviation).

Survey	Mean weight ± SD (g)	Mean length ± SD (cm)	Mean UGPLI ± SD (%)	Mean Intersex UGPLI ± SD
D1 (Sep 2004) low	3421 ± 1981	70 ± 13	1.830 ± 0.500	D1 = 15
D1 (Jan 2005) high	2562 ± 1092	72 ± 7	2.127 ± 0.205	1.959 ± 0.095
D1 (Sep 2005) low	4000 ± 2603	77 ± 11	2.473 ± 0.293	
D1 (Jan 2006) high	2566 ± 1020	71 ± 9	2.008 ± 0.249	
D2 (Sep 2004) low	3836 ± 1942	74 ± 13	2.113 ± 0.491	D2 n= 7
D2 (Jan 2005) high	3490 ± 1518	76 ± 8	2.481 ± 0.357	2.144 ± 0.187
D2 (Sep 2005) low	2283 ± 1291	62 ± 8	2.079 ± 0.395	
D2 (Jan 2006) high	4100 ± 1599	76 ± 12	2.126 ± 0.358	

7.1.3.5 Fish fat EDC levels

The mean fish fat levels (mean ± SD) of the different EDCs from both dams during the four surveys are indicated in table 7.5. Lindane, aldrin, *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDD, *o,p'*-DDE, *p,p'*-DDE, PCB153 and OcP and *p*-NP residues occured almost equally at the two dams at high levels.

7.1.3.6 11-Ketotestorone values in feral catfish plasma

The 11-KT values are summarized in Table 7.6. The concentrations in male fish from D1 ranged 4.62 ± 4.7 to 13.79 ± 6.71 while the values were between 2.45 ± 0.602 and 11.447 ± 2.22 in males of D2. No significant differences were found between 11-KT in males and intersex fish from both dams.

Table 7.5: The average levels (± SD) of selected target EDCs in *C. gariepinus* fat, sampled over two low- and high-flow seasons in D1 and D2.

Target EDC	D1 (Sep 2004) low	D1 (Jan 2005) high	D1 (Oct 2005) low	D1 (Jan 2006) high
Lindane (µg/kg)	145.61 ± 85.03	33.74 ± 18.11	60.64 ± 24.93	111.88 ± 55.45
Aldrin (µg/kg)	24.11	28.76		
2,4-DDE (µg/kg)	19.34 ± 7.56			
4,4-DDE (µg/kg)	548.04 ± 369.07		109.61 ± 74.42	302.80 ± 416.16
2,4-DDD (µg/kg)			17.70 ± 4.53	29.04 ± 15.02
4,4-DDD (µg/kg)	93.38 ± 43.25		20.31 ± 7.36	48.12 ± 47.37
o,p'-DDT (µg/kg)	213.61 ± 143.81		32.93 ± 18.69	35.38 ± 19.74
p,p'-DDT (µg/kg)	133.81 ± 49.02		46.64 ± 27.56	58.17 ± 38.45
PCB153 (µg/kg)	65.83 ± 35.28	14.17 ± 4.42	17.43 ± 4.88	34.56 ± 41.37
Octylphenol (µg/kg)	121.27 ± 122.16	87.48 ± 76.17		
Nonylphenol (µg/kg)	111.77 ± 80.08	218.10 ± 157.50	183.46 ± 120.13	1440.51 ± 682.87
Target EDC	**D2 (Sep 2004) low**	**D2 (Jan 2005) high**	**D2 (Oct 2005) low**	**D2 (Jan 2006) high**
Lindane (µg/kg)	48.71 ± 2.90	99.21 ± 51.69	90.91 ± 25.48	62.72 ± 58.10
Aldrin (µg/kg)		44.62 ± 11.78		
o,p'-DDE (µg/kg)	28.43 ± 0.91	26.15 ± 10.23		
p,p'-DDE (µg/kg)	355.14 ± 412.02	660.10 ± 835.52	56.34 ± 23.91	171.64 ± 233.08
o,p'-DDD (µg/kg)		13.82	41.33 ±21.69	26.76 ± 15.86
p,p'-DDD (µg/kg)	123.71 ± 9.41	160.05 ± 159.29	28.74 ± 10.63	32.16 ± 19.10
o,p'-DDT (µg/kg)	168.63 ± 25.59	101.31 ± 50.10	47.01 ± 15.91	48.64 ± 34.11
p,p'-DDT (µg/kg)	186.17 ± 40.76	135.43 ± 84.80	86.27 ± 47.50	58.04 ± 32.56
PCB153 (µg/kg)		67.78 ± 38.17	14.90	40.23 ± 30.54
Octylphenol (µg/kg)	49.52 ± 57.63	33.68 ± 25.67		
Nonylphenol (µg/kg)	98.24 ± 127.20	216.31 ± 134.47	145.24 ± 84.88	272.06 ± 237.82

Table 7.6: The mean ± SD 11-ketotestosterone (11-KT) values measured in fish from D1 and D2 over both the high-flow and l ow-flow seasons.

	11-KT (mg/ml)
D1 Sep 2004	8.59 ± 8.97
D1 Jan 2005	7.45 ± 7.42
D1 Sep 2006	13.79 ± 6.71
D1 Jan 2006	4.62 ± 4.7
D2 Sep 2004	7.463 ± 3.47
D2 Jan 2005	10.69 ± 1.78
D2 Sep 2005	2.55 ± 0.602
D2 Jan 2006	11.447 ± 2.22

7.1.4 Discussion

Fish are generally considered the most reliable organism for monitoring pollution in aquatic environments (Heath, 1995). This study confirmed the findings of Barnhoorn et al. (2004) that the external sexual papilla of the sharptooth catfish as a single parameter is not fully reliable to sex fish. On the other hand, if one relies only on histology, it may still be possible to fail to see testicular oocytes present, because of where the sample was taken from the gonad. Therefore, careful macroscopic inspection of the gonad for female feature is crucial as it may add to the sensitivity of sexing sharptooth catfish. Therefore it was concluded that a multisystem approach (MSA) will be used to sex fish in future studies. The MSA in sharptooth catfish includes the use of the urogenital papilla in association with laparotomy (macroscopic evaluation) and histology (microscopic evaluation).

Studies by Kirby et al. (2002), Brion et al. (2004) and Robinson et al. (2004) indicated that certain secondary sexual characteristics of fish such as male nuptial colouration or urogenital papilla development, are hormonally controlled. A number of these morphological characteristics are specific only to males and include breeding tubercles (occur in many cyprinid fish), fat pad (in mature male fathead minnows), and a modified anal fin (gonopodium in the mosquitofish and guppy) (Brion et al., 2004). These all develop as the male fish becomes sexually mature (Robinson et al., 2004). In medaka, the shape of the urogenital papilla is a female-specific sexual characteristic, which is probably under control of estrogens from the ovary (Brion et al., 2004). Specimens can be sexed by examining the sexually dimorphic urogenital papilla (Kirby et al., 2002). Robinson et al. (2004) found that secondary sexual characteristics of fish could be

manipulated through exposure to pollutants and therefore exposure of fish to estrogenic chemicals can lead to a disturbance (feminization) of these secondary sexual characteristics. This also applies to the sharptooth catfish as demonstrated above.

This study also confirmed the presence of intersex in sharptooth catfish (Barnhoorn et al., 2004). The histology from catfish showed typical testicular cells in males and typical ovarian cells in females (Steyn, 1984; de Graaf and Janssen, 1996), whereas the histology of intersex fish showed cells from both sexes in one individual (Barnhoorn et al., 2004). The occurrence of intersex has been reported in several fresh water and marine fish species from around the globe. In UK Rivers it has been reported in wild roach, *Rutilus rutilus*, (Jobling et al., 1998) and the gudgeon, *Gobio gobio* (Van Aerle et al., 2001), the three spined stickleback, *Gasterosteus aculeatus*, from Northeastern Germany (Gercken and Sordyl, 2002), the barbel, *Barbus plejebus*, Italy (Viganò et al., 2001) and in the shovelnose sturgeon, *Scaphirhynchus platyorynchus*, USA (Harshbarger et al., 2000).

However, the GSI data from table 7.2 showed that the mean GSI values of the intersex fish were in a much closer range to male GSI values than that of the female GSI values. This suggested that the intersex fish could have been a result from the feminization of male sharptooth catfish. The same tendency was noted by van Aerle et al. (2001) in *Gobio gobio* collected from the UK, downstream of receiving high-volume sewage treatment work effluent. Water in the UNR receives effluent from industries, agricultural activities, informal settlements and municipal treatment plants. Although the exact causation of intersex in fish worldwide is not known, most of the intersex fish have been caught in water near or receiving sewage treated effluent. Exposure to estrogens (Hunter et al., 1983) or estrogen mimicking chemicals (Gimeno et al., 1996) during sexual maturation has been shown to induce sex reversal and/or intersexuality.

Fish fat contained various concentrations of lindane, aldrin, *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDD, *o,p'*-DDE, *p,p'*-DDE, PCB153 and OcP and *p*-NP residues. In the water samples (Chapter 4) α-BHC, lindane, heptachlor epoxide, methoxychlor, *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE, *p*-NP, OcP were present, while the sediment contained *4,4'*-DDD and *p*-NP. Especially lindane, DDT and metabolites, as well as the alkylphenols seemed to have bio-accumulated in the fat tissue. PCB153 was not detected in water or sediment samples, but bio-accumulated in the fish. Therefore, bio-accumulated compounds in fat tissue might more closely reflect the history of chemical exposures of fish and pollutants demonstrated to be present in a water system that might not have been possible even with regular samplings as in this study.

Aldrin and dieldrin are insecticides with similar chemical structures and aldrin quickly breaks down to dieldrin in the body and environment. These chemicals are used on corn and cotton, but the EPA banned all uses of aldrin and dieldrin in 1974, except for termites and then for all use in 1987. They bioaccumulate in fat and decreased fertility was reported from some studies. The findings included decreased sperm count, degeneration of germ cells, decreased weights of seminal vesicles and prostate and coagulating glands, decreased seminiferous tubule diameter, decreased plasma and testicular testosterone, decreased prostatic fructose content and acid phosphatase activity, and decreased LH and FSH. *In vitro* studies using rat prostate tissue have shown that dieldrin blocks binding of the androgen dihydrotestosterone (DHT) (ASTDR, 2002). The drinking water limit of the EPA is 0.001 and 0.002mg/L.

Although all the EDCs were detected in the fish, it is important to note that these levels were not the cause of intersex in fish *per sé*. Intersex is a condition that has it onset at embryogenesis assuming the organism has been exposed to EDCs at that time. It is also important to note that most fish are gonochoristic (separate sexes), but the process of sex determination may be influenced by environmental conditions such as photoperiod, temperature, pH, nutrient and social interactions (Uguz et al., 2003; Hurley et al., 2004). These will affect the number of males and females and, therefore, skew the sex ratio in an environment. For example, increased temperature during early embryonic development in one species may increase the male population while in another species higher temperatures will favour female individuals (Baroiller and D'Cotta, 2001). Intersex on the other hand, is the development of testicular oocytes in a gonad (Gray and Metcalf, 1997; Metcalfe et al., 2001) as a result of exposure to an exogenous steroid during embryonic development of fish (Jobling and Sumpter, 1993). Xenogenous compounds include EDCs such as alkylphenols, DDT and derivatives and others (Jobling et al., 1998).

Aquatic organisms may accumulate persistent environmental toxicants through bioconcentration (via gills and skin), ingestion through intake of suspended particles and biomagnification, when consuming contaminated food. Biomagnification of persistent environmental toxicants enhances the distribution of chemicals throughout the food chain (van der Oost et al., 2003). Higher trophic organisms of the food chain, such as humans and fish eating birds, are more exposed to toxic chemicals by consuming contaminated fish. Catfish is an important source of protein in Africa either as a needed food source for the poor or a delicacy for the affluent. The detected levels of persistent environmental toxicants in catfish tissues not only indicated water pollution but may also be a warning signal of exposure and possible effects on higher trophic consumers in the food chain.

After HIV/AIDS, malaria, a mosquito-borne disease is the second leading cause of deaths in Africa (Seavy, 2002). Dichlorodiphenyltrichloroethane (DDT) has been the most successful pesticide in controlling the malaria vectors, but research has shown that DDT and its metabolites *p,p'*-1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (*p,p'*-DDE) and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDD) result in adverse effects in humans and wildlife (Turusov et al., 2002). *p,p*-DDE is a lipid soluble organochlorine contaminant and readily bioaccumulates in fatty tissue. *p,p*-DDE is an anti-androgen inducing anti-androgenic developmental effects (Kelce et al., 1997). DDT has been used as a pesticide for malaria control and for agricultural purposes during the 1950's and 60's in South Africa. In 1976 it was banned for agricultural use but remained in use as pesticide for control malaria. In 1996/7 DDT was replaced by pyrethroids to ensure malaria control but DDT spraying was reintroduced in certain malaria stricken areas due to the resistance of the vector mosquito to pyrethroids (Bouwman, 2000). Once released in the environment, DDT can persist as long as 15 years or breakdown to the main metabolites DDE and DDD. The presence of *p,p'*-DDE in fish tissue may not only be a result of agricultural use of DDT in the earlier years, but also through the atmosphere as a result of volatilization (from soil and water) and deposition (ASTDR, 2002). However, the presence of the *o,p'*-DDT is a reason for concern as it indicated recent exposure and the possible use of DDT upstream from D1.

PCBs are industrial chemicals used as stabilisers in paints and adhesives, lubricants and coolants in transformers as well as insulating materials in electrical transformers. PCBs include 209 individual congeners with different chemical configurations in commercial products (Solomon et al., 2001). The manufacturing of these hazardous congeners was terminated by the USA in the 1970's but in South Africa, PCBs are still manufactured and have numerous purposes in the steel industry and agriculture. Various concentrations of PCBs were found in a study of South African water in the Kruger National Park, Northwest Province and Gauteng (De Jager et al., 2002). PCBs were also found in fish tissue from the Isipingo estuary in KwaZulu-Natal in a study done by Grobler et al. (1996). The UNR partly receives effluents from industries in the upper catchment area and to the surprise of the authors none of the PCB congeners analysed for, were present in fish tissues. However, (Barnhoorn et al., 2004) reported PCBs in water and sediment samples collected in the Reserve at the same time. The possibility also remains that PCB levels may be below the detection limits of the analytical method.

From a confirmed list of suspected EDCs, it is clear that most laboratory studies were done on alkylphenols, which includes *p*-nonylphenol (*p*-NP) (Pait and Nelson, 2002). *p*-NP is a microbial breakdown product of nonylphenol polyethoxylates, industrial chemicals widely used in detergents, cosmetics, paints, herbicides (Ying et al., 2002). Although *p*-NP is generally considered an industrial chemical, significant levels were reported in effluents from municipal

sewage treatment plants (Sekela et al., 1999). *p*-NP is lipophilic and, therefore, bio-accumulate in fat of humans and animals and consequently have a more persistent effect than natural estrogens (Tapiero et al., 2002).

7.1.5 Conclusions

Numerous scientists used fish as a model system to evaluate pollution in the aquatic environment, such as metals and persistent organic pollutants (Heath, 1995; Kime, 1999; Heath and Claassen, 1999; van der Oost et al., 2003). One of the most important findings was that male fish are adversely affected by environmental estrogens and can serve as animal models for studying estrogenic activity (Kime, 1999). The use of the papilla as a tool to allocate the presence of intersex in fish is not sensitive enough. Histological investigations confirmed intersex in fish after the evaluation for intersex according to the papilla and macroscopic evaluation. Intersex might be as a result of EDC exposure of the fish in D1 and D2.

7.2 African clawed frog, *Xenopus laevis*

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7.2.1 Introduction

Considerable interest and controversy has recently surrounded the issue of potential endocrine disrupter effects of pollutants on development and reproduction in amphibians (Clark et al., 1999). Agricultural chemicals, particularly in habitats adjacent to application have been suggested as one of multiple causes for declines in populations of frogs that have occurred in several areas since the 1960s (Houlahan et al., 2000). In particular, concern has been raised about the widely used broadleaf herbicide, atrazine (6-chloro-4-ethylamino-6-isopropyl-amino-*s*-triazine) (Hayes et al., 2002a; Hayes et al., 2002b; Hayes et al., 2002c; Kiesecker, 2002; Reeder et al., 1998). The great majority of studies on the effect of EDCs on amphibians use the African Clawed Frog (*Xenopus laevis*) as test animal. Apart from the mouse and the chick, the clawed frog is probably the best studied laboratory animal. One of the reasons why *X. laevis* is so popular for contaminant studies is because the frog is a true aquatic frog and thus permanently exposed to potentially harmful chemicals in the water. In South Africa we have the unique opportunity to study this animal in its natural environment as this is a true African frog and it is found in most fresh water bodies in South Africa (Du Preez, 1996).

7.2.2 Materials and Methods

7.2.2.1 Test animal

African Clawed Frog, *Xenopus laevis*. Permits for collection were obtained from Gauteng Nature Conservation.

7.2.2.2 Sample sizes

The aim was to collect male frogs from each of two sites within the UNR (D1 and D2) and 20 from a control site on a game farm near Potchefstroom. When difficulties were encountered to meet the target number, additional sites were monitored.

7.2.2.3 Collecting method

Funnel traps (modified L buckets) were used for collecting *X. laevis*. Ten bated traps (Figure 7.3) were placed out at six sites at the UNR, indicated as sites A – F on Figure 7.2. Site E was not in the main water system, but a drinking hole in a small stream feeding into the main stream.

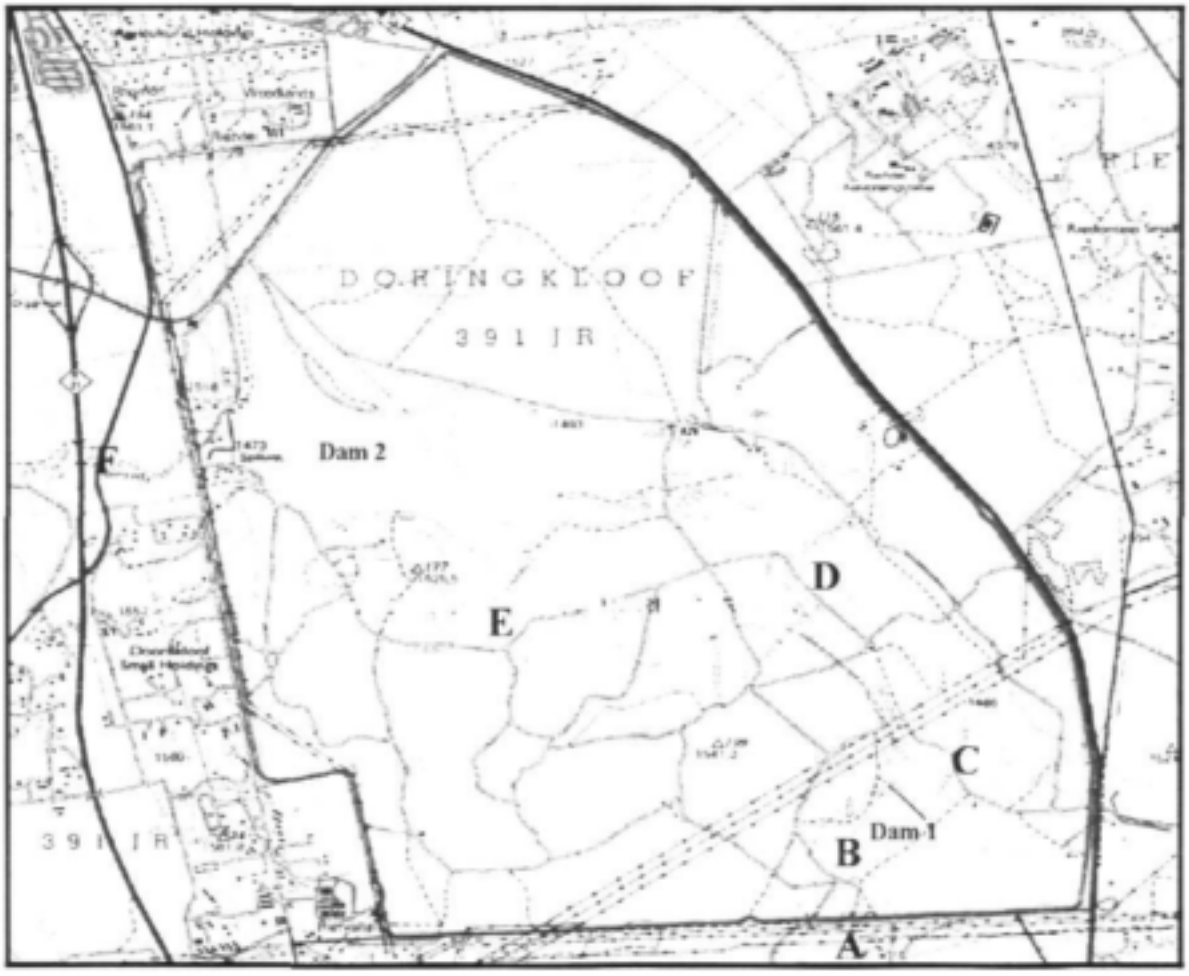


Fig. 7.2: Sampling sites in an around the UNR.

Fig. 7.3: Trap baited with ox-liver.

Traps were left for a period of 48 hours, for a total of 2400 cumulated trap hours. Collected animals were transported live to the laboratory of the African Amphibian Conservation Research Group at the North-West University in Potchefstroom.

7.2.2.4 Processing of material

Frogs were observed for external anomalies including skin lesions and limb deformities. Frogs were anaesthetised in MS222 and a blood sample taken through cardiac puncture for possible future analyses. Frogs were weighed and dissected open to expose the gonads. Gonads were examined and photographed using a Nikon Coolpix 995 digital camera mounted on a Nikon SMZ 1500 dissecting microscope. Testes were measured, weighed and fixed in Bouin's fixative for histological examination. Frog carcasses were labelled and frozen for further investigation. All specimens were processed and histologically sectioned. The preserved tissues were dehydrated in graded alcohols, embedded in paraffin-wax; serially sectioned at 6 µm, routinely stained with Meyer hematoxylin and eosin and permanently mounted. All sections (ca. 630) were examined with the aid of a Nikon E800 compound microscope.

7.2.3 Results

7.2.3.1 Frogs collected

After considerable effort during three trapping events only 7 male and 13 females were collected. Of these 20 frogs, only 9 (2M & 7F) were collected in the main stream, whereas the remaining 11 (6M & 5 F) were collected in Site E (Tables 7.7 & 7.8). In the reference site, a total of 99 frogs (41M & 58F) were collected. All but two of the *Xenopus* collected in the main stream were collected at Locality A, a well vegetated

locality with shallow water and sufficient hiding place for *Xenopus*. No *Xenopus* were collected in D1.

Table 7.7: Morphometric details of female frogs collected.

Locality	Frog ID	Frog weight
A	D2 1	45.1
	D2 2	9.0
	D2 3	16.1
	D2 6	43.9
	D2 7	7.1
B	D2 5	6.8
	D2 13	62.2
E	D2 10	47.6
	D2 11	65.3
	D2 15	21.4
	D2 16	21.4
	D2 17	44.7
	D2 19	25.6

Table 7.8: Morphometric details of male frogs collected.

Locality	Frog ID	Weight	Testis Length	Testis Width	Testis Weight	GSI
A	D2 4	16.2	7.5	2.7	0.020	0.21
			6.5	2.2	0.014	
	D2 8	8.9	6.3	1.3	0.002	0.045
			6.0	1.3	0.002	
E	D2 9	66.0	8.2	3.3	0.032	0.080
			8.4	3.2	0.021	
	D2 12	58.6	10.5	4.4	0.088	0.241
			10.8	5.7	0.053	
	D2 14	26.9	8.1	3.7	0.036	0.260
			8.0	2.9	0.034	
	D2 18	11.63	5.9	1.8	0.006	0.103
			5.8	1.8	0.006	
	D2 20	42.1	8.5	4.0	0.039	0.152
			8.8	3.9	0.025	

7.2.3.2 Male-Female ratios and Gonado-Somatic Index (GSI)

In the Main Stream, only 20% of the frogs collected were male, whereas 55% of the frogs collected at Site E were male. In the Reference Site, 42% of the frogs collected were male (Figure 7.4). The male GSI varied from 0.13 for both the Main Stream and the Reference Site while the GSI for site E was slightly higher at 0.17 (Figure 7.5).

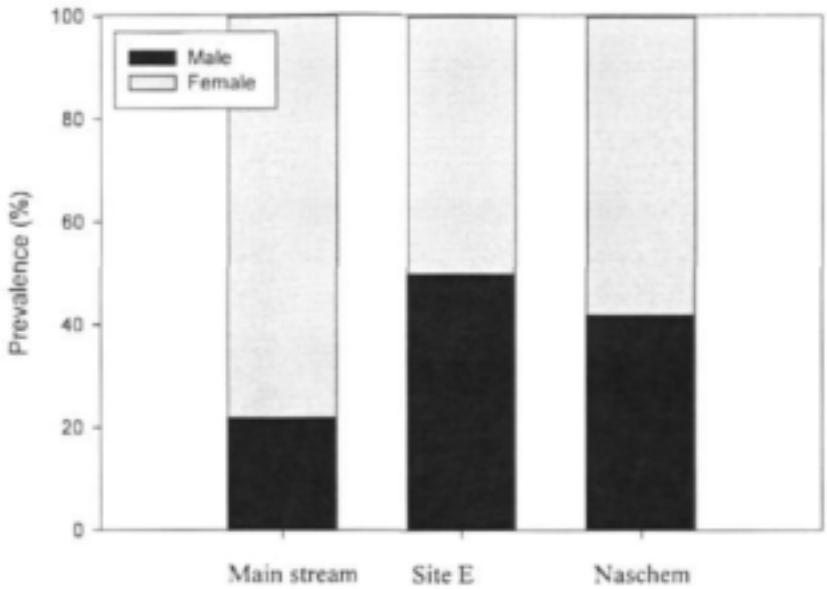


Fig. 7.4: Male-Female ratio as observed in the Main Stream, Site E and the Reference site.

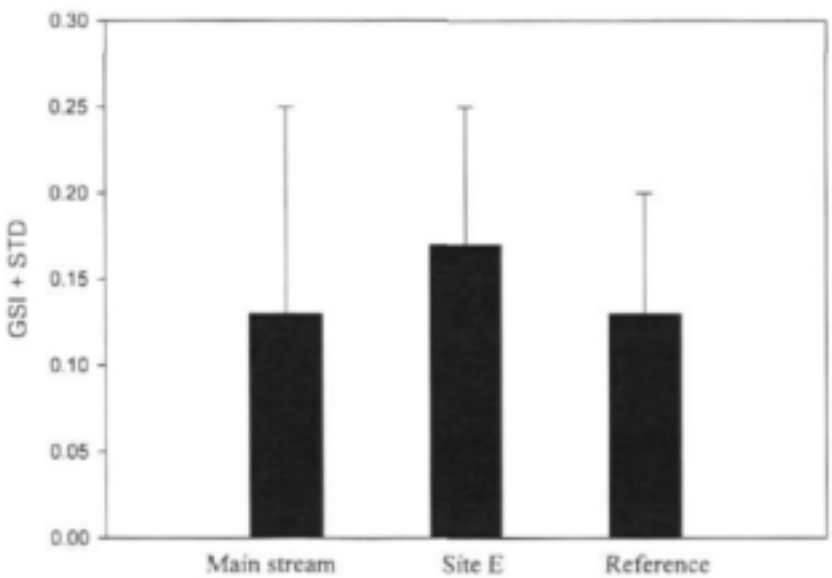


Fig. 7.5: Male gonado-somatic index as observed at the three sites.

7.2.3.3 General condition and gonadal anomalies

In general, all the frogs collected were in good condition. No deformities such as extra or missing limbs were observed. Fat bodies were swollen and bright yellow (Figure 7.6) indicating a healthy condition. No gross gonadal anomalies were observed in any of the male or female frogs from the UNR, but 3.5% of the frogs from the reference site had deformities in the form of discontinued or uneven size testes. The state of development of ovaries varied from resting ovaries to ovaries charged with fully developed oocytes (Figure 7.6).

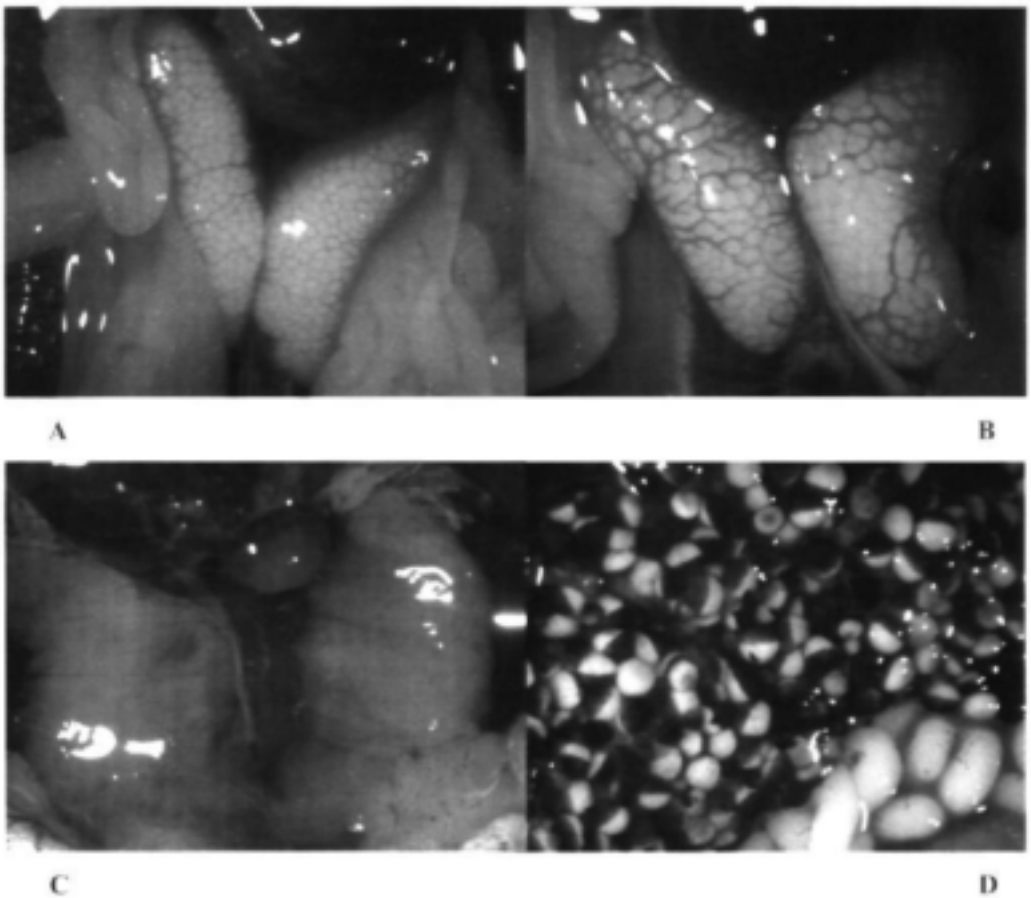
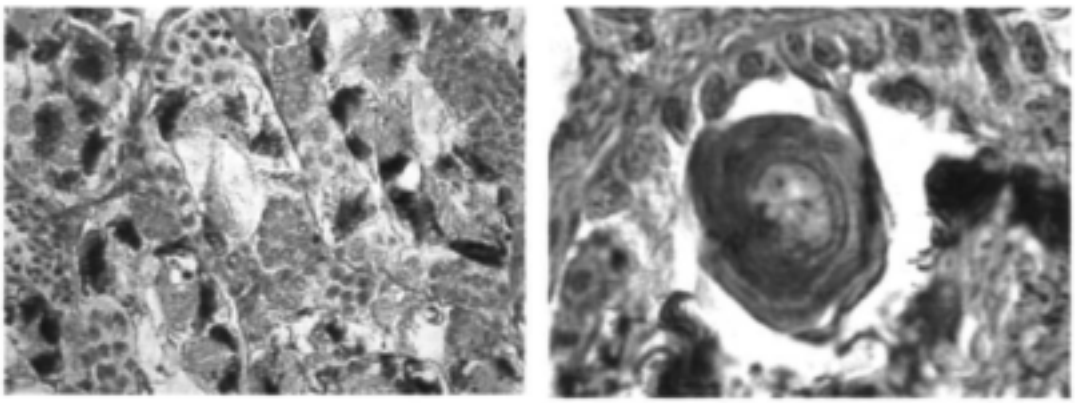


Fig. 7.6: Well developed testes (A, B), resting ovary (C) and a mature ovary (D).

At histological level we did observe regressed testicular oocytes (Figure 7.7) in two of the males (40% at that site) collected at Site E. One animal had a single oocyte while the second had 18. None of the animals from the Main Stream had any testicular oocytes. At the reference site, 13% of the animals had testicular oocytes.



A B

Fig. 7.7: Micrographs of a section through testis tissue (A) and a regressed testicular oocyte (B).

7.2.4 Discussion

All the frogs collected were generally in a good condition. The difficulty experienced collecting *Xenopus* in the main stream at the UNR is most likely due to the presence of the predatory catfish that are, according to the reserve management and anglers, quite common in the system. Although the frogs appeared to do fine in the UNR system with no gross morphological or testicular deformities observed, the skewed sex ratio observed in the main stream is of concern. Although the sample size is too small to come to a conclusive answer, the ratio of 4 females for each male is abnormal and of concern. The normal picture for *Xenopus* is a 50-50 ratio $\pm$ 10% (Du Preez et al., 2005). It is known that pollutants in water can cause demasculinization or complete sex reversal in frogs (Clark et al., 1999) and is a matter of concern. Testicular oocytes at a prevalence of around 6% have been reported for *X. laevis* at impacted and reference sites in the central parts of South Africa, while no oocytes were reported from any sites in the Western Cape. A possibility could be that there are genetic differences between *X. laevis* from the two regions but this remains to be tested. All *Xenopus* strains used outside South Africa in Europe and the USA originated in the Western Cape and this may explain why no testicular oocytes occur in these frogs.

Clawed frogs are widely used as model to test the effect of pesticides on amphibians under controlled laboratory conditions. This species have been shown to serve as bio-indicator under natural conditions in South Africa (Du Preez, et al., 2005). The fact that it is primarily water living and has a very permeable skin, ensures that they get full exposure to potentially harmful chemicals in the aquatic environment. The present study highlighted the fact that catfish is a ferocious predator of African Clawed Frogs and this should be taken into account when designing or repeating a similar study.

7.3 Freshwater snail *(bulinus tropicus)*

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7.3.1 Introduction

An *in loco* investigation of the freshwater snail species diversity was conducted in the spring of 2004 in D1, as well as in D2, located in the UNR. The North-West University hosts the National Snail Collection. From historical data, we have made a summary of the snails that have previously been collected at the UNR before.

1963

Bulinus tropicus, B. depressus, Lymnaea natalensis, L. collumella, Physa acuta, Cerotaphallus natalensis, Gyraulus connollyi, Pysidium sp.

1994

Burnupia sp., *Unio caffer*

1995

B. tropicus, P. acuta

From this data it was obvious that the species diversity had declined drastically since 1996. Dam 2, for some reason, never seems to have contained many snails. From this it became apparent that, even though some specimens of *Physa acuta* (known as the sewage snail) and *Ceratophallus natalensis* were found, only one species, *Bulinus tropicus*, was found in D2 in sufficient numbers to justify further investigation. In D1, some specimens of *C. natalensis* and *B. tropicus* were found, while some specimens of *Burnupia transvaalensis* were found in the connecting stream.

7.3.2 Materials and Methods

7.3.2.1 Snail collection

Approximately 200 specimens of *B. tropicus* were sampled with the help of scoop-nets in D2, and were transported in habitat water to the laboratory.

7.3.2.2 Narcotization

The snails were subsequently narcotised for 24 hours in a water solution of chloral hydrate and menthol according to the Van Eeden method. During this period the snails were left

undisturbed in order for the soft tissue to relax completely, so that it can protrude from the shell. The narcotic is then decanted and the snails are fixed.

7.3.2.3 Fixing

Fixing is accomplished by pouring pre-heated (60°C) 4% hexamine-buffered formaldehyde over the snails. The snails were left in this solution for 24 hours, after which they were preserved.

7.3.2.4 Preserving

The snails were then finally preserved in 70% ethanol with a final concentration of 5% glycerol, and then stored for dissection. This method of narcotisation, fixing and preserving was followed because it is the standard working method for snail specimens that has been applied since the beginning of the National Snail Collection, and still enjoys international recognition. This method also allowed comparable measurements of the snails exposed to the conditions in D2, as well as the measurements of the living control snails, to the material obtained from the National Snail Collection.

7.3.2.5 Dissecting

The soft tissue of 150 D2 snails was carefully removed from the shells with a watchmaker's forceps under a stereo microscope provided with cold lighting and dissected under a magnification of 80X, in a Petri dish filled with the standard preservative, to remove the penis complex, consisting of the penis, preputium, penis sheath and vas deferens (Figure 7.8).

7.2.3.6 Measurement

The dissected complex of each of the snails was then removed and pinned with micro-pins, in a Petri dish, provided with a cork base. The preputium- and penis sheath lengths were separately measured in millimetres by means of a stereomicroscope provided with a calibrated eyepiece. The data was statistically analysed, with the assistance of the Statistical Consultation Services at NWU. This analysis should be seen as preliminary.

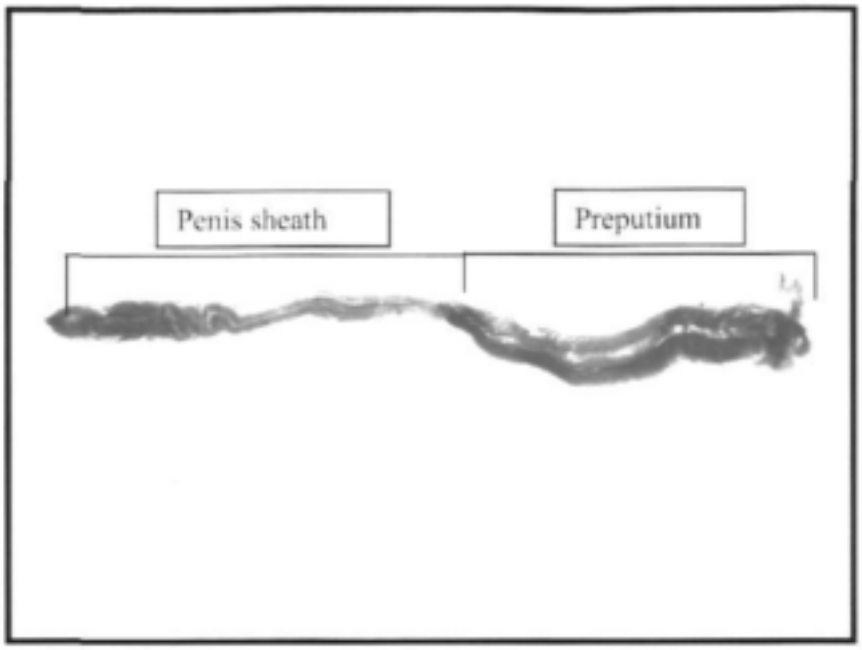

Fig. 7.8: Dissection of the penis complex, consisting of the penis, preputium, penis sheath and vas deferens. The sheath contains the penis.

7.3.3 Results

As seen in Table 7.9 the mean penis sheath length of snails collected at the UNR was 6.13mm and that of the preputium was 3.75mm, with a mean ratio of 1.68. In six snails no observable penises were found.

Table 7.9: The mean penis sheath- (A) and preputium lengths (B) (mm) of the iving snails collected in Dam 2 as well as the ratio of A:B.

Number of Snails	**n = 150**	**n = 150**	
	Sheath (A)	**Preputium (B)**	**Ratio**
Mean	6.13	3.75	1.684
SD	2.0	1.3	0.404

7.3.3.1 Living control snails

7.3.3.1a Snail collection

After several fruitless attempts to find specimens of *B. tropicus* in the so-called undisturbed habitats bordering the Suikerbosrand Nature Reserve and the Vredefort Dome, specimens of this species was found in a natural habitat close to Potchefstroom. The absence of snails in the so-called undisturbed habitats was expected because this species occurs mostly in habitats that are organically enriched. The habitat close to Potchefstroom where the snails were found is also accessible to farm animals and water birds and animal

excreta were found that could lead to organic enriching in the habitat and bordering areas. Sixty specimens that were large enough to be dissected and were sexually mature were collected and brought to the laboratory. Preparation of the snails for dissection as well as the measurements of the relevant structures was conducted as discussed. In this instance 48 of the snails were dissected as discussed above, while the remaining snails were allowed to lay eggs. The results are given in Table 7.10. The mean penis sheath length of the control snails was 8.31mm, and the preputium was 4.61mm, while the ratio between A:B was 1.84.

Table 7.10: The mean penis sheath- (A) and Preputium lengths (B) (mm) of the living control snails as well as the ratio of A:B.

Number of Snails	**n = 46**	**n = 64**	
	Sheath (A)	**Preputium (B)**	**Ratio**
Mean	8.31	4.61	1.84
SD	1.40	1.16	0.32

7.1.3.1b Control specimens from the National Snail Collection

Pre-mounted penis complexes of 608 *B. tropicus* specimens were extracted from the Snail Research Collection for additional comparison and measurement. Care was taken so that the slides represent snails from habitats as far as possible removed from industrial areas. They were mainly from isolated farm dams with little or no external inflow from larger rivers and streams. Water that was impounded here was primarily rainwater or water from boreholes. The lengths of the relevant structures were determined in the same manner as for the wet specimens.

The results are indicated in Table 7.11. The mean penis sheath length of the snails from the National Snail Collection was 5.26mm and that of the preputium was 2.89mm, while the ratio A:B was 1.84, the same ratio as in Table 7.18 for the live-caught control specimens.

Table 7.11: The mean penis sheath- (A) and preputium lengths (B) (mm) of the control snails from the National Snail Collection, as well as the ratio of A:B.

Number of Snails	**n = 608**	**n = 608**	
	Sheath (A)	**Preputium (B)**	**Ratio**
Mean	5.25	2.89	1.84
SD	1.13	0.89	0.34

7.3.3.2 Statistical comparisons

The statistical evaluations of comparisons made between the A:B ratios of the different groups are given in Table 7.12. From this it is clear that the ratios of the penis sheath length (A) to the preputium length (B) of D2 snails differed significantly from both the living control snails from Potchefstroom (P= 0.0119), as well as the samples from the National Snail Collection (P<0.0001). No significant difference could be indicated between the two snail control groups (P= 0.9522). Even though the mean lengths of the measured structures of the three groups also differ, this could be ascribed to the mean size of the snails. The 608 specimens selected from the National Snail collection mainly came from the Free State and the mounted structures, in general, were smaller than the Potchefstroom wet specimens and those from D2. It was however clear however, that the ratio between the two structures of D2 snails was significantly smaller than those of the control groups. Whether this phenomenon affects the reproductive ability of this species is not certain, but in view of the large number of snails present in D2 during the survey, as well as the relatively high *in vitro* hatching percentage of the eggs of these snails (see later), it would appear that the influence, if any, on the snails' reproductive ability may be limited, but requires further investigation.

Table 7.12: Statistical comparisons between D2 snails, the living control specimens and the control specimens from the National Snail collection.

Dam 2 / Control (Potchefstroom)	
P	0.0119
T	*2.518108*
Degrees of Freedom	194
Dam 2 / NSC Slides	
P	<0.0001
T	4.570881
Degrees of Freedom	756
NSC Slides / Control (Potch)	
P	0.9522
T	0.060689
Degrees of Freedom	652

7.3.3.3 Egg hatching studies

7.3.3.3a In vitro studies

For this investigation the remaining 50 snails collected from Dam 2 were kept in an aquarium. The egg clutches that were deposited daily were removed from the holding

tanks, and each egg clutch was transferred to a separate cell of a multi-cell container that was kept in a waterbath at 25°C. A total of 101 egg clutches were collected over a period of 14 days. The development of embryos was microscopically monitored on a daily basis by counting the number of eggs per egg clutch, and recording the number of eggs with developing embryos. Even though research information indicates that the development of eggs of this species occurs completely within 7 days at a temperature of 25°C, each egg clutch was still monitored for 21 days after which the number of hatchlings was recorded and the hatching percentage calculated.

These results are presented in Table 7.13. As can be seen from Table 7.13, the mean hatching percentage was 88.4%. This percentage would in all likelihood have been higher, had not been necessary to mechanically remove the egg clutches from the captivity tanks (according to experience).

Table 7.13: The *in vitro* hatching percentage of Dam 2 eggs.

% Hatching of eggs of D2 snails *in vitro*

	Eggs	**Embryos**
Mean	14 6	13.4
SD	4.76	5.16
Percentage hatched embryos	88.4	

7.3.3.3b In vivo studies (active biomonitoring)

For this investigation a number of egg clutches from the control snails that were found close to Potchefstroom and kept in the aquarium, were collected and the number of eggs per egg clutch determined. Nine snail containers were specially prepared for D2, the stream that connects the two dams (stream) and D1. Three containers containing 77, 82, and 90 eggs respectively were suspended in an inconspicuous place in the shade at D2, three more containers with 91, 97 and 94 eggs respectively were suspended in the stream, while the remaining 3 containers with 93, 98 and 95 eggs respectively were suspended in D1. The containers were all covered with 80% shade netting and attached to floats. Each float was in turn attached to an iron pole that was anchored to the bottom of the habitat. Care was taken to allow two thirds of the snail containers to be submerged at all times, irrespective of the water level, and therefore the developing embryos were at all times submerged in water. The containers were removed after three weeks and the number of snails that have hatched in each container was determined. The results are given in Table 7.14. From this it is clear that 31%, 37% and 76% of the eggs hatched in D2, stream and

D1, respectively. This is in stark contrast with the *in vitro* hatching percentage of D2 that was 88%. The fact that the hatching percentage of the snails from D2 was significantly lower could be ascribed to the limited flow of water through the mesh of the snail containers due to the mesh-clogging effect of the high algae population in D2. This flourishing of algae could probably be ascribed to organic pollution present in this dam, but the real cause is not known.

The presence of organic enrichment is supported by the presence of significant numbers of the so-called sewage snail, *P. acuta* in this dam. The low hatching percentage in the stream could probably also be ascribed to the fact that the anchored float came adrift due to unknown circumstances (animal activity or human tampering) and during the collection thereof was found at a place in the stream where it was exposed to direct sunlight.

Table 7.14: The mean number of eggs and snails that hatched per habitat, as well as the hatching percentage per habitat.

% Hatching of eggs of the exposed snails *in vivo*

	Mean Eggs	**Mean snails hatched**
Dam 2	83	25
Stream	88	34.5
Dam 1	95.33	72
Percentage hatched embryos		
Dam 2	31.43	
Stream	37.31	
Dam 1	76	

7.3.4 Conclusion

The finding that the sheath:preputium ratio of snails from D2 was significantly different when compared with similar measurements from both live-caught and preserved specimens, must be seen as a strong indication of an impact on the male reproductive system, although the causality in this case has yet to be established. As a first approximation, these results can be compared with Tillmann et al. (2001), who also found similar effects on male organ parameters, in *Marisa cornuarites*, a freshwater gastropod, after exposure to antiandrogens. As far as we are aware of, this is the first indication of such an effect in hermaphroditic freshwater snails. As such, this relatively easy parameter should be further investigated, as a number of possibilities are now open for further investigation. The absence of penises in six specimens from D2 is a very rare

phenomenon in *B. tropicus* (a characteristic that is common in *B. depressus*) (Brown, 1994) and should be further investigated.

The active biomonitoring introduced a number of variables that probably stressed the snails in addition to the presumed chemical stressors, and this data should be treated with caution. It will take additional work to refine this method for this species. The penis sheath:preputium ratio, at this stage, seems as the best parameter to develop further.

7.4 Small mammals

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7.4.1 Introduction

Although several studies have been published on the effects of potentially and known endocrine disruptors in laboratory settings (Lintelmann et al., 2003), the number of studies that have investigated these effects in actual field situations have been quite small. Most of the field studies on free-living mammals involved larger Arctic mammals, such as polar bears, whales and seals (Lintelmann et al., 2003). Perhaps not surprisingly, most field studies on the possible disruption of the endocrine system of free-living vertebrates have focused on frogs and fish, where indeed a number of effects, mostly with weak causative linkage to chemicals, have been found (Vos et al., 2000). Quite number of studies though, has been published on the levels of pollutants found in small mammals (Harrison et al., 1997), but most have not investigated effects relevant to this project.

7.4.2 Materials and Methods

7.4.2.1 Sample collection

To determine the reproductive health of the small mammals in the Urban Nature Reserve (UNR) specimens were caught at Dam 2 (D2) by scientists from the University of North West (Potchefstroom), using standard research mouse traps (Figure 7.9). Two species were sampled, ten Striped Mice (*Rhabdomys pumilio*), and eight Musk Shrews (*Crocidura mariquensis*). A second sampling took place in November 2005. These specimens were caught in mid-May of 2005. Two species were then sampled, seventeen striped mice and three Vlei rats (*Otomys irroratus*). The specimens were taken to the Andrology laboratory (Department of Urology) at the University of Pretoria for sample taking and analyses.

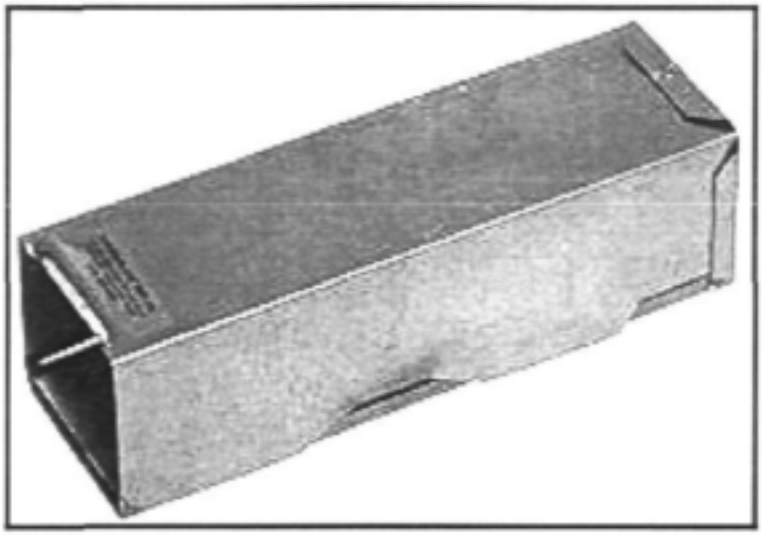

Fig. 7.9: A typical mouse trap used to capture the specimens.

The mice and shrews were anaesthetizing, using Halothane. The ano-genital distance was measured in all animals. Once the specimens were teminated their body mass and reproductive organs were removed and weighed. The mass of the left testis and left epididymis, and right testis and right epididymis, the liver and the body mass of each specimen were recorded. The prostate and seminal vesicles were weighed where possible. Histological preparations of the testes were done at the Department of Anatomical Pathology (UP) at Onderstepoort.

7.4.2.2 Testis Histology (STAGES)

A histological evaluation of the testis, including measurements of the seminiferous tubules and identification of the different stages of spermatogenesis, were used as a tool to identify any EDC effects on the rodent testes. The fixation time for the testicular samples in the Bouin's fixative was 3 to 5 days. After this period, the Bouin's was washed from the samples with 70% ethanol.

Fixed cross-sections of the testes were then embedded in paraffin wax and the testicular tissue was then dehydrated in a graded series of ethanol. Thin sections, 3 µm, were cut on a microtome and stained with a modified periodic acid-Schiff's reaction (PAS) and counterstained with Alum hematoxylin (H&E) (Brady and Schoonhoven, 1985).

These histological slides were used to do staging of spermatogenesis using a Nikon Optiphot photomicroscope with 10x, 40x and 100x objectives. A computer software program on spermatogenesis, STAGES^{TM} 2.1 (Vanguard Media Inc., Illinois, USA), were used in the

staging process together with a histological atlas by Russel et al. (1990). Slides were analyzed for any presence of active spermatogenesis and as many stages as possible were identified.

For each individual rodent, thirty randomly selected seminiferous tubules were staged to identify and classify all 12 stages of spermatogenesis for the mice and 14 stages of spermatogenesis for rats. The tubular diameter, seminiferous epithelium and lumen diameter for all thirty tubules were then measured horizontally and vertically. The mean values of the horizontal and vertical measurements for each parameter were used for statistical analyses of the tubular diameter, seminiferous epithelium and lumen diameter.

There were various forms of degeneration that were looked for within the testis. These included apical sloughing, degeneration of spermatogonia and spermatocytes, vacuolization, and seminiferous tubule shrinkage. All specimens were analyzed and the presence of these characteristics determined for the different species.

7.4.2.3 Cauda epididymal sperm count

As a decreased sperm count and semen quality is associated with EDC exposure, it was included in the study to get baseline data on the semen quality of the rodents in the UNR. The following procedures were followed:

The left cauda epididymis was used to determine the sperm concentration. The cauda epididymis was separated from the caput-corpus and placed in 2 ml of phosphate buffered saline (PBS) medium (cat. no. BR14a, Oxiod, Hampshire, England) in a Petri dish. The cauda epididymis was then cut up into very small pieces to free the sperm. The Neubauer method (WHO, 1999) was used to determine the sperm concentration, expressed as million/mL.

7.4.2.4 Computer-Assisted Sperm Analysis (CASA)

Sperm motility was additionally evaluated with a semi-mobile Hamilton-Thorn sperm motion analyzer. CASA is being used increasingly in reproductive toxicology, as some CASA parameters are sensitive to reproductive toxins (ESHRE Andrology Special Interest Group, 1998). The percentage of motile sperm, progressive motility, linear velocity and curvilinear velocity were all analyzed in duplicate. For each suitable sample, eight fields and a minimum of 150 sperm were selected and automatically analyzed (Schrader et al., 1992).

Only recently there has been a positive and concerted action to define the role of CASA instruments in both the clinical andrology laboratory and in the research laboratory (Mortimer et al., 1995). CASA is potentially a very powerful research and clinical tool. Indeed, its

inclusion in reproductive toxicology studies indicates its relevance (Slott et al., 1993, 1995, 1997), but its features and limitations must be understood quite clearly before attempting to make clinical diagnoses on the basis of its results (ESHRE Andrology Special Interest Group, 1998).

The Hamilton-Thorn Research third generation system named IVOS (Integrated Visual Optical System) was used. This system is capable of analyzing up to 60 frames/second with internal data storage on hard disk. Measurements of velocity, linearity, mean and maximum amplitude of the lateral head displacement and cross-beat frequency were evaluated utilizing a Leja (20μm) chamber.

CASA systems cannot provide an adequate estimate of curvilinear velocity (VCL), straight-line velocity (VSL), amplitude of later head displacement ALH), or linearity (LIN) in suspensions with sperm concentrations over 40 million/mL. More concentrated semen samples were diluted to a concentration of less than 40 million/mL with iso-osmotic buffer. Five frames were analyzed to have a consistent degree of accuracy. In diluted semen samples an analysis rate of 30 frames/second is acceptable. Kinematics, "time-varying geometric aspects of motion" (Drobnis et al., 1988) are used to differentiate sperm movement patterns. The kinematic values determined for each spermatozoon cover the velocity of movement, the width of the sperm head's trajectory and frequency of the change in direction of the sperm head (David et al., 1981; Serres et al., 1984).

The velocity values that were determined are the curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP). The VCL refers to the total distance that the sperm head covers in the observation period and is always the highest of the 3 velocity values. The VSL is determined from the straight-line distance between the first and the last points of the trajectory and gives the net space gain in the observation period. This is always the lowest of the 3 velocity values for any spermatozoon. The VAP is the distance the spermatozoon has travelled in the average direction of movement in the observation period. This is conceptually the most difficult velocity value to understand because it might appear that it should be similar to the VSL. The amplitude of lateral head displacement (ALH) is the width of the lateral movement of the sperm head. It is calculated as the total width of the head trajectory. The beat-cross frequency (BCF) is the number of times the sperm head crosses the direction of movement and is expressed in hertz and is calculated by counting the number of times the curvilinear path crosses the average path per second. BCF is of value in the estimation of gross changes in the flagellar beat pattern (Mortimer, 2000).

7.4.3 Results

7.4.3.1 First collection

During the rodent sampling of May 2005 two species were sampled, ten Striped Mice (*Rhabdomys pumilio*), and eight Swamp Musk Shrews (*Crocidura mariquensis*). From the testes size it was obvious that the rodents were not sexually active and hardly any sperm were found. The prostate and seminal vesicles were too small to remove and no sperm count or CASA analyses could be performed. Histological evaluations were done, but are of no real scientific value as the spermatogenesis was found to be dormant.

7.4.3.2 Second collection

During the second rodent sampling in November 2005 two species were sampled, seventeen Striped Mouse (*Rhabdomys pumilio*), and three vlei rat (*Otomys irroratus*). The mice were all sexually mature and all the analyses could be performed (Table 7.15).

Table 7.15: Summary of the data for the second sampling of the striped mouse (*Rhabdomys pumilio*).

Parameter	N	Mean	95% CI (+/-)	SE	SD
Weight (g)	17	35.84	2.10	0.99	4.08
Ano-genital distance (mm)	17	14.71	0.83	0.39	1.61
Testes (g)	34	0.32	0.11	0.05	0.30
Epididymis (g)	34	0.10	0.03	0.01	0.08
Seminal Vesicles (g)	34	0.06	0.04	0.02	0.12
Prostate (g)	17	0.03	0.01	0.00	0.02
Liver (g)	17	1.63	0.24	0.11	0.46
Sperm concentration (10^6/ml)	17	22.1	15.54	7.33	30.23
Histology					
Seminiferous tubule	680	178.94	2.83	1.44	37.57
Epithelium	680	60.59	1.15	0.59	15.26
Lumen	680	58.75	1.49	0.76	19.79
CASA					
VAP	91	97.52	12.49	6.29	59.96
VSL	91	75.16	10.23	5.15	49.12
VCL	91	169.04	22.57	11.36	108.35
ALH	91	7.07	0.80	0.40	3.86
BCF	91	24.22	1.25	0.63	5.98
STR	91	71.27	2.84	1.43	13.63
LIN	91	46.08	3.10	1.56	14.89
Motility (%)	91	11.19	3.38	1.70	16.24

The mean cauda epididymal sperm count for the mice was 22.1 x 10^6/ml with two animals without any sperm. Sperm motion parameters were successfully analyzed with the CASA system and will form part of the database for comparison. The histology of the testes of both these animals (M5 & M11) confirmed that spermatogenesis was not complete. Both animals also had spots on the liver. Figure 7.10 and 7.11 are representative of the abnormal histology of these mice.

Figure 7.12 is an example of normal spermatogenesis with all 12 stages in the Striped Mouse. A database is available with all the measurements and photos for the individual animals.

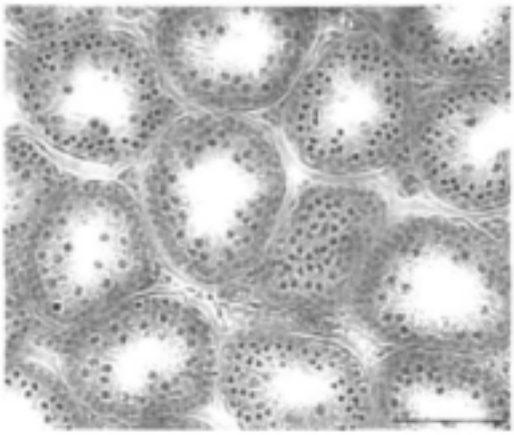

Fig. 7.10: Seminiferous tubules with no active active spermatogenesis in M11.

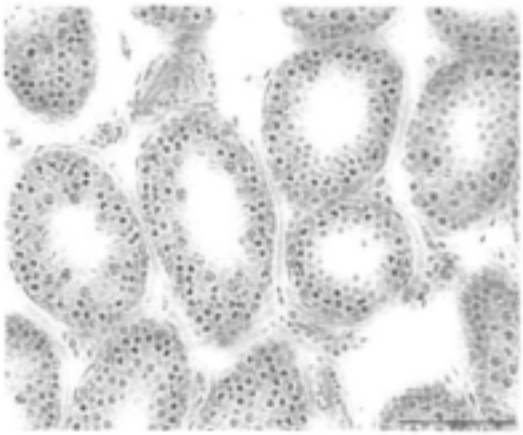

Fig. 7.11: Seminiferous tubule with no spermatogenesis in M5.

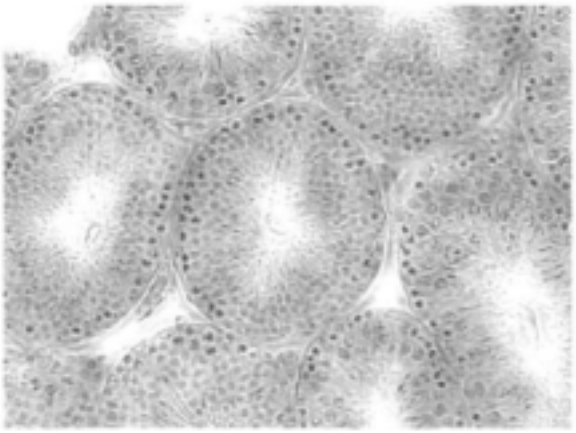

Fig. 7.12: Seminiferous tubules with normal spermatogenesis in the Striped Mouse.

During the week of capturing only three vlei rats were captured. The data are summarized in Table 7.16. Sperm counts were done and sperm motility was analyzed successfully.

The rats had normal spermatogenesis. A database is available with all the measurements and photos for the individual animals.

7.4.4 Discussion

The aim of this study was to identify suitable sentinel species, build up a database for small mammals and to look for any reproductive abnormalities that might be as result of EDC exposure. The species that were captured was the Striped Mouse (*Rhabdomys pumilio*), the Swamp Musk Shrew (*Crocidura mariquensis*) and the vlei rat (*Otomys irroratus*). The shrews were very small and difficult to work with. Although the vlei rats were much bigger and easy to work with, very few were caught. The striped mouse can be regarded as the species of choice due to their size, distribution and the results obtained.

The time of sampling was found to be very important, as was seen from the results (Addendum). It is suggested that sampling of small mammals takes place during summer when the animals are sexually active. Histological parameters can give clear results as far as the different stages of spermatogenesis are concerned. Exposure to EDCs and toxic effects can be seen in cases where the Sertoli cells are affected. The histological slides can also be used to confirm abnormalities of the testes, as was seen in some of the striped mice.

The histopathology was an important part of the project. Clear evidence of toxin damage was observed. The various forms of degeneration looked for in the histopathological analysis were apical sloughing, degeneration of spermatogonia, vacuolization and seminiferous tubule shrinkage. These four forms of degeneration are typical symptoms of exposure to chemicals such as *p*-NP, PCBs and other EDCs. Apical sloughing occurs as a result of the Sertoli cells damage. These Sertoli cells then release the germ cells that they are nurturing into the lumen. Apical sloughing was quite common in most of the specimens and would lead one to believe that exposure levels to these chemicals might be relatively high. Interestingly apical sloughing was absent in the one shrew with active spermatogenesis. Another clear sign of exposure to estrogenic compounds is the degeneration of spermatogonia and spermatocytes present in some specimens. Yet again though, the degeneration of spermatogonia was not as prevalent in the specimens with active spermatogenesis.

Table 7.16: A summary of the data for the second sampling of the vlei rats (*Otomys irroratus*).

Parameter	N	Mean	95% CI (+/-)	SE	SD
Weight (g)	3	130.91	78.06	18.14	31.42
Ano-genital distance (mm)	3	24.67	7.17	1.67	2.89
Testes (g)	6	0.77	0.55	0.21	0.52
Epididymis (g)	6	0.19	0.15	0.06	0.14
Seminal Vesicles (g)	6	0.19	0.21	0.08	0.20
Prostate (g)	3	0.16	0.23	0.05	0.09
Liver (g)	3	4.40	0.47	0.11	0.19
Sperm concentration (10^5/ml)	3	62.9	106.94	24.85	43.05
Histology					
Seminiferous tubule	120	222.11	8.02	4.05	44.39
Epithelium	120	74.33	2.88	1.45	15.91
Lumen	120	73.56	5.57	2.81	30.83
CASA					
VAP	18	271.61	27.31	12.94	54.92
VSL	18	183.09	18.50	8.77	37.20
VCL	18	516.14	45.23	21.44	90.95
ALH	18	18.38	1.22	0.58	2.46
BCF	18	21.59	2.58	1.22	5.20
STR	18	64.22	1.83	0.87	3.69
LIN	18	36.67	2.29	1.08	4.60
Motility (%)	18	7.12	2.80	1.33	5.63

The third form of toxic damage is vacuolization. Some of the mice showed clear signs of vacuolization. This phenomenon was also more common in the mice than the other species. However, in general this phenomenon was not as common as the other three forms of degeneration. Seminiferous tubule shrinkage was very common in the mice but not nearly as common in the shrews or the rats. This of course could be due to the different habitats that the two different species inhabit or due to their different diets.

Through the analysis of the spermatogenesis and the histopathology it is clear that the mice were more affected than the other species. Whether this is due to the shrews being more tolerant of the EDCs, or whether the mice are in fact exposed to higher levels of these chemicals, is not clear. Further research investigation the amount of these chemicals accumulated in the lipids of these

animals could clarify this. One could also take samples from the respective environments of these two species to ascertain as to the levels of exposure in these different habitats.

Although this is a descriptive study, the striped mouse (*Rhabdomys pumilio)* was found to be the best species to use for studying EDC exposure. Typical signs of EDC exposure were observed on histological level and some animals were severally affected. Further research is needed to do target exposure analyses on individual animals to confirm the results.

CHAPTER 8
ACTIVE BIOMONITORING

8.1 Sharptooth catfish, snails and midgets

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8.1.1 Introduction

Since pollutant concentrations in aquatic systems are highly variable and often below detection limit, the permanent control of water quality is indispensable. There is an increasing trend to use the behaviour of pollutants (e.g. bioavailability and bioaccumulation) as well as pollution-induced biological effects on aquatic organisms to evaluate or predict the impact of complex chemicals on aquatic ecosystems (van der Oost et al., 2003). To reveal the presence of pollutants over time and to measure their toxic effect, the use of selected organisms as biomonitors or bioindicators can play a prominent role in the monitoring (i.e. active biomonitoring (ABM)) of aquatic ecosystems (Salánki et al., 2003). The ABM approach consists of the deliberate exposure of organisms to (polluted) environmental conditions within aquatic environments. Organisms are transplanted from a "clean", unperturbed site and are caged at various sites of the study (monitoring) area (Baumard et al., 1998; Wepener et al., 2005).

The need to detect and assess the effects of pollutants, such as heavy metals and EDCs at low concentrations and in complex mixtures, has led to the development of a wide range of biological markers (biomarkers) of exposure and effect. Assessing the energetics of organisms provide an early warning system for acute events, because the toxic response is usually instantaneous and/or sensitive to low pollutant concentrations. Monitoring changes in the major components of the energy budget of an organism have the potential to be used as a toxicological tool, as a positive net energy balance must be maintained for growth, reproduction and fitness (Verslycke et al., 2003; Smolders et al., 2004). The use of cellular energy allocation (CEA) methodology as a biomarker of exposure, provides an integrated quantification of an organism's net energy budget that can link (sub)cellular effects to more ecological relevant individual responses (De Coen and Janssen, 2003).

This study evaluated the use of ABM and the biomarker methodology as a measure of the water quality status of the Urban Nature Reserve. The diptera midge (*Chironomus* sp.), prosobranch mollusc, *Melanoides tuberculata* and the sharptooth catfish, *Clarias gariepinus* were selected as test organisms to compare interspecies differences in chemical (i.e. metal and EDCs) bioaccumulation and biomarker response, following the monitoring study.

8.1.2 Materials and Methods

8.1.2.1 Study area

The midge larvae (± 20mm) were obtained from a breeding culture (Aquaculture, Meyerton), whereas the molluscs (adult) and fish (± seven months old) were bred and reared under controlled aquarium conditions at the University of Johannesburg. The ABM study was conducted in a low-flow period (October 2005). Organisms were placed into self-designed, polyethylene cages of 40 x 40 x 40cm at three sites in duplicate (Figure 8.1). The midges were placed in a biomonitoring tube (20 x 10cm), to separate them from the other organisms, in the same cage. Cages were in contact with both sediment and water, except at site 2. The organisms were exposed for a four week period, except for the midge larvae (two week period), due to their short life-span. The test organisms, *M. tuberculata* and *C. gariepinus* were fed on a weekly basis with lettuce and frozen midges respectively, to ensure that a shortage on food supply and nutritional requirements did not cause any additional stress on the organisms during the field exposure study. The laboratory-reared organisms were used as control groups.

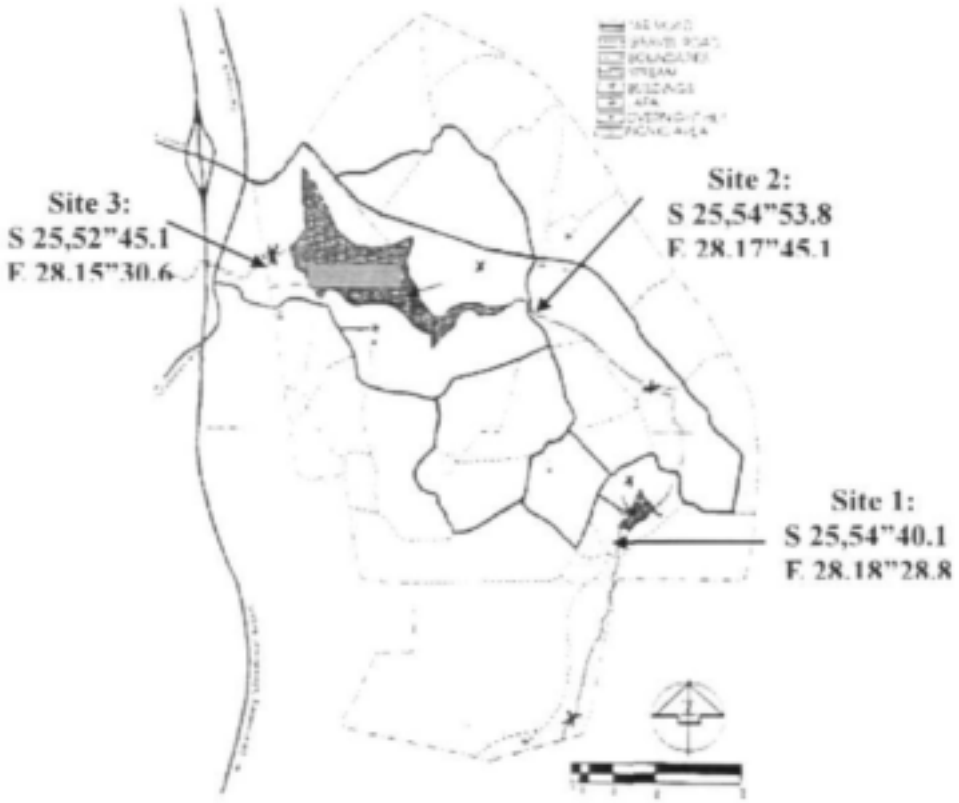

Fig. 8.1: Map of the catchment area indicating the sampling sites for the ABM study.

8.1.2.2 Water sampling and analysis

Physico-chemical water quality variables, such as temperature (°C), dissolved oxygen (mg/L and %), conductivity (µS/cm), total dissolved solids (TDS) (ppm) and pH were determined for the different exposure periods. The readings were taken using the following field instruments: Cyberscan DO100-Dissolved oxygen/temperature meter, Cyberscan DO100- Conductivity/TDS meter and waterproof pHScan pH meter. Separate water samples were taken for metals, target chemicals (Chapter 4) and nutrient content. The samples for nutrient content were collected in plastic containers and preserved using $HgCl_2$ and analysed as soon as possible.

Nutrient spectroquant content was determined with a Merck SQ118 photometer. The following test kits were used NO_2^- (14776), NO_3^- (14773), NH_4^+ (14752), P (14848).

8.1.2.3 Sediment sampling and analysis

Sediment samples were collected at all three sites, and taken back to the laboratory for moisture and organic content analysis according to standard procedures.

8.1.2.4 Metal analysis

Liver tissue, whole organisms (snails and chironomids) were prepared for metal analysis according to the method of Blust et al. (1988). Sediment samples were prepared and metals extracted according to the method of Bervoets and Blust (2003). Samples were selected for screening and high metal (chromium (Cr), nickel (Ni) and lead (Pb)) concentrations (ppm, mg/L) were found in sediment, water and tissue samples.

8.1.2.5 Biomarker analysis

Whole body tissues of *Chironomus sp.* and *M. tuberculata* and the liver tissue of *C. gariepinus* were used for biomarker analysis. Tissues were thawed and homogenised on ice, in the appropriate homogenising solution or buffer. All the samples were analysed in triplicate.

8.1.2.5a Available energy reserves (E_a)

The methodology of the CEA technique was carried out according to De Coen and Janssen (1997). The samples were homogenised on ice in 1mL of ice-cold distilled water. The energy reserves determined were: the carbohydrate, the lipid and the protein content of the samples. Whole body carbohydrate content was determined using a glucose test kit (GOD-PAP 1 448 668, Roche) and glucose standard (C FAS 759 350, Roche) at a wavelength of 560nm using an automated microplate reader (ELx180 Universal microplate reader, Bio-TEK Instruments, USA). Total lipids were extracted following the method of Bligh and Dyer (1959), and the absorbance was read at 340nm using tripalmitin as a standard. The protein content was

determined using Bradford's reagent (Bradford, 1976). The absorbance was measured at 630nm using BSA as a standard.

8.1.2.5b Energy consumption (E_c)

The energy consumption or cellular respiration rate was determined by measuring the electron transport activity (ETS). Each sample was homogenised in 1mL homogenising buffer (0.1 M Tris-HCl pH 8.5, 15% (w/v) Poly Vinyl Pyrrolidone, 153 µM $MgSO_4$ and 0.2% (w/v) Triton X-100). After centrifugation (at 3000 r.p.m. for 10 minutes at 4°C), 25µL supernatant was added to 75µL buffered substrate solution (0.13mM Tris-HCl and Triton X-100, pH 8.5) and 25µL NAD(P)H solution (1.7mM NADH and 250µM NADPH). The reaction was started by adding 100µL INT (*p*-IodoNitroTetrazolium) and the absorbance was measured kinetically at 49 nm for 10 minutes at 20°C. The amount of formazan formed was calculated using ε=15900/M.cm.

8.1.2.5c CEA

The different energy reserves (E_a) were transformed into energetic equivalents, using the enthalpy of combustion values used by De Coen and Janssen (1997). The values are as follows: 17 500mJ/mg glycogen, 39 500mJ/mg lipid and 24 000mJ/mg protein. The cellular respiration rate (E_c) was determined using the theoretical stoichiometrical relationship that for each 2µmol of formazan formed, 1µmol oxygen was consumed in the ETS system. The amount of oxygen was transformed into energetic equivalents using an average oxyenthalpic equivalent of 484kJ/mol O_2. The total energy budget was calculated using the following equation:

$$\mathbf{CEA = E_a - E_c}$$

where: $E_a = E_{carbohydrate} + E_{lipid} + E_{protein}$

$E_c = E_{ETS}$

8.1.2.6 Statistical analysis

Statistical analysis was performed using the program, SPSS 12.0 and the data reported as an average in this report. The Levene's test was used to test all data for homogeneity of variance. Significant differences were tested for between the various CEA components among the species, using an ANOVA one-way. The Scheffé's multiple comparison test (for homogenous data) as well as Dunnett's-T3 test (for non-homogenous data) were applied as post-hoc criterion, when significant differences were found amongst the variables. The significant level of $p<0.05$ was used.

8.1.3 Results

The effects of the ABM study on the available energy reserves and energy consumption are shown in Table 8.1. The exposed diptera midges showed an increase in the total energy reserves at sites 2 and 3, contributing to an increased CEA compared to site 1 and the control organisms. As for the prosobranch snails, a slight decrease in all of the CEA components, were observed at all the sites. The catfish revealed a significant increase in all the CEA components at site 1. However, both sites 2 and 3 showed to have a negative effect on the CEA, since a decrease in E_a was obtained. The increase in E_c contributed to the decreased net energy budget for the catfish.

8.1.4 Discussion

The detoxification or removal of a toxicant, cellular damage repair and avoidance of toxicants, are energetically costly and would be expected to result in the relocation of energy expenditures together with an increased metabolism to maintain physiological integrity (Wicklum and Davies, 1996; Sivaramakrishna and Radhakrishnaiah, 2000; Fent, 2004). Thus, the energy budget will reflect how energy availability and consumption changes within an organism under exposed conditions. The results indicated that the ABM study interfered with energy metabolism, specifically the E_a of all the species. The organisms showed a distinct difference in biomarker response during the field exposure. The increase in E_a of the midges could be explained by their short life-span and that they were only exposed for a two week exposure period. Even though these organisms were exposed to possible polluted environments, they seemed to compensate or adapt rapidly to prevailing conditions. However, the energy consumption of the midges showed an increase at sites 2 and 3, compared to the control, which could lead to an increased toxicant uptake (Smolders, 2003). In a recent study, Wepener et al. (2005) found an increased organic content at both these sites, which could suggest that the nutritional availability was high for the midges, as benthic-detritus feeders and caused the increased E_a.

The reduced available glucose and lipid content of the exposed molluscs, contributed to a decreased CEA. Toxicant stress preferentially causes a depletion of the more readily available carbohydrate and lipid reserves instead of protein (Smolders et al., 2003). It is known that certain molluscs are able to exploit special metabolic mechanisms to attain states of greatly reduced energy utilisation and exchanges of gases and fluids (Luchtel et al., 1991). The prosobranch mollusc revealed a lower respiration rate, which would result in a lower metabolic fuel demand and so a lower CEA. Lower energy reserves available together with decreased respiration rates were observed for the mysid shrimp, *Neomyis tiger*, following tributyltin exposure (Verslycke et al., 2003). Sivaramakrishna and Radhakrishnaiah (2000) observed that a decrease in oxidative metabolism induced an elevation in anaerobic glycolysis, in order to derive the required energy to resist toxic stress in the freshwater gastropod, *Pila globosa* and mussel, *Lamellidens marginalis*, exposed to mercury.

Table 8.1: Effect of the AMB study on the total energy available reserves and the energy reserves of the selected test organisms (data presented as an average).

		GLUCOSE Energy(mJ/g)	LIPID Energy(mJ/g)	PROTEIN Energy(mJ/g)	Ea Energy(mJ/g)	Ec Energy(mJ/g)	CEA Energy(mJ/g)
Chrinonmus sp.	Control	360 309.186	11 087.44[illegible]	345 598.147	716 994.782	54 763.44[illegible]	662 231.340
	Site 1	335 024.981	11 186.23[illegible]	341 727.477	687 938.695	49 212.97[illegible]	638 725.715
	Site 2	403 080.538	9 757.71[illegible]	1679 198.843	2 092 036.913	148 246.51[illegible]	1 943 790.394
	Site 3	190 391.662	10 279.05[illegible]	1662 179.17[illegible]	1 862 849.892	144 008.18[illegible]	1 718 841.705
M. tuberculata	Control	213 442.354[ab]	11 361.73	736 178.344	960 982.429	39 520.43[illegible]	921 461.990
	Site 1	[a] 90 236.705	10 542.69[illegible]	600 465.345	701 244.749	35 147.63[illegible]	666 097.117
	Site 2	[a] 32 831.093[a]	9 695.86[illegible]	691 456.565	733 983.529	24 956.53[illegible]	709 026.990
	Site 3	42 915.209[b]	7 986.56[illegible]	635 613.030	686 514.807	26 356.58[illegible]	660 158.222
C. guriepinus	Control	[a] 192 142.314[a]	11 589.25[illegible][a]	334 800 [a]	538 531.567[a]	34 600.15 [a]	503 931.410
	Site 1	[ab] 380 053.490[ab]	12 419.74 [ab]	408 630.204[ab]	801 103.442	40 848.86[illegible][ab]	760 254.577
	Site 2	[b] 163 713.189[ab]	12 096.31 [b]	323 342.85[illegible][b]	499 152.358	37 618.17[illegible][ab]	461 534.179
	Site 3	[ab] 59 988.73[illegible][b]	11 561.91[illegible][ab]	282 145.56[illegible][ab]	353 696.221[a]	44 854.34[illegible][ab]	308 841.871

Letters a,b represent significant differences between the groups (Control and sites 1-3) for different organisms

The biomarker, CEA, showed that the test species, *M. tuberculata* and *C. gariepinus* were adversely affected, since energetic costs can be increased through various repair and compensatory mechanisms (i.e. induced antioxidant systems) to combat toxic stress at cellular level (Wicklum and Davies, 1996). Although an increase in energy reserves of the exposed juvenile catfish at site 2 was found, the respiration rate was increased possibly due to uptake of pollutants via the gills (Smolders, 2003). All the CEA components of the control catfish group were significantly different from the exposed groups at sites 1 and 3. Similar results observed between the control group and site 2 corresponded with the low mortality rate found at site 2.

8.1.5 Conclusions

Since the catchment area is affected by several anthropogenic activities (see Chapter 3), it can be concluded that the exposed test organisms, appeared to be affected in the ABM study as well. Changes in the energy budget (CEA) can be used as a good indicator of toxicity during sub-lethal exposure and should be used in future biomonitoring programs in as part of a battery of biomarker assays. Results showed that the selected organisms reveal to have distinct interspecies as well as inter-individual differences in their energy metabolism. Juveniles of *C. gariepinus* showed to be the most sensitive test organisms and thus, can be considered as the preferred bioindicator species in this study.

8.2 Cell death in feral sharptooth catfish (*clarias gariepinus*)

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8.2.1 Introduction

In this section the possible effect of pollutants on the biomolecular events in the testis of sharptooth catfish inhabiting the water at that time, was investigated.

Fish is useful biosentinel species in surveys of environmental pollution of landlocked water sources, such as these described above. In this project, we used the feral sharptooth catfish, *C. gariepinus*, as a biomarker species. The testes of species such as *Clarias* and other anamniotes are useful model systems to study toxicant actions during spermatogenesis, because they are organisationally less complex than those of mammals and other amniotes since spermatogenesis proceeds in a cystic mode, i.e. a germ cell clone in any given stage is associated with stage-synchronised supporting cells (Sertoli cells) in a discrete follicle-like unit, i.e. the spermatocyst.

This anatomical arrangement further indicates that the various germ cell stages have different hormonal requirements and may, therefore, have different sensitivities to any given EDC, all of which

may be manifested as apoptotic cell death if the immediate milieu of the germ cell clone is disturbed. Germ cell development occurs in the context of a complex three-dimensional relationship with supporting testicular somatic cells (Sertoli and Leydig cells), whose behaviour also change during the course of spermatogenesis. An example of such change is the spermatogenic stage-dependent tight junctions between adjacent Sertoli cells, which create an evolutionary conserved blood-testis barrier that sequestrates late spermatocytes, spermatids and spermatozoa into a special microenvironment (Dym and Fawcett, 1970; Bergmann et al., 1984).

The morphological definition of apoptosis has traditionally centred on specific nuclear events, such as pyknosis, chromatin condensation and margination against the nuclear periphery (Wyllie et al., 1980; Cohen et al., 1992; Dini et al., 1996). These morphological expressions of apoptosis are in most cases regulated by caspases, which are cysteine aspartyl-specific proteases that function either as initiators (caspase-2, -8, -9, -10) or executioners (caspase-3, -6, -7) of apoptotic death (Reed, 2000; Slee et al., 2001). Regardless of the various pathways that may activate caspases, it is established that all of them converge on caspase 3, recognised as the major executioner of the nuclear and cytoplasmic events in apoptosis (Woo et al., 1998; Slee et al., 2001), including chromatin condensation (Sahara et al., 1999).

Despite the popularity of *in situ* end-labeling techniques such as the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling assay (TUNEL, Gavrieli et al., 1992) and *in situ* nick translation (ISNT, Oberhammer et al., 1996), to detect apoptotic cells in normal and toxicant-exposed tissues, their spurious labelling of nonapoptotic phenomena, such as necrotic cells, DNA repair, or non-labelling of distinctly apoptotic cells have however, increased controversy around the use of these two assays (Oberhammer et al., 1993; 1996; Negoescu et al., 1996; Kanoh et al., 1999).

The commercial availability of antibodies to the cleaved forms of most of the caspases, has opened up new avenues to investigate these controversies at the cell and tissue level (Barrett et al., 2001; Gown and Willingham, 2002).

By taking advantage of these technical features in this teleost fish, the aims of this study were to assess testicular apoptosis in *C. gariepinus* as an indicator of xenoestrogen pollution in freshwater sources and to compare the efficacy of TUNEL and cleaved caspase 3 immunostaining of apoptosis following xenoestrogen exposure in *C. gariepinus*.

8.2.2 Materials and methods

8.2.2.1 Animals and dissection

The specimens used in this study were captured from Dam 2 in January 2005 and processed as previously described (Barnhoorn et al., 2004). Sexually mature *C. gariepinus* (n=5) raised under

laboratory conditions in the Department of Zoology at the University of Johannesburg, Johannesburg, South Africa served as a reference group. The laboratory fish were raised as part of a study to breed catfish in an EDC-free environment and to serve as reference specimens because no source of unpolluted fish could be found. Parents and offspring were kept in glass tanks containing circulating reverse osmosis water (Van Dyk, 2006). The fish were decapitated, and the testes excised.

The salient morphological features of the testis of *C. gariepinus* have been described before (Cavaco et al., 1998). The paired elongated and relatively flattened testis is medially attached to a connective tissue rich central portion, which also contains the spermatid duct. Blocks (25mm x 15mm) were cut of the middle section of the testis. Although the spermatogenically active catfish testis has a simple medial to lateral zonation, individual members of the fish population caught at any given time in the spawning season are not all synchronised in terms of their testicular development, and the testicular zonation may vary between specimens as depicted in Figure 8.2. Thus, the zone containing the spermatogenic tubules, include tubules that are lined by single primary spermatogonia, spermatogonial, primary and secondary spermatocyte, and early spermatid cysts, and whose lumens are partially filled with spermatozoa. Mature tubules are fully distended with spermatozoa, and are lined with a scattering of single spermatogonia and occasionally with spermatocyte cysts. Spent tubules are largely devoid of spermatozoa, and lined with Sertoli cell remnants and the occasional spermatogonium.

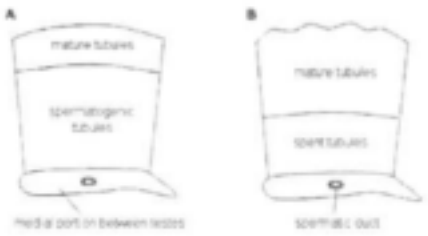

Fig. 8.2: A diagram of a block of spermatogenically active catfish testicular tissue. Showing the simple medial to lateral zonation. The testicular development is not exactly synchronised in individual members of the population, and males caught in the spawning season often possess either spermatogenic tubules and mature tubules (A), or mature tubules and spent tubules (B). Testes containing only one zone were also occasionally observed.

8.2.2.2 Tissue preparation

Blocks (25mm x 15mm) catfish testes were fixed in 10% neutral buffered formalin in PBS for several days, after which they were embedded in Paraplast. Sections of 4µm thick were floated onto a distilled water bath (45°C), collected on Superfrost Plus slides, deparaffinized and rehydrated stepwise through an ethanol series.

8.2.2.3 TUNEL immunohistochemistry

Deparaffinized and rehydrated sections were subjected to a pre-treatment protocol as described in Labat-Moleur (1998), which involved microwave oven treatment (800W) in 10 mM citrate buffer (pH 3.0) for 14 min. Following treatment for 2 min in 0.1 Triton X-100 in 0.1% sodium citrate on ice, sections were treated with proteinase K (20µg/mL) for 15 min at room temperature (RT), after which they were washed twice with PBS-A (50mM sodium phosphate, pH 7.4; 200mM NaCl). Endogenous peroxidase was quenched by treating sections in darkness with 3% hydrogen peroxide in PBS-A for 5 min, followed by two washes in PBS-A. Subsequent steps for TUNEL staining were carried out using the ApopTag-Peroxidase Kit according to the supplier's instructions (Chemicon, USA). Following incubation of the sections with the TUNEL reaction mixture in a humidified chamber at 37°C for 1 hr, antidigoxigenin-peroxidase complex was added for 30 min at RT. Sections were then treated with 3,3'-diaminobenzidine (DAB) for 1-2 min according to the supplier's instructions (Vectorlabs, Burlingame, CA) such that positive cells stained brown. Sections were counterstained with 0.5% crystal violet-containing methyl green in 100mM sodium acetate (pH 4.0), dehydrated in 100% butanol, cleared in xylene and mounted with Entellan (Merck, SA). To generate negative controls, TdT was replaced with sterile water, whereas for positive controls, sections were pretreated with DNase I (10µg/ml in distilled water) to generate DNA fragments.

8.2.2.4 Cleaved caspase 3 immunohistochemistry

Deparaffinized and rehydrated sections were subjected to an antigen retrieval procedure, i.e. heating in a microwave oven (800W) in 10mM sodium citrate buffer (pH 6.0) for 10 min. After cooling for 20 min at RT, sections were rinsed in distilled water and treated in darkness for 10 min with 3% hydrogen peroxide to quench endogenous peroxidase. Following a rinse in distilled water, the sections were placed in 0.1% Tween 20 in phosphate buffered saline (PBS-B, pH 7.4) for 5 min. Sections were then incubated with blocking solution (5% normal goat serum in 0.1% Tween 20-PBS-B) for 1 hour at RT to reduce non-specific binding, and then overnight at 4°C with a rabbit polyclonal antibody to cleaved caspase 3 (17/19kDa fragment of activated caspase 3, Cell Signaling Technology, Beverly, MA, USA) diluted 1:100 in blocking solution. After three washes in 0.1% Tween 20-PBS-B, sections were incubated with biotinylated antirabbit IgG for 30 min at RT, washed in PBS-B, and then incubated with the Vectastain avidin-biotin-complex (Vectorlabs,

Burlingame, CA, USA) for a further 30 min at RT. Following three 5 min PBS-B washes, the antigen was finally detected by treating the sections with 3,3'-diaminobenzidine, counterstained, dehydrated, cleared and mounted as described above TUNEL. Negative controls were generated by omitting the primary antibody and by serial dilution of the primary antibody, as well as by using a rabbit monoclonal antibody to cleaved caspase 3. Human testicular sections processed in the same way served as positive controls.

8.2.2.5 Microscopy

Quantitative measurements were done on a Nikon Optiphot microscope fitted with an ocular grid in one eyepiece. For each testicular zone and using a 10x objective, each one of the 50 tubules intersecting each of two widely separated lines parallel to the adjacent testicular zone were scored for the number of caspase 3-positive and TUNEL-positive cells it contains, and the average number was taken of the two lines. For the spermatogenic tubule zone and using a 20x objective, each one of a 100 randomly counted spermatogenic tubules were scored as caspase 3-postive or –negative. A tubule was designated caspase 3-positive if it contained at least one cluster of four caspase 3-postive cells. Sections were photographed with a Nikon Optiphot microscope, fitted with a Nikon DXM1200F digital camera connected to a PC computer equipped with image-capturing software.

8.2.2.6 Statistics

The data was expressed as the mean number of caspase 3-positive and TUNEL-positive cells per 50 tubules in each testicular zone, and the mean number of caspase 3-positive tubules per 100 spermatogenic tubules. Data were examined by two-way ANOVA using SPSS Version 13. Tukey's multiple-comparisons test was used to determine which values differed significantly ($p < 0.05$).

8.2.3 Results

Normal spermatogenic development in the spermatogenically active laboratory-reared fish was characterised by the occasional observation of scattered degenerate cells in routine hematoxylin and eosin-stained sections (Figure 8.3). Dying cells were conspicuous by their isolated appearance and separation from adjacent cells. They were mostly single cells with either pyknotic nuclei (Figure 8.3A,B) or irregularly shaped intensely stained nuclei, whose appearance were distinctly different from mitotically dividing cells (Figure 8.3B), or were, more rarely, groups of coalesced germ cells with characteristic horse-shoe shaped nuclei (Figure. 8.3B). Besides the occasional spermatogonium (not shown), most dying cells were primary spermatocytes and more frequently secondary spermatocytes (Figure 8.3B,C). Dying testicular somatic cells (Sertoli cells, Leydig cells and peritubular cells) were not observed. These results suggest that apoptosis is part of normal

spermatogenetic activity in the catfish.

Fig. 8.3: Low (A) and high (B, C) magnification photomicrographs of hematoxylin and eosin-stained control catfish testicular sections. In (A), the complete spermatogenic sequence could be observed in spermatogenic tubules, which were lined by cysts that each contained germ cells of only one particular stage. At the end of spermiogenesis, the spermatozoa were gathered in the tubule lumen. Dying cells (*) were sporadically observed, either as single cells (with either pyknotic nuclei (A,C) or with irregularly shaped, intensely stained nuclei, B) or rarer, as coalesced groups of cells (B). Dying cells could also be observed in cysts in which other members of the cysts were mitotically active (M). Psg, primary spermatogonia; Ssg, secondary spermatogonia; Psc→Ssc, cyst undergoing primary spermatocyte→secondary spermatocyte transition; Ssc→Est, cyst undergoing secondary spermatocyte→early spermatid transition; St, spermatids sz, spermatozoa; SC, Sertoli cell nuclei; Li, lipid granules; Ptc, peritubular cells. Bar = 10μm.

In order to determine whether habitation in a xenoestrogen-polluted environment was associated with an alteration in the rate of DNA fragmentation in spermatogenically active catfish, TUNEL

immunohistochemistry was performed on the testes of the catfish. The overall incidence of TUNEL-detected DNA fragmentation in Dam 2 catfish testes was surprisingly low, as shown by the pattern of labelling shown in Figure 8.4. Clearly degenerate cells, including coalesced germ cell masses were partially TUNEL-stained in some instances, whereas they were completely TUNEL-labelled in other instances (Figure 8.4A). Likewise, degenerate-looking germ cells with nuclei having the same intense colour as that of the heads of adjacent spermatozoa, were conspicuously TUNEL-negative (Figure 8.4B). However, all cell types were labelled after DNase I treatment of tissue sections (not shown), evidence that the observed negative labelling was actually due to the absence of fragmented DNA rather than a lack of access of TUNEL reagents *in situ*. Besides the sporadic occurrence of vacuoles associated with cell death in routine histological sections of Dam 2 catfish testes (see Figure 8.3), the morphology of the UNR catfish testes was otherwise similar to that of the control fish (not shown).

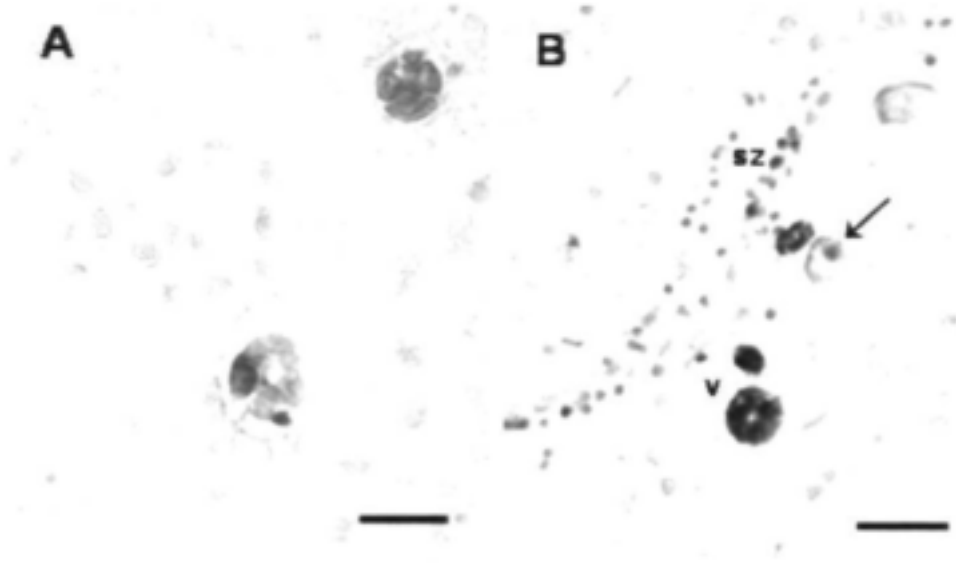

Fig. 8.4: Photomicrographs of TUNEL-stained testicular sections of Dam 2 catfish. (A) Coalesced cell masses were either partially TUNEL-stained in some instances or completely TUNEL-labelled in other instances (insert). Take note chromatin or other nuclear material are indistinguishable. (B) Likewise, single germ cells were sometimes TUNEL-positive, whereas adjacent degenerate-looking cells with nuclei that stained the same colour as the adjacent spermatozoa were distinctively TUNEL-negative in other instances (arrow). sz, spermatozoa; v, vacuole. Bar = 10μm.

To investigate the possibility that the TUNEL method was selective rather than specific in its detection of the various stages of the apoptotic process, cleaved caspase 3 immunohistochemistry was performed on the testes of Dam 2 catfish. Aging human testes were similarly processed and served as positive controls. Unlike with TUNEL, cleaved caspase 3 immunoreactivity was very common in the

spermatogenically active xenoestrogen-exposed catfish, was limited to germ cells, and was found in scattered isolated cells, groups of cells, and groups of coalesced germ cells (Figure 8.5). Entire primary spermatocyte and secondary spermatocyte cysts were often caspase 3-labelled. Caspase 3 immunoreactivity was particularly associated with germ cells at the secondary spermatocyte – early spermatid transition, such that the occasional early spermatid was also caspase-positive (Figure 8.6B). Caspase 3-labelling varied intracellularly, though with a distinct pattern: labelling was either (a) weakly cytoplasmic in cells that had a nucleus with a characteristic ring-like appearance (Figure 8.5A), or (b) moderate but extensively cytoplasmic in cells with translucent nuclei (Figure 8.5A,B), or (c) intense throughout the whole cell with the outlines of the nuclei barely discernible (Figure 8.6A), or intensely nuclear (Figure 8.5B), or (d) moderately cytoplasmic coincident with intense blue staining of the nuclei, such that the colour of the nuclei of such cells was identical to that of the heads of spermatozoa (Figure 8.5B), or (e) was complete lacking in clearly degenerate cells with intensely counterstained nuclei (Figure 8.5B).

To assess whether the observed differences between TUNEL- and cleaved caspase 3-immunostaining patterns may be testicular zone-related, we took advantage of the pronounced zonation of the spermatogenically active *C. gariepinus* testis and compared the occurrences of TUNEL- and caspase 3-positive cells in the spermatogenic, mature and spent regions of Dam 2 catfish. As shown in Figure 8.6, the number of labelled cells (both caspase 3- and TUNEL-positive) varied significantly ($p<0.0001$) according to testicular region, i.e. it was highest in the spermatogenic region, and very low in the mature and spent regions of the testis.

Although the numbers of caspase 3- and TUNEL-positive cells were comparable in the mature and spent tubules, the number of caspase 3-positive cells in the spermatogenic tubules was 3-fold higher ($p<0.05$) higher than the number of TUNEL-positive cells (Figure 8.7). These results suggest that cleaved caspase 3 immunohistochemistry was a superior method to detect apoptotic germ cells during active spermatogenesis in D2 catfish.

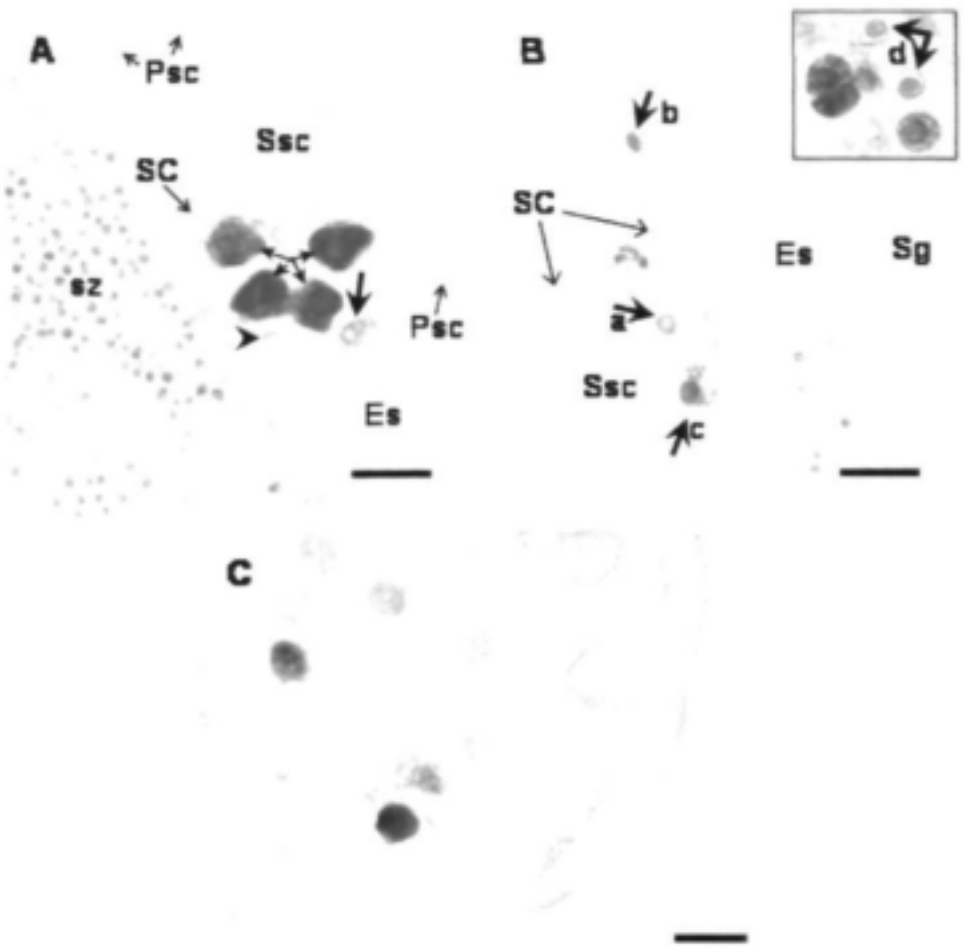

Fig. 8.5: Photomicrographs of cleaved caspase 3 immunolabelling in spermatogenic tubules of D2 catfish. (A) In many labelled secondary spermatocyte spermatocysts, intensity of caspase 3-labelling varied intracellularly, being initially cytoplasmic in cells that had a nucleus with a characteristic ring-like appearance (arrowhead), followed by extensive cytoplasmic labelling of cells with translucent nuclei (large arrow) and later, complete labelling of the cell (small arrows). (B) Besides cytoplasmic immunoreactivity associated with normal-looking nuclei, in this case an early spermatid (a→), labelling was also nuclear (b→), cytoplasmic coincident with intense blue staining of nucleus such that the colour of the nucleus was similar to that of the heads of spermatozoa (c→), or was lacking in degenerate-looking cells containing nuclei that reacted intensely with the methyl-green counterstain (inset, d→). (C) Positive control showing nuclear caspase 3 immunoreactivity of spermatocytes in human testis. Bar = 10μm.

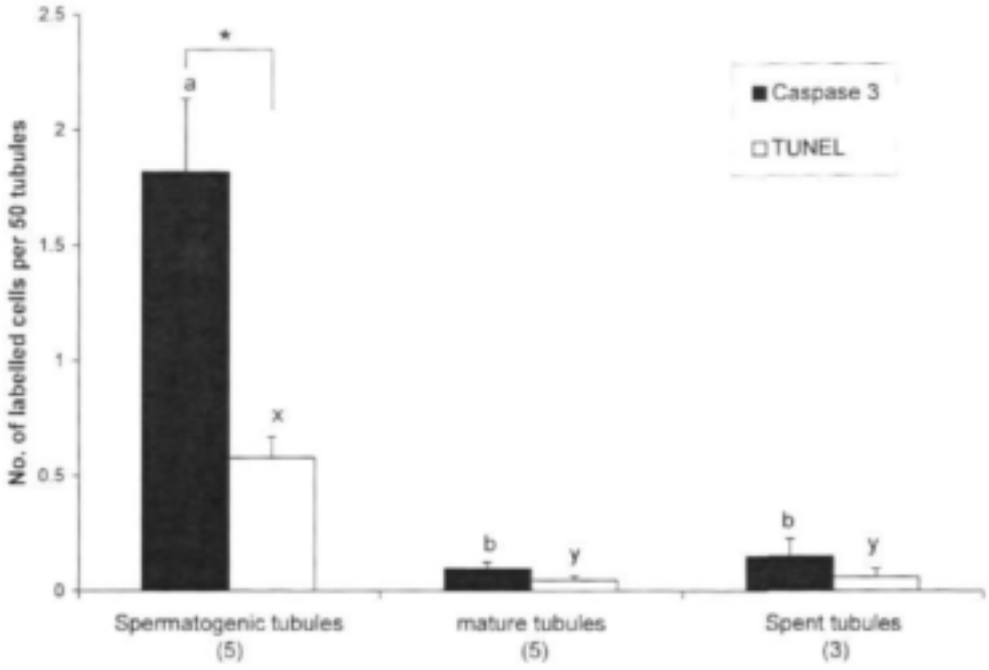

Fig 8.6: Testicular zone-related changes in caspase 3-and TUNEL-labelling of germ cells in D2 catfish. Values represent the mean number ± S.E.M of labelled cells per 50 tubules. Animals with any given testicular zone were pooled and shown as numbers at the bottom of the figure. Differences among zones within a given method were significant (P<0.0001), and is indicated with a different letter (caspase 3, a-b; TUNEL, x-y). Differences between methods in a given zone are indicated with * (p<0.05).

To finally confirm whether the relatively high incidence of caspase 3-labelling in the spermatogenic tubules of Dam 2 catfish reflected the effects of inhabiting a xenoestrogen-polluted environment, cleaved caspase 3 immunohistochemistry was used to assess cell death ratios in control vs Dam 2 catfish (Figure 8.7). The number of caspase 3-positive cells in the spermatogenic tubules of Dam 2 catfish was 1.9-fold higher ($p<0.05$) than those in the control catfish (Figure 8.7A). In addition, the number of caspase 3-positive spermatogenic tubules in the testes of Dam 2 catfish was more than 4-fold higher ($p < 0.01$) than those of the control catfish (Figure 8.7B). These results suggest that xenoestrogen pollution did not only increase the number of caspase 3-positive cells, but also increased the number of spermatogenic tubules containing at least one cluster of apoptotic cells.

The water collected at the same time contained per L 14.04ug lindane, 3.18mg DEP, 3.93mg DMP and 0.46mg DEHP respectively (Chapter 4). The fish fat samples contained lindane, aldrin, DDT and all metabolites as well as PCB153 (Table 8.2).

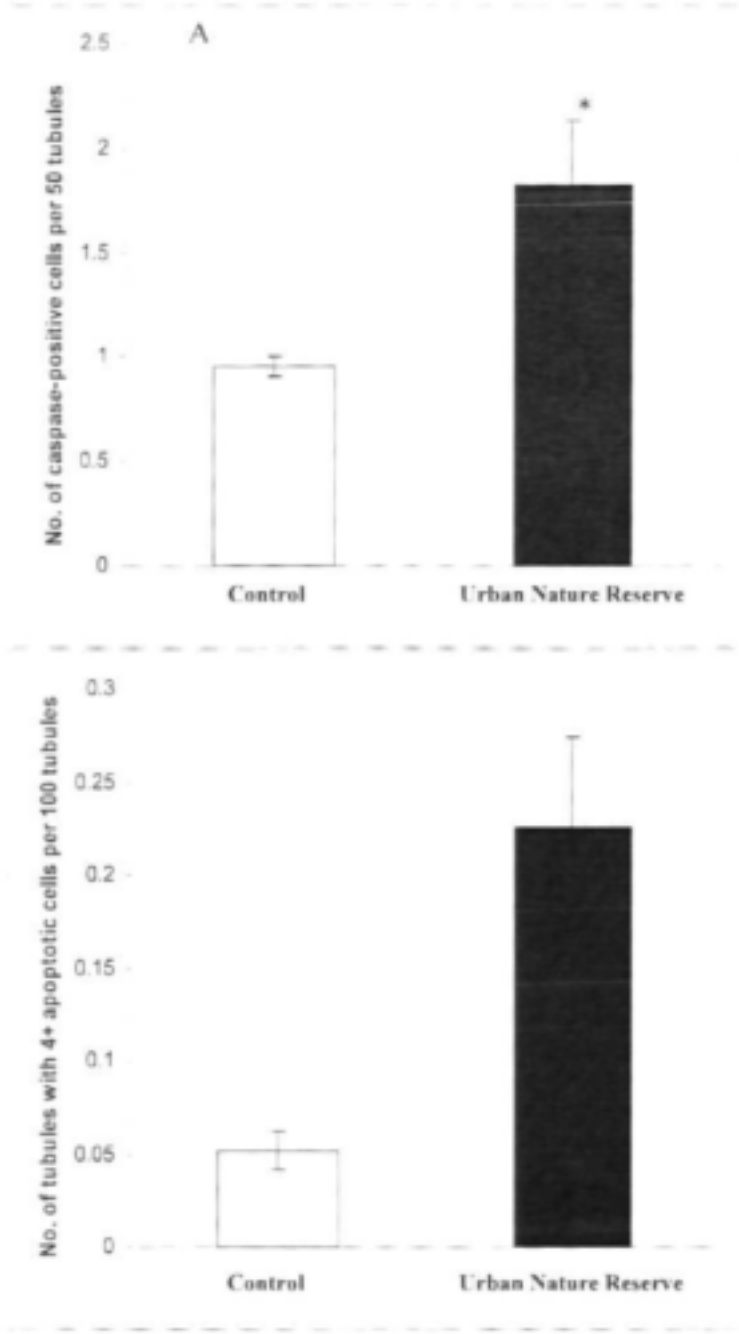

Fig. 8.7: Effects of a xenoestrogen-polluted habitat (UNR) on apoptosis in the spermatogenic tubules. Values represent (A) the mean number of caspase 3-positive cells ± SEM per 50 tubules, and (B) the mean number of caspase 3-positive tubules per 100 tubules, as described in Material and Methods (5 animals per group). Means of the two groups differed significantly (*, $p < 0.05$; **, $p < 0.01$).

Table 8.2: Chemicals present in fat of sharptooth catfish (µg/kg)(All empty spaces indicate not detected).

Fish	Lindane	Aldrin	*o,p'*-DDE	*p,p'*-DDE	*o,p'*-DDD	*p,p'*-DDD	*p,p'*-DDT	*p,p'*-DDT	PCB 153
1				84.882		133.412		78.872	
1	150.429		40.847	1759.169	13.820	523.295	199.430	321.429	40.787
2	70.861	60.446		84.577		104.835	73.576	91.465	
3			17.124	97.901		79.860	89.016	98.423	
4						22.390			
5	50.161	33.056				94.462	86.842		
6	56.353			220.077		157.999	58.962	97.583	
7	186.744	45.871	23.786	1714.002		416.550	100.028	146.916	94.766
8	106.272	39.108	22.850			100.053		113.301	
9	73.669					78.838			
						48.903			
mean	**99.2**	**44.6**	**26.2**	**660.1**		**160.1**	**101.3**	**135.4**	
sd	**51.7**	**11.8**	**10.2**	**835.5**		**159.3**	**50.1**	**84.8**	
med	**73.7**	**42.5**	**23.3**	**159.0**		**100.1**	**87.9**	**98.4**	

8.2.4 Discussion

Through analysis of testicular apoptosis, which is described here for the first time in a clariid teleost, we show that *C. gariepinus* inhabiting xenoestrogen-polluted water sources do not only have a higher number of caspase 3-positive germ cells in the testis, but also show a higher number of spermatogenic tubules containing at least one cluster of four or more apoptotic spermatocytes when compared to laboratory reared catfish. By taking advantage of the prolonged duration of apoptosis in this species, we also provide qualitative and quantitative evidence that DNA fragmentation (as indicated by TUNEL-staining) and early nuclear changes (as indicated by cleaved caspase 3 immunoreactivity) are distinctly separate events during the process of apoptosis. A qualitative demonstration of this was the clear distinction between the cytoplasmic and nuclear compartments in caspase 3-positive coalesced cell masses, whereas these two compartments were indistinguishable in TUNEL-positive coalesced cell masses in TUNEL-stained sections. Likewise, using the intense blue-stained heads of spermatozoa, indicative of the condensed state of chromatin in these cells, as a reference standard, our findings show that the nucleus becomes condensed after cleaved caspase 3 has translocated back into the cytoplasm from the nucleus. Furthermore, germ cells with condensed nuclei are not TUNEL-labelled, suggesting that chromatin condensation and DNA fragmentation are not coincident.

An interesting observation was the germ cell stage-dependent nature of apoptotic death in Dam 2 catfish. Several possible explanations are plausible. The hormonal requirements of meiotic and postmeiotic stages are well-documented for some fish species. Thus, preferential deletion of spermatocytes via apoptosis in the Dam 2 catfish may reflect, among others, these hormone sensitivities. Earlier work on the hypophysectomised catfish, *Heteropneustes fossilis*, demonstrated the near absolute requirement of androgen for the appearance of spermatocytes and spermatids in spermatogenic progression after hormonal therapy, whereas high doses of follicle-stimulating hormone were inconsequential for the development of these two germ cell stages (Sundararaj et al., 1967). Although similar hypophysectomy experiments have not yet been performed on *C. gariepinus*, it is established, however, that 11-ketotestosterone is the biologically active androgen in *C. gariepinus* (Cavaco et al., 1998), and rising plasma 11-KT levels are coincident with the appearance of spermatids in *C. gariepinus* (Cavaco et al., 1997). NP exposure has been reported to decrease plasma androgen concentrations and increase plasma 17β-estradiol concentrations in goldfish, observations that are consistent with increased aromatisation (Soverchia et al., 2005). Likewise, xenoestrogens also reduced plasma 11-KT levels in male goldfish (Maclatchy and Van der kraak, 1995) and suppressed the mRNA levels of testicular cytochrome P450 11β-hydroxylase, a key enzyme in the production of 11-ketotestosterone in male medaka (Yokota et al., 2005). Thus, a synthesis from these findings would suggest that xenoestrogen-pollution may disrupt androgen synthesis, and thus lead to caspase 3-dependent apoptosis in androgen-sensitive stages in *C. gariepinus*, a notion that agrees with findings of caspase 3-dependent apoptosis in rat spermatocytes following reduced intratesticular testosterone

levels (Kim et al., 2001). The discovery of elevated amounts of both estrogen receptor-α RNA and estrogen receptor-β RNA, specifically in secondary spermatocytes and spermatids in the channel catfish (Wu et al., 2001), supports the notion of the role of aromatization and thus estrogen for the secondary spermatocyte – spermatid transition at this phyletic level. Interestingly, maximal aromatase activity in the shark testis occurs also in primary and secondary spermatocyte cysts (Callard et al., 1985), and estrogen receptor-α knock-out mice have disrupted spermatogenesis and reduced sperm counts (Lindzey and Korach, 1997). By extension, and assuming these estrogen receptors are also expressed in *C. gariepinus*, one explanation could thus be that xenoestrogens may alter the immediate steroidal milieu, e.g. the androgen – estrogen ratios required for optimal spermiogenesis. Further studies are needed to clarify the hormonal requirements of *C. gariepinus* germ cells.

Alternatively, the increased incidence of clusters of apoptotic germ cells at mainly the secondary spermatocyte – spermatid transition could be the result of indirect inhibitory or disruptive actions of *p*-NP on germ cells, i.e. via Sertoli cells, an idea that is consistent with our observations that Sertoli cells were never apoptotic, but contrary to that reported for the medaka testis (Weber et al., 2002). In fact, clusters of apoptotic cells were rare in control catfish testes. *p*-NP has also been proposed to exert its endocrine disrupting actions via non-receptor-mediated mechanisms on the estrogen receptor (Kirk et al., 2003). Thus, it possible that *p*-NP could disrupt any of the numerous Sertoli cell endocrine-related functions, several of which are Ca^{2+}-dependent, by either disrupting endoplasmic reticulum Ca^{2+} pumps (Hughes et al., 2000), or inhibiting IP_3-sensitive Ca^{2+} channels that are particularly abundant in Sertoli cells (Khan et al., 2003). Thus, any diminishing in Sertoli cell function will negatively impact development of especially germ cells that are sequestered behind the blood-testis barrier, such as spermatids and sperm (Bergmann et al., 1984). Conversely, non-specific killing of Sertoli cells would have caused random and non-stage dependent germ cell death.

Quantitatively, the number of caspase 3-positive cells in the spermatogenic region was at least 3-fold higher than the number of TUNEL-positive cells, indicating that the TUNEL method underestimated the rate of testicular apoptosis in xenoestrogen-exposed *C. gariepinus*. The discordances between the TUNEL- and caspase 3-immunostaining reported here are, in part, consistent with reported uncertainties regarding the exact relationship between chromatin condensation and DNA fragmentation (Kerr, 1995). A meaningful interpretation is perhaps provided by Walker et al. (1994) who showed that DNA cleavage occurs in two stages, firstly by endonuclease activities associated with high molecular weight DNA fragmentation and which may cause chromatin condensation, but that this enzyme activity is distinct from that causing the subsequent internucleosomal DNA fragmentation, which is usually detected by the TUNEL method. Thus, our findings agree in principle with the notion that chromatin margination and condensation, and internucleosomal DNA fragmentation are separate events, the latter occurring at the very end of apoptosis, as was also

reported in thymocytes (Cohen et al., 1992) and hepatocytes (Oberhammer et al., 1993; 1996; Sun et al., 1994).

We also provide evidence of different pathways of apoptosis progression in the teleost testis. Besides affecting isolated germ cells, groups of coalesced germ cells were also apoptotic, an observation that was also made in other vertebrates with cystic spermatogenesis, i.e. cartilaginous fishes (Prisco et al., 2003; McClusky, 2005). The findings of the present study, together with those reported for the spiny dogfish shark (McClusky, 2005), clearly suggest that, unlike in other tissue systems, germ cell coalescence is a very early marker of apoptotic death in lower vertebrates, and that DNA fragmentation occurs much later. It is unknown why germ cell coalescence is so prevalent at these phyletic levels. Given the intimate environment in a cyst, it may be that the cytoplasmic bridges joining germ cells, known to be present in another catfish species at least (Batlouni et al., 2005), open up and lead to coalescence of a group of cells if the apoptotic stimulus is large enough. Conversely, a temporary interruption in cytoplasmic continuity [e.g. by way of the appearance of multiple transverse cisternae in the intercellular bridges (Dym and Fawcett, 1971)] among clone members may prevent the spread of abortive or death signals to the entire germ cell clone provided the magnitude of the apoptotic signal does not exceed a certain threshold within the cyst. Such local control might explain the observations of scattered isolated apoptotic cells in otherwise healthy spermatocysts and the coincidence of apoptotic and mitotic activities in the same spermatocyst in *C. gariepinus* (this study) and in the spiny dogfish shark (McClusky, 2005).

CHAPTER 9

AQUATIC PLANTS

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9.1 Introduction

Research has indicated the usefulness of aquatic plants for removal of insecticides from water (Weinberger et al., 1982). Processes important for the removal of non-point source pesticide runoff in wetlands may include adsorption (accumulation on the surface), decomposition, and microbial metabolism (Rodgers et al., 1999). Macrophytes present in a wetland may play an important role in providing an increased surface area for sorption as well as for microbial activity (Karen et al., 1998; Hand et al., 2001). Furthermore they may contribute directly to chemical metabolism (Wetzel, 1993). Emergent vegetation (rooted plants that have most of their leaves and stem tissue above the water surface *e.g. Phragmites australis, Typha latifolia, Sagittaria spp.*) was demonstrated to reduce the resuspension of sediments in wetlands (Dieter, 1990). Therefore, the use of wetlands for the removal of pollutants involves a complex variety of biological processes, involving microbiological transformations and physio-chemical processes such as adsorption, precipitation or sedimentation (Dunabin and Bowmer, 1992). The need now exists to link wetland characteristics such as the presence of emergent macrophyte vegetation with the transport of non-point-source (pesticide) contamination and the resulting biological effects (Schulz et al., 2002).

Aquatic plants are an important component of aquatic communities due to their roles in oxygen production, nutrient cycling, controlling water quality, sediment stabilization and providing habitat and shelter for aquatic life (Harris and Davidson, 2002). The immobile nature of macrophytes makes them an effective bioindicator of metal pollution as they represent real levels present at the site. In emergent plants metals and nutrients are mainly absorbed by the roots or rhizomes and translocated within the plant to the shoots and reproductive structures. In submerging species (rooted plants with most of their vegetative tissue beneath the water surface e.g. *Potamogeton*), the soil is usually the main pathway for metal and nutrient uptake, but depending on the species there can also be a direct absorption from the water in highly eutrophic lakes (Bole and Allan, 1977; DeMarte and Hartman, 1974).

Plants can directly affect two metal mobility-regulating soil factors – redox potential (Eh) and pH. As an adaptation to waterlogged, anoxic conditions, the roots of some plant species allow oxygen to diffuse into the adjacent soil. This radial oxygen loss (ROL) may enable plants to reduce soil toxins via chemical, enzymatic and microbial oxidation (Armstrong, 1975).

Plants and sediment are the major accumulators of nutrients in wetlands. For example, *Schoenoplectus validus* can reduce biological oxygen demand, nitrogen (N) and phosphorous (P) in wastewater (Tanner, 1994), while *Typha* spp. has been shown to enhance the removal of heavy metals (zinc, lead, silver) and salts from underground mine drainage water in Northern Australia (Noller et al., 1994). The water, sediments and plants in wetlands receiving urban runoff contain higher levels of heavy metals than wetlands not receiving urban runoff. Large aquatic plants (eg *Typha latifolia, Iris pseudacornis* and *Phragmitis australis*) are known to accumulate heavy metals in their tissues (Noller et al., 1994). Concentrations in their roots systems were found to be higher than in their leaves and stems. As the macrophytes die and decay, there can be an increase in the concentration of heavy metals in sediments. Hydrogen sulfide, produced as a by-product of bacterial anaerobic processes is also responsible for the sedimentation of dissolved heavy metals from water (Noller et al., 1994).

Hansel et al. (2000) applied scanning electron microscopy to elucidate metal precipitation processes at the soil-root interface in the aquatic plant *Phalaris arundinacea*. The chemical form of metals affects their bioavailability, toxicity and mobility through food chains so it is important to have an understanding of metal complexation within the rhizosphere of aquatic plants. Observations on elevated metal concentrations within the root relative to shoot tissue, suggests that wetland plants may adopt exclusion mechanisms (external or internal) to hinder translocation of metals to aerial tissue (Hansel et al., 2000). Iron (Fe) and lead (Pb) are concentrated on the exterior of the root, forming a superficial rind on the root epidermis. Concentration gradients allow for metal diffusion toward the root surface and potential uptake and subsequent toxicity to plants and biota (Hansel et al., 2000).

9.2 Materials and Methods

9.2.1 Sampling sites

Macrophyte sampling as well as water and sediment (refer to Chapter 4.2) sampling was carried out at the following sites:

9.2.1a Site 1

GPS location: S25^0 54' 671" E28^0 18' 482"

Sampling at site 1 was done just below the Dam 1(D1). The location is a shallow channel, approximately 3m wide. It is characterized by fast flowing water and a solid rock bed. Grasses and exotic weeds dominate the fringing vegetation.

9.2.1b Site 2

GPS location: S25^0 54' 414" E28^0 18' 509"

Sampling was done in Dam 1 (D1). The banks are lined with reeds, bulrush and exotic weeds. Surface sediment samples were taken from the depositional zones.

9.1.2c Site 3

GPS location: S25^0 52' 911"E28^0 17' 727"

This site is characterized by fast-flowing waters and large boulders on the substratum, above D1. The water depth is greater than at site 1. The fringing vegetation is mainly bulrush and grasses.

9.1.2d Site 4

GPS location: S25^0 52' 910"E28^0 17' 728"

Sampling was done here in Dam 2(D2). The substratum is characterized by coarse sediment. Regular activities on the dam include fishing, boating (sailing and rowing) and camping.

9.1.2e Site 5

GPS location: S25^0 52' 701"E28^0 15' 385"

This sampling location is outside the reserve on the Sesmylspruit. The substratum is heavily silted and the water is discoloured. The riparian vegetation is mainly trees and shrubs. It is also a cattle drinking point.

9.2.2 Plant sampling

A brief preliminary vegetation survey was done with the assistance of a botanist, to identify three plant species; the bulrush (*Typha capensis*), the common reed (*Phragmites australis)* and the exotic weed *Persicaria lapathifolia*, a characteristic wetland macrophytes. At each sampling site, an area located within the wetland's emergent zone was selected. Identification of the same sampling points was done in subsequent sampling seasons with the aid of a Global positioning system (GPS) instrument. Collection of plants was done by hand or when the water level had risen, using a garden fork. The plants were rinsed in water at the site and then placed in Ziploc plastic bags. Specimens for chemical analysis were wrapped in aluminium foil before being sealed in the Ziploc bags and stored in a cooler box on ice for transport to the laboratory. The sample consisted of both fine and coarse root structures.

Fig. 9.1: The sampling sites 1-5 where the roots of the bulrush (*Typha capensis*), the common reed (*Phragmites australis)* and the exotic weed *Persicaria lapathifolia*, a characteristic wetland macrophyte were collected.

9.2.3 Sediment and water sampling

Surface sediment samples were collected from the depositional zones of the channel. A grab sampler was used during the high flow season when the water level had risen. The sediment was placed in Ziploc bags pending metal analysis, and in methanol pre-washed glass jars for EDC analysis and stored at 4°C. Water samples were also collected in plastic and glass containers for

metal and EDC analysis respectively. The water samples were filtered and acidified to pH 2 before undergoing ICP-MS analysis for metals (Waterlab, Pretoria)

9.2.4 Microwave digestion for sediments and plants

9.2.4.1 Recoverable (Environmentally Available) Metals

9.2.4.1a Principle

Total metals analysis requires the complete destruction of the sample in order to release all forms of metal. These include metals adsorbed on the sediment particles, metals in the form of insoluble salts and organic complexes, and metals in the mineral phase of the sediment. The method below employs strong acid digestion to extract recoverable or environmentally available metal. Addition of hydrofluoric acid would result in release of metals trapped in the silicate matrix of the sediment.

9.2.4.1b Procedure for sediment

Weigh 0.5g dried sediment into a Teflon vessel. Add 2ml HCL + 9ml HNO_3 + 1ml 30% H_2O_2.

9.2.4.1c Procedure for plant tissue

Weigh 0.5g dried plant root tissue into a Teflon vessel. Add 2ml of 30% H_2O_2 + 7mL of 65% HNO_3. In both cases the Teflon vessel was introduced into the safety shield. The acids were added; if part of the sample stayed on the inner wall of the Teflon vessel, it was washed down by adding acids drop by drop, then gently swirling the solution to homogenize the sample with the acids. The vessel was closed and introduced into the rotor segment, then tightened using the torque wrench. The segment was inserted into the microwave cavity and connected to the temperature sensor. The microwave temperature regime at power up to 1000W was as follows:

4 min 85°C

9 min 145°C

4 min 200°C

14 min 200°C

The microwave program was run to completion, and after the solution reached room temperature it was to a marked flask. The sample was then diluted to 100mL with milli-Q water and analyzed by ICP-MS (Department of Geology, University of Johannesburg) for Cadmium (Cd), Aresenic (As), Lead (Pb) and Mercury (Hg).

9.3 Results

9.3.1 Wetland macrophytes used for the study

In this study the roots of the bulrush (*Typha capensis*) (Figure 9.2A), the common reed (*Phragmites australis)*(Figure 9.2B), and the exotic weed *Persicaria lapathifolia*, a characteristic wetland macrophyte (Figure 9.2C) were used.

9.3.2 Analysis of heavy metals in plant root tissue

9.3.2.1 Metal analyses form the roots of *Typha capensis*

There were high As levels at site 5 during the low-flow season (Figure 9.3). The concentration of Cd remained relatively constant at all the sites in both seasons (Figures 9.3 and 9.4). Figure 9.3 shows that there was no variation in the concentration of Pb and As at sites 1 and 2 from the low flow season (Fig. 9.4). The Cd concentration was low in *Typhia capensis* compared to the other metals during both seasons (Figures 9.3 and 9.4). *Typhia capensis* were not available at all the sites during the high flow and low flow seasons.

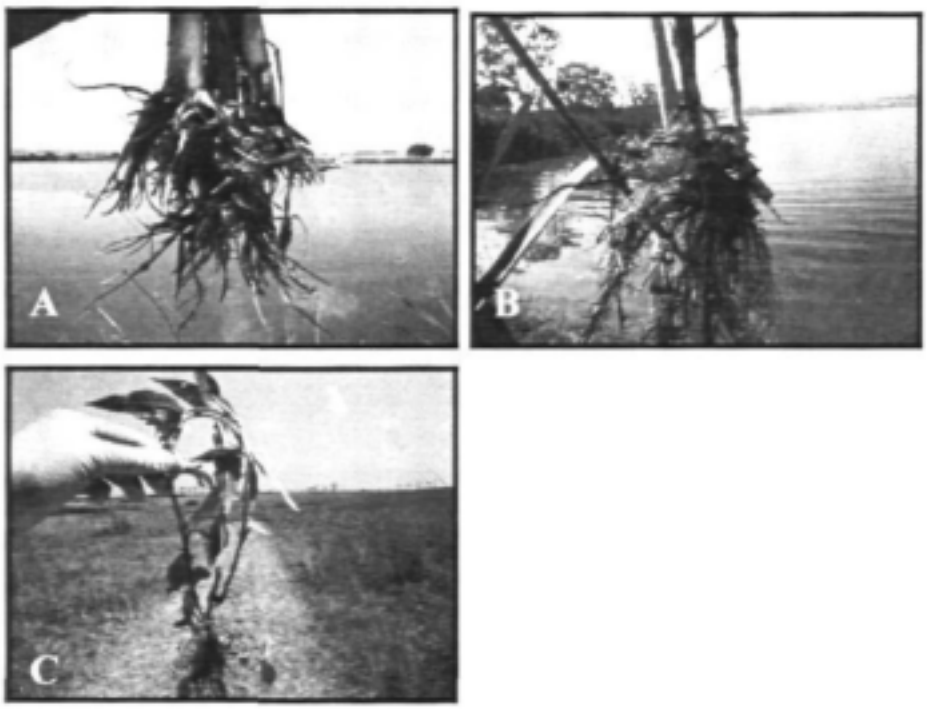

Fig. 9.2A, B and C: A. The roots of the bulrush, *Typha capensis*; (B) The roots of *Phragmites australis* and (C) The exotic weed *Persicaria lapathifolia*.

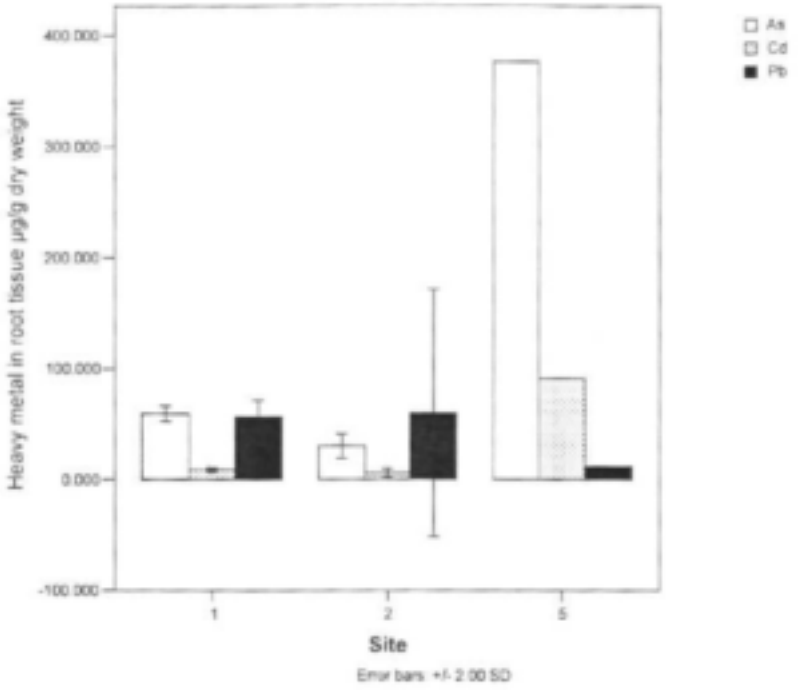

Fig. 9.3: Mean metal concentration (+/S.D.) in root tissues of *Typhia capensis* at different sites during a high-flow season (µg/g dry weight).

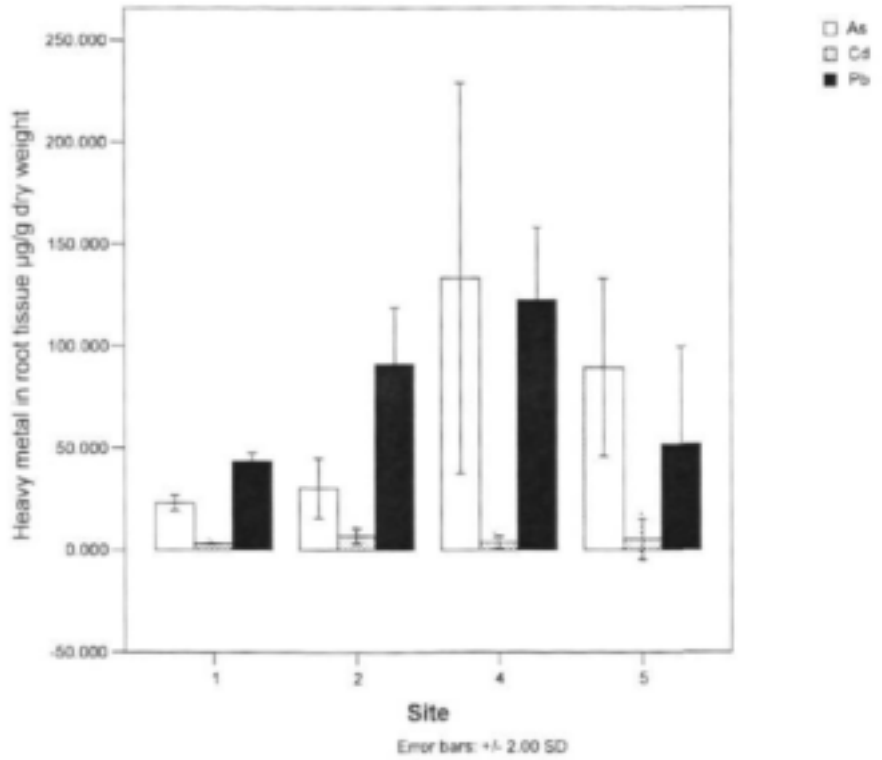

Fig 9.4: Mean metal concentration (+/S.D.) in root tissues of *Typhia capensis* at different sites during a low-flow season (µg/g dry weight).

9.3.2.2 Metal analyses form roots of the common reed *Phragmites australis*

In figures 9.5 and 9.6 the concentrations of As and Pb recorded at sites 1 and 2 were in the same range as those for *T. capensis* in the same season. *Phragmites australis* is a common reed characteristic of wetlands. The heavy metal concentration reflected an almost similar pattern to that observed for the *T. capensis* in the two seasons, except for the high Pb concentrations observed during the high flow seasons at sites 4 and 5. *P. australis* was available at all the sites during the high flow and low flow seasons.

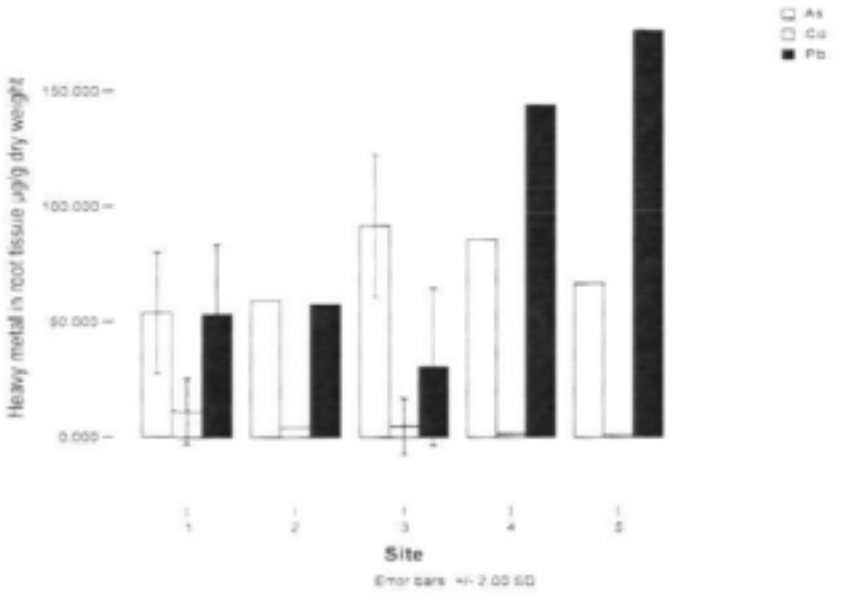

Fig 9.5: Mean metal concentration (+/S.D.) in root tissues of *Phragmites australis* at different sites during a high-flow season (µg/g dry weight).

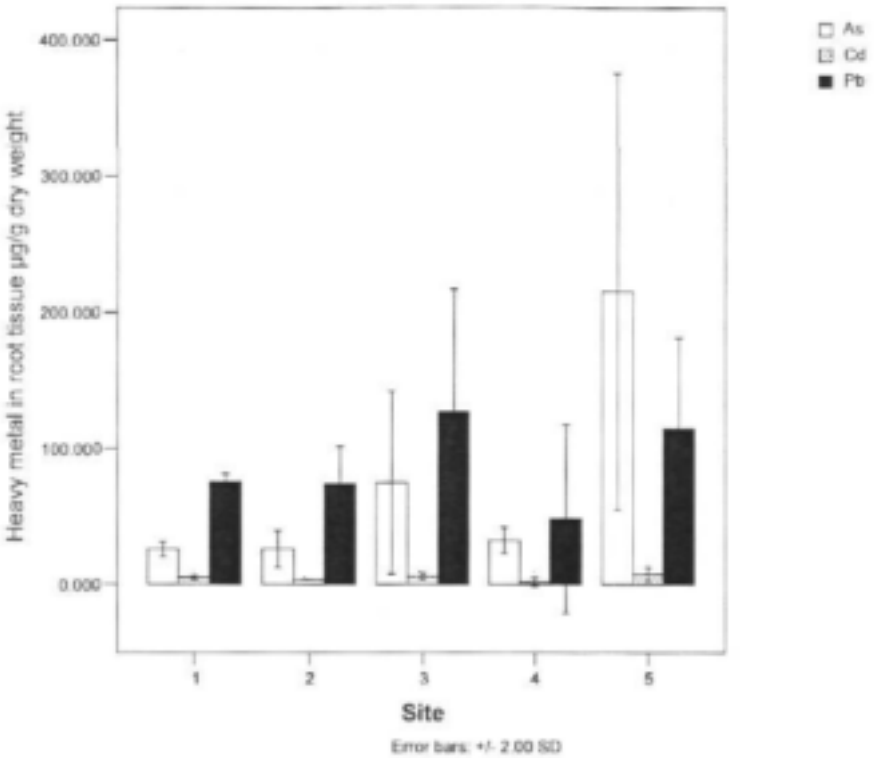

Fig. 9.6: Mean metal concentration (+/S.D.) in root tissues of *Phragmites australis* at different sites during a low-flow season (µg/g dry weight).

9.3.2.3 Metal analyses from *Persicaria lapathifolia*

Figure 9.7 shows that the high arsenic concentration in *P. lapathifolia* was in the same range as that recorded during the same season for *T capensis* (Figure 9.3). The results also reflected the same pattern of lower concentrations at sites 1 and 2. Although the concentrations detected for the exotic weed were much lower than for the other plants. According to figure 9.7 As concentration was higher again at site 5 in the roots of *P. lapathifolia*. Lead was also found in high concentrations (Figure 9.8) during the low flow season.

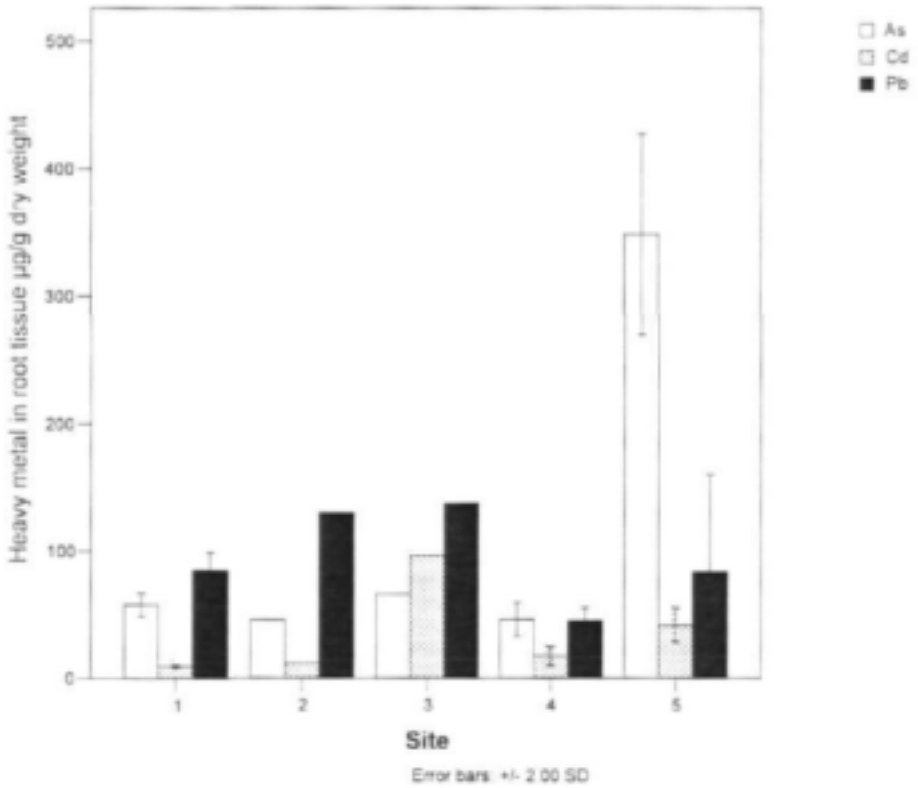

Fig. 9.7: Mean metal concentration (+/S.D.) in root tissues of *Persicaria lapathifolia* at different sites during a high-flow season (µg/g dry weight).

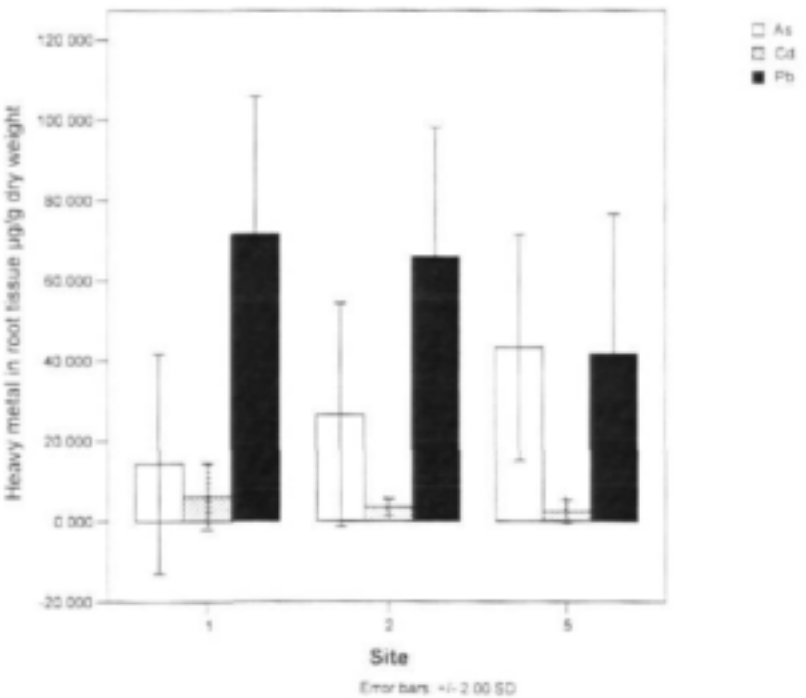

Fig. 9.8: Mean metal concentration (+/S.D.) in root tissues of *Persicaria lapathifolia* at different sites during a low-flow season (µg/g dry weight).

9.4 Discussion and conclusion

9.4.1 Heavy metal analysis in plants

After site 2, which is D1, the water passes through the wetland before discharging into D2, and the results imply possible settling of metals as they pass through the wetland. The results also suggest that there is no significant re-suspension of metals into the water column from the sediment, because the results from plant analysis show high lead and arsenic levels at sites 3, 4 and 5. The lower concentrations in the high-flow season can be attributed to dilution as the water level rose significantly.

T. capensis is a characteristic wetland macrophyte, commonly referred to as the bulrush. The arsenic concentrations in *T. capensis* were significantly lower in the high flow than the low flow at site 5. However this cannot be attributed to dilution of the water as the metals are in the plant tissue. The variation could have arisen from other environmental factors not accounted for in the study. *T. capensis* was most useful for this purpose because there is always sufficient root mass from the plant during both the high flow and the low flow season. *P. lapathifolia* is an exotic weed that is also characteristic of wetlands. It was observed that the plant flourished during the high flow season and dried out during the low flow seasons, which made it a difficult plant to use for these purposes.

At Site 5, where plants and sediment were sampled, there was a heavily silted location where there was a build up of fine sediment rich in organic matter and the flow of water is partially blocked. The flow however varied along the channel with certain parts having fast flowing waters and a solid bedrock. Biological monitoring of metal pollution requires knowledge of the spatial distribution of metals among biota to establish the association between metals in biota, metals in the environment and the availability of metals to biota (Vesk and Allaway, 1997). Plant metal levels are often found to be highly correlated with levels in the sediment, although other studies have produced contrasting results (Vesk and Allaway, 1997).

CHAPTER 10

SOUTH AFRICAN SCORING SYSTEM (SASS)

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10.1 Introduction

The South African Scoring System (SASS) is a highly successful rapid bioassessment technique, which has formed the backbone of the River Health Programme in South Africa (Uys et al., 1996). Using this method, macroinvertebrate families are scored according to their sensitivity to deterioration in water quality. SASS scores have been shown to relate directly to water quality and are particularly sensitive to organic pollution (Chutter, 1994), hence the relevance of applying this technique in the overall assessment of endocrine disrupting chemicals and heavy metals in the aquatic environment. SASS has been recommended for the determination of the flow requirements of rivers (O'Keeffe and Dickens, 2000), and has also been used for many impact assessments.

The SASS is broadly based on the water quality monitoring system developed by the Biological Monitoring Working Party (BMWP) in the UK in the early 1980s, but the scores have been adapted to local (South African) conditions and therefore differ slightly from those used in the BMWP system. The scores range from 1-15, with highly pollution tolerant species scoring low, and intolerant/highly sensitive species scoring high. The three principal indices calculated for SASS are: SASS score, number of taxa (No. Taxa) and average score per taxa (ASPT). ASPT is the sum of the 'quality' scores for each taxon noted, divided by the number of taxa. Each taxon has been assigned a 'quality' score, based on its susceptibility or resistance to pollution and perturbations. The lowest scores are assigned to the taxa that are resistant and the highest scores to those susceptible to pollution. Additional information relating to site description and any signs of disturbances is captured on the sheet to help in the later interpretation of results (Dickens and Graham, 2002).

SASS is designed to assess river health or condition as influenced by water quality, even though other minor factors may come into play. Macroinvertebrate communities play an important role in many ecological and ecotoxicological processes in lakes and rivers (Brinkhurst, 1974). Advantages of using macroinvertebrates as water quality indicators include sensitivity to chemical and physical perturbations, limited mobility to avoid discharges, ability to detect intermittent discharges, abundance and ease of collection, and vital position in the food chain. Changes in taxonomic richness and composition of macroinvertebrate communities are considered sensitive tools for detecting alterations in aquatic ecosystems (Pinel-Alloul et al., 1996). The toxicity of effluent discharged into water and sediment is complex, with synergistic and antagonistic effects. Benthic and fish communities respond accordingly, but over the long term, fish may be eliminated and the benthos are dominated by

pollution tolerant forms like tubificid worms and the chironimid larvae. Macroinvertebrate communities will give an indication of pollution problems no longer evident in water samples (Williams and Feltmate, 1992).

Plecoptera, a highly pollution sensitive group whose presence is a hallmark of stream health, are usually absent from wetlands (The Volunteer Monitor, 1998). Benthic macroinvertebrates are a highly diverse group, which makes them excellent candidates for changes in biodiversity. The time required for insect assemblages to return to their natural state, following disturbances such as those of point source industrial pollutants, can be the order of many years for streams, decades for lakes (Williams and Feltmate, 1992).

10.2 Materials and Methods

10.2.1 Sampling sites

10.2.1a Site 1 (Figure 10.1)

GPS location: S25^0 54' 671" E28^0 18' 482"

Sampling at site 1 was done just below D1. The location is a shallow channel, approximately 3m wide. It is characterized by fast flowing water and a solid rock bed. Grasses and exotic weeds dominate the fringing vegetation.

10.2.1b Site 3 (Figure 10.1)

GPS location: S25^0 52' 911"E28^0 17' 727"

This site is characterized by fast-flowing waters and large boulders on the substratum, above D1. The water depth is greater than at site 1. The fringing vegetation is mainly bulrush and grasses.

10.2.1c Site 5 (Figure 10.1)

GPS location: S25^0 52' 701"E28^0 15' 385"

This sampling location is outside the vlei on the Sesmylspruit. The substratum is heavily silted and the water is discoloured. The riparian vegetation is mainly trees and shrubs. It is also a cattle drinking point.

Fig.10.1: Sites 1-3 where SASS was performed during this study.

10.2.2 Macroinvertebrate sampling

Kick sampling was done using a SASS net from the full range of biotopes available at each site:

-stones in current, stones out of current

-sand, mud and gravel

-marginal vegetation

The collected sample was tipped into a large rectangular tray filled with stream water. Identification is done on site within 15 minutes. Unidentified organisms are preserved in Rose

Bengal for later identification in the laboratory. All the invertebrate families present were scored according to the scores allocated on the standard field sheet. The sample score, number of taxa and the ASPT were calculated from the completed field sheet. Other physical and chemical parameters at each site were recorded. When all biotopes had been sampled, an integrated habitat assessment (IHAS) was done at each site. Water and sediment were also collected at each site.

10.2.3 Using SASS5 score sheet

An example of a SASS Score sheet and how to use it during SASS analyses is given in Figure 10.2 and described below.

On the score sheet a score can be entered for each taxon of invertebrates identified in the field in each biotope. A list of indicator organisms whose sensitivity has already been determined is given on the SASS sheet. For example if the taxon Corixidae was identified in all of the three biotopes the predetermined score of 3 given on the sheet and this value is entered under the biotopes Stone (S), Vegetation (VG) and Gravel Sand and Mud (GSM). At each site, and for every taxon identified, a total score for all the biotopes is given. In the example of Corixidae the total is 3. At the bottom of the list a total is given of all the scores under each biotope (e.g. Sand) per site. To get the average score per taxa (ASPT), the SASS total at the bottom is divided by the number of taxa. Further explanations on ASPT are given in the results and discussion.

10.3 Results

10.3.1 Low-flow sampling 2004 (LF1)

Table 10.1 shows SASS results from the first low-flow season. The lowest ASPT for all biotopes was at site 3. Site 5 had the lowest number of taxa.

5. SASS Version 5 Score Sheet -

Version date: Feb 2005

Date:	/ /	Grid reference (dd.mmm ss.s) Lat: S	(dd.ddddd)
RHP Site Code:	-	Long: E	
Collector/Sampler:		Datum (WGS84/Cape):	
River:		Altitude (m):	m
Level 1 Ecoregion:		Zonation:	
Quaternary Catchment:			
Site Description:	Temp (°C):	Cond (mS/m)	
	pH:	Clarity (cm):	
	DO (mg/L):	Turbidity:	
	Flow:	Colour:	
	Riparian Disturbance:		
	Instream Disturbance:		

Biotopes Sampled	Rating (1 - 5)	Time (min)
Stones In Current (SIC)		
Stones Out Of Current (SOOC)		
Bedrock		
Aquatic Veg		
Marg/Veg In Current		
Marg/Veg Out Of Current		
Gravel		
Sand		
Mud		
Hand picking/visual observation		

Taxon		S	Veg	GSM	TOT
PORIFERA	5				
COELENTERATA	1				
TURBELLARIA	3				
MOLLUSCA					
Gastropoda	1				
Hirudinea (Leaches)	3				
CRUSTACEA					
Amphipoda	13				
Potamonautidae*	3				
Atyidae (Shrimps)	8				
Palaemonidae	10				
HYDRACARINA (Water	8				
PLECOPTERA					
Nemouridae	14				
Perlidae	12				
EPHEMEROPTERA					
Baetidae 1 sp	4				
Baetidae 2 sp	8				
Baetidae > 2 sp	12				
Caenidae	6				
Ephemeridae	15				
Heptageniidae (Flatheaded	13				
Leptophlebiidae	9				
Oligoneuriidae (Brushlegged	15				
Polymitarcyidae (Pale	10				
Prosopistomatidae (Water	15				
Teloganodidae	12				
Tricorythidae (Short Winged	9				
ODONATA (Dragonflies & Damselflies)					
Calopterygidae	10				
Chlorocyphidae	10				
Coenagrionidae	4				
Lestidae (Emerald	8				
Platycnemididae (Stalk	10				
Protoneuridae	8				
Aeshnidae (Hawkers &	8				
Corduliidae	8				
Gomphidae	8				
Libellulidae	4				
MEGALOPTERA (Aquatic Dobsonflies)	12				

Taxon		S	Veg	GSM	TOT
HEMIPTERA					
Belostomatidae* (Giant water	7				
Corixidae* (Water	3				
Gerridae* (Pond skaters/Water	5				
Hydrometridae* (Water	6				
Naucoridae* (Creeping water	7				
Nepidae* (Water	3				
Notonectidae*	3				
Pleidae* (Pygmy	4				
Veliidae/M. veliinae* (Ripple	5				
MEGALOPTERA (Alderflies, Dobsonflies & Alderflies)					
Corydalinae (Families &	6				
Sialoidea (Alderflies)	6				
TRICHOPTERA					
Dipseudopsidae	10				
Ecnomidae	6				
Hydropsychidae 1 sp	4				
Hydropsychidae 2 sp	5				
Hydropsychidae > 2 sp	13				
Philopotamidae	10				
Polycentropodidae	12				
Psychomyiidae/Ecnomidae	8				
Cased caddis:					
Barbarochthonidae	13				
Calamoceratidae	11				
Glossosomatidae	11				
Hydroptilidae	6				
Hydrosalpingidae	15				
Leptoceridae	10				
Petrothrincidae	11				
Pisuliidae	10				
Sericostomatidae	13				
COLEOPTERA					
Dytiscidae/Noteridae* (Diving	5				
Elmidae/Dryopidae* (Riffle	8				
Gyrinidae* (Whirligig	5				
Haliplidae* (Crawling water	5				
Hydrophilidae (Marsh	12				
Psephenidae* (Aquatic moss	8				
Scirtidae* (Water scavenger	5				
Limnichidae	10				
Ptilodactylidae (Water	10				

Taxon		S	Veg	GSM	TOT
DIPTERA (Flies)					
Athericidae	10				
Blephariceridae (Mountain	15				
Ceratopogonidae (Biting	5				
Chironomidae	2				
Culicidae*	1				
Empididae* (Dance	6				
Ephydridae (Shore	3				
Muscidae (House flies, Stable	1				
Psychodidae (Moth	1				
Simuliidae	5				
Syrphidae* (Rat-tailed	1				
Tabanidae (Horse	5				
Tipulidae (Crane	5				
GASTROPODA					
Ancylidae (Limpets)	6				
Bulinus*	1				
Hydrobiidae*	3				
Lymnaeidae* (Pond	3				
Physidae* (Pouch	3				
Planorbinae* (Orb	3				
Thiaridae*	3				
Viviparidae* ST	5				
BIVALVIA					
Corbiculidae	5				
Sphaeriidae (Pills	3				
Unionidae (Perly	6				
SASS Score					
No. of Taxa					
ASPT					
Other biota					
Comments/Observations					

Procedure:

Kick SIC & bedrock for 2 mins, max. 3 mins. Kick SOOC & bedrock for 1 min. Sweep marginal vegetation (SIC & OOC) for 2m total and aquatic veg 1m. Sh & sweep gravel, sand, mud for 1 min total. * = acceptable
Hand picking & visual observation for 1 min - record in biotope where found (by circling estimated abundance on score sheet). Score for 15 macroinvertebrates but also if no new taxa seen after 5 mins.
Estimate abundances: 1 = 1, A = 2-10, B = 10-100, C = 100-1000, D = >1000 S = Stone, rock & solid objects, Veg = Kill vegetation, GSM = Gravel, sand, mud SWC = South western Cape, T = Transvaal, KT = Sub-tropical
Rate each biotope sampled: 1=very poor (i.e. limited diversity) 5=highly suitable (i.e. wide diversity)

Fig. 10.2: An example of a SASS score sheet to be completed during assessment.

Table 10.1: Summary results for SASS indices over the three sites from the UNR wetland system during the low flow season in 2004 (LF1).

Biotope	Indices	Site 1	Site 3	Site 5
All biotopes combined	Score	41	33	12
	No. Taxa	10	10	3
	ASPT	4.1	3.3	4
Stones	Score	37	25	20
	No. Taxa	9	7	4
	ASPT	4.11	3.57	5
GSM	Score	13	22	3
	No. Taxa	3	6	1
	ASPT	4.33	3.67	3
Vegetation	Score	3	16	11
	No. Taxa	2	5	2
	ASPT	1.5	3.2	5.5

10.3.2 High-flow sampling 2005 (HF1)

Table 10.2 shows that in all biotopes site 5 still had the lowest number of taxa, as recorded in the first sampling season.

Table 10.2: Summary results for SASS indices over the three sites from the UNR wetland system during the high flow season in 2005 (HF1).

Biotope	Indices	Site 1	Site 3	Site 5
All biotopes combined	Score	42	40	7
	No. Taxa	11	12	3
	ASPT	3.81	3.3	2.33
Stones	Score	34	9	4
	No. Taxa	9	3	2
	ASPT	3.8	3	2
GSM	Score	23	17	3
	No. Taxa	6	5	1
	ASPT	3.8	3.4	3
Vegetation	Score	24	21	3
	No. Taxa	6	6	1
	ASPT	4	3.5	3

10.3.3 Low-flow sampling 2005 (LF2)

Table 10.3 shows SASS scores from the second low flow season. The ASPT had decreased markedly particularly at sites 1 and 5, compared to the previous low-flow season.

Table 10.3: Summary results for SASS indices over the three sites from the UNR wetland system during the low flow season in 2005 (LF2).

Biotope	Indices	Site 1	Site 3	Site 5
All biotopes combined	Score	**22**	**29**	**11**
	No. Taxa	**7**	**7**	**4**
	ASPT	**3.14**	**4.14**	**2.75**
Stones	Score	**19**	**25**	**8**
	No. Taxa	**6**	**6**	**2**
	ASPT	**3.16**	**4.16**	**4**
GSM	Score	**9**	**12**	**0**
	No. Taxa	**3**	**4**	**0**
	ASPT	**3**	**3**	**0**
Vegetation	Score	**12**	**17**	**3**
	No. Taxa	**4**	**4**	**2**
	ASPT	**3**	**4.25**	**1.5**

10.3.4 High-flow sampling 2006 (HF2)

The ASPT values recorded in Table 10.4 for the second high-flow season did no show great variation from those recorded in the first high-flow seasons.

Table 10.4: Summary results for SASS indices over the three sites from the UNR wetland system during the high flow season in 2006 (HF2).

Biotope	Indices	Site 1	Site 3	Site 5
All biotopes combined	Score	**28**	**34**	**7**
	No. Taxa	**9**	**10**	**3**
	ASPT	**3.11**	**3.4**	**2.33**
Stones	Score	**21**	**9**	**6**
	No. Taxa	**6**	**4**	**2**
	ASPT	**3.5**	**2.5**	**3**
GSM	Score	**19**	**16**	**3**
	No. Taxa	**7**	**5**	**1**
	ASPT	**2.71**	**3.2**	**3**
Vegetation	Score	**14**	**17**	**4**
	No. Taxa	**4**	**5**	**2**
	ASPT	**3.5**	**3.4**	**2**

10.3.5 Summary of ASPT for all biotopes

According to figure 10.3 the lowest ASPT was recorded during the second high flow season at all three sites. At sites 1 and 5 the highest Apt was recorded during the first low flow season, suggesting that it may have decreased over the last three seasons. Over the last three sampling seasons the lowest ASPT was at site 5. In the last two seasons site 3 had higher ASPT than site 1.

10.3.6 Integrated habitat assessment (IHAS)

Table 10.5 indicates the integrated habitat assessment at all the sites with highest score (100) found at site 1 during the first low flow season while the lowest score (64) was found at site 5 during the second high flow season. The IHAS scores are recorded as part of SASS, at every site where invertebrates have been sampled. The total IHAS score is obtained from adding the Habitat total and the Stream Condition value. The habitat total is obtained by adding the values for Stones in current, Vegetation and Other general habitat. The maximum possible score at any site is 100.

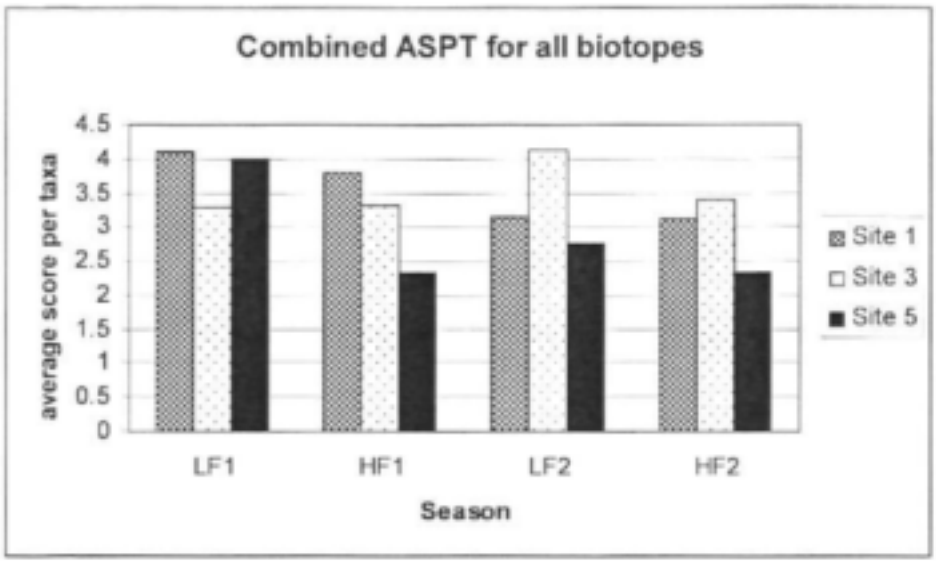

Fig. 10.3: Average score per taxa for all biotopes from the four sampling seasons at the three sampling locations.

Table 10.5: Summary of the IHAS scores of the three sites from the UNR Wetland System during the four sampling seasons.

SAMPLING HABITAT	Site 1				Site 3				Site 5			
	LF1	HF1	LF2	HF2	LF1	HF1	LF2	HF2	LF1	HF1	LF2	HF2
Stones in current	9	11	14	17	14	17	20	20	13	20	20	15
Vegetation	7	11	11	11	10	15	8	11	15	14	15	10
Other habitat general	20	18	16	16	20	17	14	13	20	18	14	9
Habitat total	38	40	41	44	44	49	42	44	48	52	49	34
Stream condition	26	33	39	37	26	31	31	36	32	28	28	30
Total IHAS score	100	73	80	81	70	80	73	80	80	80	77	64

10.4 Discussion and conclusion

Benthic macroinvertebrate species are differentially sensitive to many biotic and abiotic factors in their environment. Hence macroinvertebrate community structure has commonly been used as an indicator of the condition of the aquatic ecosystem. SASS 5 is one of the numerous biotic index systems that have been developed, which give numerical scores to specific 'indicator' organisms at a particular taxonomic level. SASS data are meaningful only when assessed together with various factors that may influence the scores. Most important of these are measures of habitat quantity, quality and diversity (Dickens and Graham, 2002). Chutter (1998) points out that ASPT is a more reliable measure of the health of good quality rivers (as opposed to poor quality river) than the SASS score is.

The average water depth and flow rates were higher during the high flow seasons. The ASPT values recorded during HF2 indicated that there had not been a significant change on a temporal scale of four seasons, when compared with the data recorded at the beginning of the survey in LF1, especially with regards to site 3. This may be explained by Williams and Feltmate's (1992) statement that the time required for insect assemblages to return to their natural state, following disturbances such as those of point source industrial pollutants, can be the order of many years for streams, decades for lakes. Therefore, variations between seasons could not have been expected to be by high magnitudes. There was however, a steady decline in ASPT at site 1 from the first sampling LF1 to the last season HF2.

A high density of chironomids was noted at site 1 during LF1 and HF1 and this, with an absence of mayflies and stoneflies in water bodies is usually indicative of industrial pollution (Williams and Feltmate, 1992). Presence of numerous families of highly tolerant organisms usually indicates poor water quality (Graham and Dickens, 2002). The Baetis species (Family *Baetidae*) consistently appeared at sites 1 and 3 in all the seasons. In the last two seasons (LF2 and HF2), it was identified at site 1 only. It is one of the pollution tolerant organisms and has a low score on the SASS sensitivity scale. Also notable was the abundance of leeches at site 5. Leech abundance is highly variable in the natural ecosystem, but generally increases in more productive fresh waters. They also have a low quality score on the sensitivity scale.

The results obtained at site 3 during LF2 and HF2 indicate that site 3 supports a more diverse population of macroinvertebrates than site 1, suggesting that the water is less polluted at site 3. Site 3 is likely to be less polluted because after site 1 the water passes through a wetland. A high ASPT for site 5 was recorded only during the first sampling. In the last three seasons the ASPT at site 5 was the lowest. Site 5 is the last point of discharge after D2 and it showed poor diversity in the macroinvertebrate community structure. This may be due to the disturbance caused by the impoundment. Anthropogenic control over the flow of running water, usually by means of dams and reservoirs, has influenced nearly all of the world's major rivers. In most cases impoundments have caused mayflies, caddisflies and stoneflies to immediately disappear and be replaced by a high density of midges (William and Feltmate, 1992). Important to note is the fact that where habitat diversity is poor, there will be less biotic diversity and consequently a lower SASS score. However, ASPT will be less affected because the few organisms present may have the appropriate sensitivity.

Where habitat is poor a low SASS score cannot be attributed to poor water quality. An IHAS score below 60 indicates poor habitat. In this survey the habitat scores were within the normal range (Table 3.5). Therefore, all the observations made pertaining to the macroinvertebrate structure can be attributed to poor water quality in the system.

CHAPTER 11

HEALTH RISK ASSESSMENT

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11.1 Introduction

This forms part of a broader project looking at the application of biological and chemical assays for measuring endocrine disruption activity and the potential health risks in the Urban Nature Reserve (UNR).

The broader study examined water, sediment and biota samples for endocrine disruptors. This component of the study examines the potential human health risks as a result of the endocrine disruptors encountered, using a health risk assessment approach.

11.2 The Health Risk assessment process

The general human health risk assessment process consists of four distinguishable but interacting phases, generally referred to as ❶ hazard identification, ❷ dose-response assessment, ❸exposure assessment, and ❹ risk characterisation (United States Environmental Protection Agency (US EPA), 1998; National Research Council (NRC), 1983).

The interrelation of these phases is depicted in Figure 11.1 following.

Fig. 11.1: Integrated Health Risk Assessment Process.

11.2.1 Hazard Identification

The first step in risk assessment is the identification or recognition of potential health hazards. There should be substantial evidence that the chemical constituent is found in drinking-water. Combined with occurrence information, there should also be evidence of potential or actual toxicity of the chemical constituent (World Health Organization (WHO), 2004).

Based on this, potentially hazardous chemicals are divided according to their effects and response (mode of action). For risk estimation purposes, chemicals are divided into three main groups (WHO, 2003):

- **Toxic chemicals** – Toxic chemicals have a threshold effect, below which no harmful effect is expected. These chemicals may have a wide range of effects and can act on different biological systems such as the immune, reproductive, respiratory or neurological systems.
- **Carcinogens** – Carcinogens are assumed to have no threshold where no adverse effect is expected. Exposure to any concentration of chemical is thought to have some associated risk.
- **Endocrine disruptors** – Endocrine disrupting compounds are suspected to interfere with the normal functioning of the endocrine system. The endocrine system regulates the development, growth, reproduction, and behaviour of humans and wildlife. Although endocrine disrupting compounds cause serious concerns, the data necessary to apply **a quantitative** chemical risk assessment is unavailable at this stage.

It is often very difficult to conclude with certainty that a chemical is carcinogenic. Based on this uncertainty, the International Agency for Research on Cancer (IARC, 1982; 1987; 1991) classified carcinogens into four groups (Groups 1 – 4) according to their potential carcinogenicity (Table 11.1). Classification of carcinogens into one of these groups depends on whether sufficient dose-response data were derived from human or animal studies. The evidence portrays the certainty of the data that a chemical is carcinogenic based on animal or human studies. The IARC approach was adopted by the WHO (2004). Similarly, the US EPA (1998) developed their "weight-of-evidence" approach, which divides chemicals into Group A - E.

Table 11.1: Carcinogen classification groups (International Agency for Research on Cancer (IARC), 1982; 1987; 1991; US EPA, 1998).

IARC APPROACH (ADOPTED BY WHO)		US EPA "WEIGHT-OF-EVIDENCE" APPROACH	
1	The agent is carcinogenic to humans	A	Known human carcinogen
2A	The agent is probably carcinogenic to humans	B1	Probable human carcinogen, limited data
2B	The agent is possibly carcinogenic to humans	B2	Probable human carcinogen, inadequate data
3	The agent is not classifiable as to its carcinogenicity to humans	C	Possible human carcinogen
4	The agent is probably not carcinogenic to humans	D	Not classified as human carcinogen
		E	Evidence that not carcinogenic to humans

11.2.2 Dose Response Assessment

The second step examines background information available for dose-response studies. Sensitivity/vulnerability of the study community should always be taken into consideration when performing a risk assessment. Sensitive subpopulations would include infants (below five years of age), elderly, immuno-compromised individuals (e.g., HIV/AIDS infected people or people with TB, cancer patients receiving chemotherapy), and also people suffering from existing debilitating disease or infection, factors associated with lifestyle (e.g., smoking), etc. For example, the target organ for various hazardous agents may be the liver and, as the liver has a detoxifying function, malfunctioning of the liver may lead to aggravated adverse health effects. Although all these factors cannot be accounted for, health managers and risk assessors should keep these in mind by using conservative or protective estimates.

11.2.2.1 Toxic (threshold) approach

For chemicals classified as "toxic", it is assumed that there is a dose (threshold) below which no adverse effect will occur even if a person is exposed to this concentration on a daily basis for the rest of his / her life (lifetime exposure) (WHO, 2004; US EPA, 2002(a)). It causes an increase in the likelihood or accelerates the onset of cancer. As such the risk assessment approach for toxic effects involves estimating an exposure that is below this threshold level. This is done by applying uncertainty factors to exposures that appear to be near this threshold to result in a safe dose (Committee on Carcinogenicity (COC), 2004; US EPA, 2003).

For chemical constituents giving rise to such toxic effects, a tolerable daily intake (TDI) or reference dose (RfD) or reference concentration (RfC) is calculated (WHO, 2004, US EPA 2002a,b). The reference dose is protective of sensitive subpopulations. Since the safe dose is derived for exposure of humans during their lifetime, it is not so precise that it cannot be exceeded for short periods of time. Short-term exposures exceeding the safe dose are not a problem. However, an individual's intake of a chemical toxicant averaged over a longer period should not appreciably exceed the set level (WHO, 2004; enHealth Council, 2002).

The safe dose derived is based on different datasets available in the literature (Toxicology Excellence for Risk Assessment (TERA), 2005; Integrated Risk Information System (IRIS), 2004). Studies may have observed adverse effects at different concentrations of chemical toxicants. Other studies report on concentration of chemical toxicants where no adverse effect is observed. This is known as the 'no-observed-adverse-effect-level' (NOAEL). Similarly, the data available can be from the "lowest-observed-adverse effect-level" (LOAEL), which is the lowest observed dose or concentration of a substance at which there is a detectable effect (TERA, 2004).

Figure 11.2 illustrates a typical non-cancer dose response curve. Toxicologists have calculated the safe dose of hazardous toxicants. Since their results are based on human studies combined with toxicology studies on test organisms used to determine a safe dose, a large uncertainty factor is introduced that should be addressed during risk estimation.

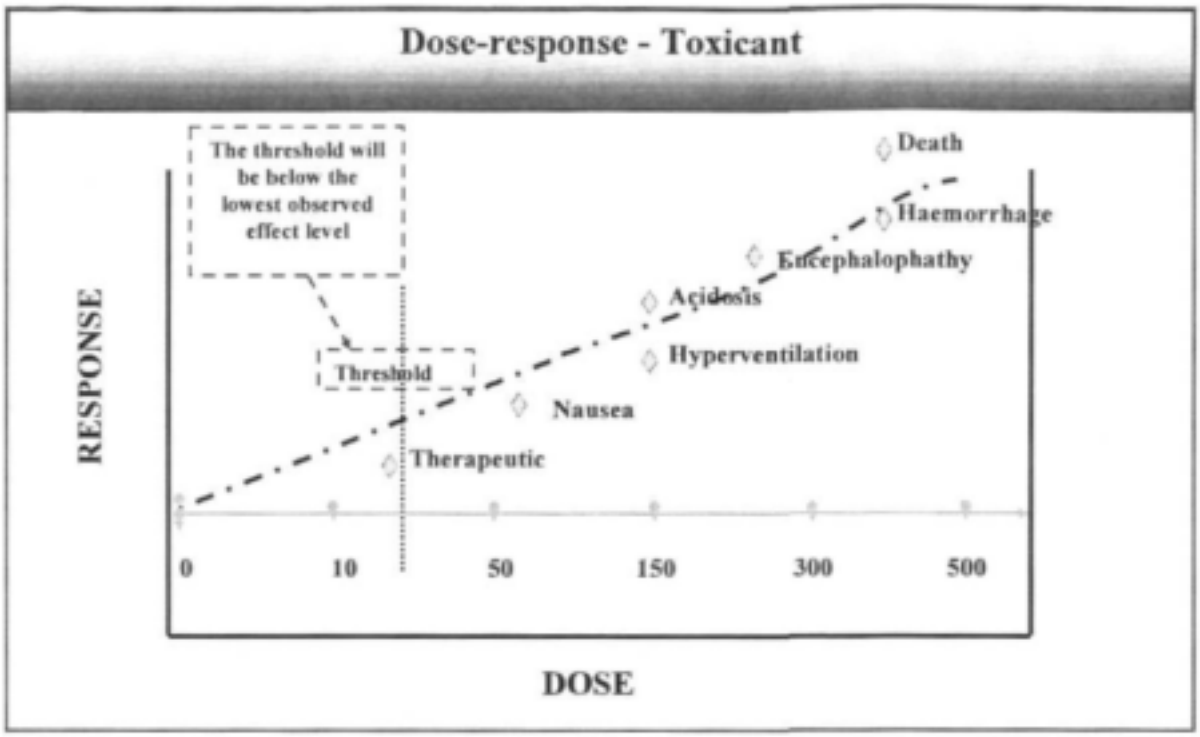

Fig. 11.2: Threshold dose response for non-carcinogens (US EPA, 2002(b)).

11.2.2.2 Uncertainty factor (safety factor)

The derivation of uncertainty (safety) factors requires expert judgement and careful consideration of the available scientific evidence (WHO, 2004). The uncertainty factors are used with toxicity data to take into account differences in sensitivity to toxic effects within and between species (interspecies and intraspecies related) and differences in toxic effects between chronic and sub-chronic exposures. Other factors that cause major uncertainty are the quality of the database and the nature and severity of the effects (International Programme on Chemical safety (WHO, 1994; US EPA, 2003).

The uncertainty factors applied to determine the safe dose generally range between 1 – 10 000. The WHO (2004) recommends that if the uncertainty factor exceeds 1 000 that permissible guideline levels be set. These would need to be revised as new data becomes available.

11.2.2.3 Carcinogenic (non-threshold) approach

Carcinogens are assumed to have no threshold value (WHO, 2004; US EPA, 2003). This means that there is a probability of adverse health effects in humans at any level of exposure. This adverse effect is normally expressed as a risk of producing tumours. For the non-threshold approach a potency factor (slope factor) is determined based on mathematical extrapolation models. Over the years various extrapolation models have been developed (e.g.

multistage model, linearised multistage model, strict threshold model, log probit model, Weibull model and the one-hit model). The multistage linearised model is the most frequently used extrapolation model (COC, 2004; US EPA, 1992; US EPA, 2002(a)).

Figure 11.3 shows a typical example of an extrapolation model with a slope factor (β). The dashed blue line indicates the actual observation data, while the dotted red line is the 95th % confidence limit of the solid line. The dash-dotted pink line is then extrapolated to low dose exposures to represent the effects expected at low doses. This extrapolation causes uncertainty as to the quality of data. The slope factor is that part of the 95th % confidence interval extrapolated to represent the effects at low doses and is indicated by the purple solid line. The slope factor therefore has an inherent uncertainty or safety factor.

The slope factor is expressed as per mg/kg/day and can be used to compare the relative potency of different chemical substances.

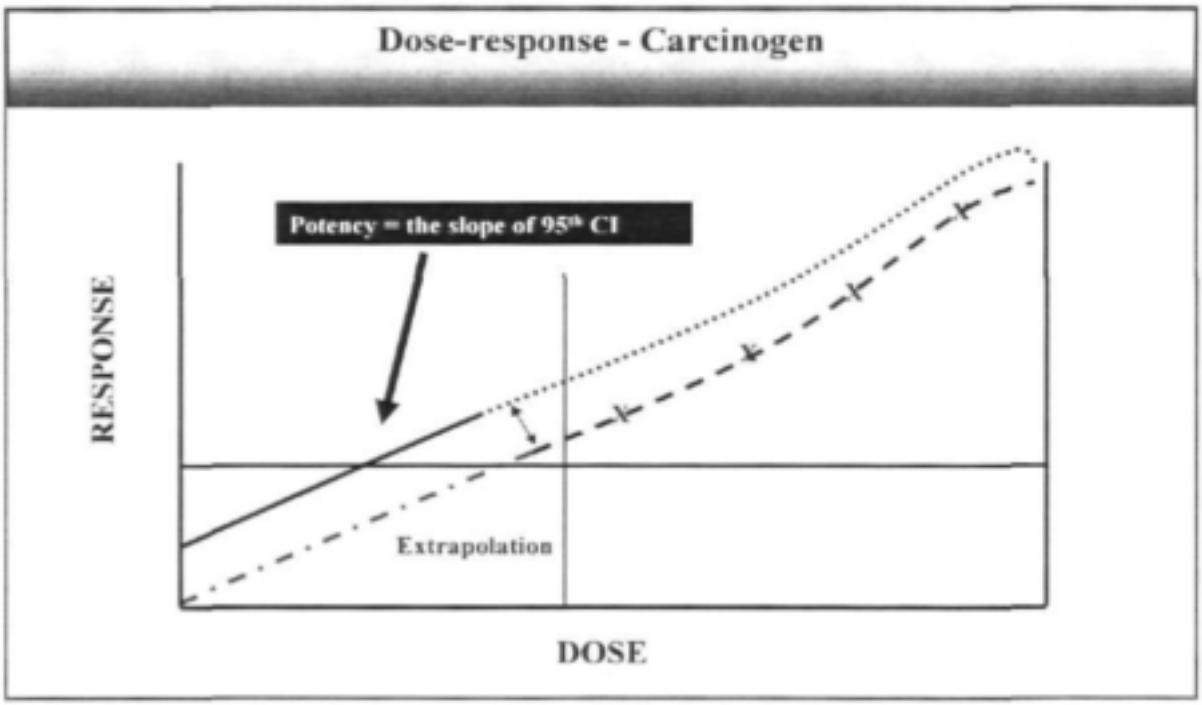

Fig. 11.3: Typical dose-response curve for non-threshold effects with a slope factor (US EPA, 2002(b)).

11.2.2.3a EDC approach (no quantitative method yet)

Contrary to the above, responses to hormones are different. High doses of hormones and chemicals can block rather than stimulate some responses resulting in what is called a non-monotonic dose-response relationship (Myers et al., 2003; Welshons et al., 2003). This

non-monotonic dose-response relationship is illustrated in the figure below (Figure 11.4). Effects initially increase and then decrease with increasing dose.

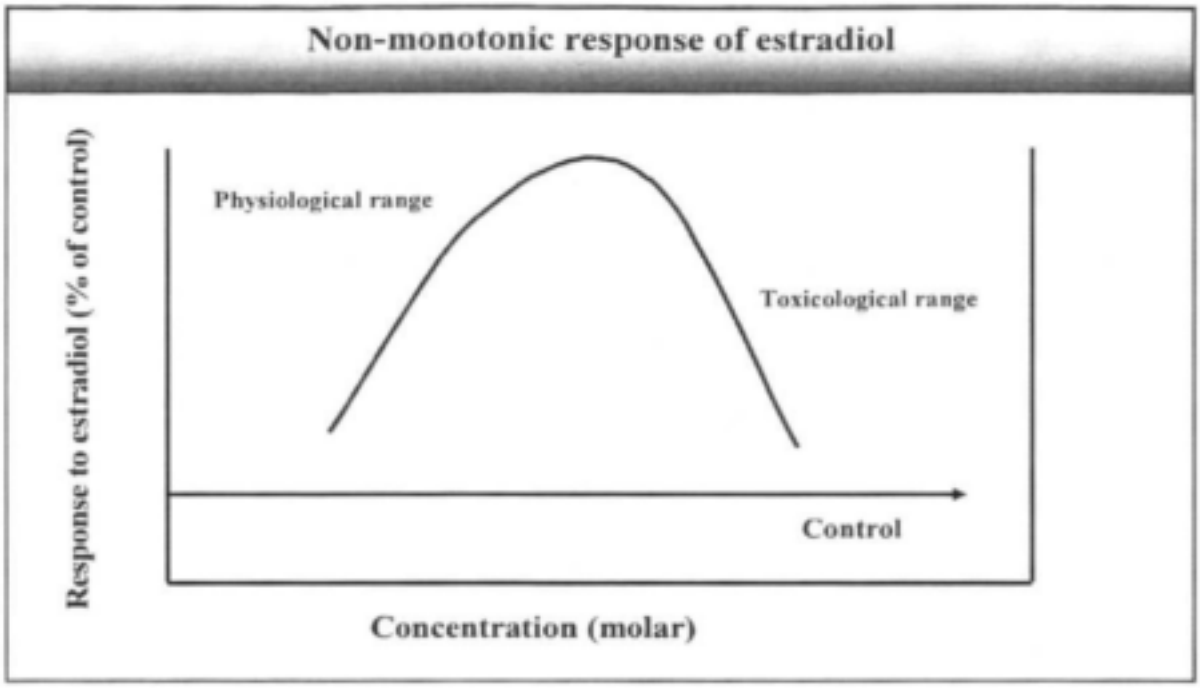

Fig. 11.4: Example of a non-monotonic dose-response curve.

Population based epidemiological studies relevant to endocrine disruption are few and often limited by factors such as the time lag between exposure and clinical disease (Myers et al., 2003; Solomon and Schettler, 2000). ***The ability of epidemiological studies to identify the cause of an adverse effect decreases as the prevalence of the effect and the number of causal factors increase*** (Myers et al, 2003). It is difficult to establish cause and effect relationships for human exposure of endocrine disruptors and incident disease (Myers et al., 2003). We have no control since everyone has been exposed to endocrine disrupting chemicals at sometime or the other.

We encounter a broad range of environmental exposures throughout our lives and frequently to a mixture of chemicals at any one time. Much work remains to be done on the study of the human health impact of exposure to endocrine disruptors particularly in view of the exposure experienced by people to a mixture of these chemicals (WHO, 2003). Developmental toxicity can result from exposure of either parent prior to conception from exposure of the embryo *in utero* or from exposure of the progeny after birth. *In vivo* studies on pregnant animals and their progeny have been widely used in developmental toxicity assessment (WHO, 2003). Developmental effects of endocrine disruptors tend to

be **latent** where traditional endpoints of toxicity may not be detectable until sexual maturity (Daston et al., 2003).

It is essential that the correct end-point be examined when conducting the dose-response assessment of a chemical thought to be an endocrine disruptor (Mantovani et al., 1999; Mantovani, 2002). The elucidation of molecular mechanisms of endocrine disrupters should make for the reasonable risk assessment and for defining the endpoints of endocrine disruption. This would help classify which compounds be removed from the environment.

11.2.3 Exposure assessment

Humans can be exposed to chemicals via carrier media other than water such as air, soil and food. Generally, exposure can occur by means of three routes / pathways which include ingestion (oral), inhalation and dermal absorption. Important exposure factors related to humans include body weight, food and drinking water consumption, and skin surface areas related to different types of exposure (WHO, 2003; US EPA, 1992).

Often humans are exposed to chemicals via more than one carrier media or exposure pathway. It should be noted that a source in one medium (e.g. potable water) may lead to additional intake from other routes (e.g. dermal and inhalation) (WHO, 1994).

The application of an integrated risk assessment framework makes provision for estimating the risk of hazardous toxicants to human health via different routes and media. The proposed method not only provides for exposure to organic toxicants in water through ingestion, but also via inhalation and dermal absorption (e.g., while showering or bathing), but also for exposure to the organic toxicant via other media (e.g. air, food and or soil) as humans may be exposed to organic toxicants via a variety of routes at any given time.

11.2.3.1 Calculations of chemical concentrations in the environment

Monitoring data of the concentrations of various hazardous chemicals in environmental carrier media (air, food, soil, and water) is necessary to calculate the total dose a person may be exposed to.

11.2.4 Risk characterization

Risk characterisation is the process of calculating the incidence of the health effect under the conditions of exposure described in the exposure assessment, using the identified dose-response relationship. Guidelines provided by the US EPA (1987) distinguish two components of risk characterisation, namely:

- presentation of a numerical estimate of risk, and
- analysis of the significance of the risk estimate.

Risk estimates may be presented in a number of forms, depending on the context of the risk assessment (US EPA, 1987; National Institute of Public Health and the environment (RIVM), 1997).

11.2.4.1 Toxicants

For chemicals that cause **toxic effects,** the risk is calculated comparing the estimated exposure to an exposure assumed to be safe. The risk is approximated using the following formula:

$Risk = \frac{ADD}{RfD}$ A risk estimate over 1 is considered to be unacceptable (US EPA, 1987).

11.2.4.2 Carcinogens

For chemicals that cause **cancer**, risk is calculated as a function of oral potency factor (β) and dose. This is approximated by the following equation:

$Risk = \beta \times LADD$ The risk estimates represent the theoretical excess cancer risk.

This is the risk of developing cancer in addition to the background cancer incidence. An example of this can be described as follows: If the cancer risk is described as 1 e-5 = 0.00001 = 1/ 100 000, then it can be said that there is an excess risk of developing cancer of 1 in a hundred thousand.

The WHO (2003) defines the acceptable risk level as "an estimated upper-bound excess lifetime cancer risk of one additional cancer per 100 000 of the population ingesting drinking water containing the substance at the set guideline value for 70 years (life expectancy)". Regulatory authorities should, however, make an informed decision based on the collected risk estimation information and decide on an acceptable risk level for local circumstances.

The WHO (2004) and various countries world-wide have set their acceptable risk level at 10^{-5}. In cases where an excess lifetime cancer risk of 10^{-5} is not feasible or practical because of inadequate analytical or treatment technology, the WHO recommends that a provisional guideline value is set at a practical level and the estimated associated cancer risk presented.

11.3 Endocrine disrupting chemicals in the UNR

The US EPA methodology (1998) was used to calculate both toxic and carcinogenic risks to humans. A number of targeted organic chemicals and metals were tested for in Dam 1 (D1) and Dam 2 (D2)

(Chapter 4). Only a selection was found to be present in the water above the detection limits of the test. These included lindane, DDT, DDD, DDE, endrin aldrin, BHC, DEHP, DEP, DBP, PCBs and heptachlor. The metals Cd, As, Pb, Hg were also tested for and included in the health risk assessment. In addition to being toxic in their own right, they are also known to have additional additive effects on endocrine disrupting chemicals (Chapter 2.3).

11.3.1 Hazard assessment

The following chemicals were found in either water or sediment samples from D1 or D2, or in the fat collected from fish in these dams. The description of the various chemicals was collected from either the Agency for toxic substances and diseases registry (ATSDR) (2006) or BPBC (2004).

Lindane is produced and used as an insecticide on fruit, vegetables, and forest crops, and animals and animal premises. It is a white solid whose vapour may evaporate into the air. The vapour is colourless. It may also be used as a prescription medicine (lotion, cream, or shampoo) to treat and/or control scabies (mites) and head lice in humans.

BHC (benzene hexachloride or gamma-HCH) is an insecticide mainly used for soil and seed treatment for controlling leaf-eating and soil inhabiting insects. It is used on a wide range of crops

Endrin is a pesticide to control insects, rodents, and birds. Endrin does not dissolve very well in water. It has been found in groundwater and surface water, but only at very low levels. It is more likely to cling to the bottom sediments of rivers, lakes, and other bodies of water.

Aldrin is a non-systemic insecticide used for the control of soil dwelling insects such as termites and ants. It is an insecticide with contact, stomach and respiratory action as well as being a wood preservative. Aldrin and dieldrin are insecticides with similar chemical structures. Aldrin quickly breaks down to dieldrin in the body and in the environment.

Dibutyl phthalate (DBP) is an odourless and colourless or faintly yellow oily liquid that is added to hard plastics to make them soft (phthalate ester plasticizer). They are used to make many products such as carpets, paints, glue, insect repellents, hair spray, nail polish, and rocket fuel. In 1994, more than 7.8 million kilograms of di-*n*-butyl phthalate were made. Most of the time, the largest source of exposure is from air.

1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) is a pesticide used to control insects that carry diseases such as malaria. DDT is a white, crystalline solid with no odour or taste. Dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD) are

chemicals similar to DDT that contaminate commercial DDT preparations. DDE has no commercial use. DDD was also used to kill pests, but its use has also been banned. One form of DDD has been used medically to treat cancer of the adrenal gland. Commercial DDT comprises several isomers, the main one (75-80%) being *p, p'*-DDT. DDT is metabolised in the body to DDE, and both these compounds persist in the body fat. The metabolite p, p'-DDE is capable of blocking the action of androgens (the male hormones) in male rats by inhibiting androgen binding to the androgen receptor, androgen-induced transcriptional activity, and androgen action in developing, pubertal and adult male rats (Kelce et al., 1995).

Diethylhexyl phthalate (DEHP) is also a phthlate ester and a manufactured chemical that is commonly added to plastics to make them flexible. DEHP is a colourless liquid with almost no odour Exposure to DEHP is generally very low. Increased exposures may come from intravenous fluids delivered through plastic tubing, and from ingesting contaminated foods or water DEHP is present in plastic products such as wall coverings, tablecloths, floor tiles, furniture upholstery, shower curtains, garden hoses, swimming pool liners, rainwear, baby pants, dolls, toys, shoes, automobile upholstery, packaging film and sheets, sheathing for wire and cable, medical tubing, and blood storage bags. DEHP does not evaporate easily or dissolve in water easily and it attaches strongly to soil particles.

Cadmium (Cd) metal is used mainly as an anticorrosive, electroplated on to steel. Cd- sulfide and -selenide are commonly used as pigments in plastics. Cd compounds are used in electric batteries, electronic components, and nuclear reactors. Fertilizers produced from phosphate ores constitute a major source of diffuse Cd pollution. In natural waters, Cd is found mainly in bottom sediments and suspended particles. Food is the main source of cadmium intake for nonoccupationally exposed people. Crops grown in polluted soil or irrigated with polluted water may contain increased concentrations, as may meat from animals grazing on contaminated pastures. Animal kidneys and livers concentrate Cd. Release of Cd from human activities is estimated at from 4,000 to 13,000 tons per year, with major contributions from mining activities, and burning of fossil fuels. Cd can enter the air from the burning of fossil fuels (e.g., coal fired electrical plants) and from the burning of household waste.

Arsenic (As) is a naturally occurring element widely distributed in the earth's crust. In the environment, arsenic is combined with oxygen, chlorine, and sulphur to form inorganic arsenic compounds. As in animals and plants combines with carbon and hydrogen to form organic As compounds. Inorganic As compounds are mainly used to preserve wood. As compounds are used as pesticides, primarily on cotton plants.

Mercury (Hg) occurs naturally in the environment and exists in several forms. These forms can be organized under three headings: metallic Hg (also known as elemental Hg), inorganic Hg, and organic Hg. Several forms of Hg occur naturally in the environment. The most common natural forms of Hg found in the environment are metallic Hg, mercuric sulfide (cinnabar ore), mercuric chloride, and methyl mercury. Some microorganisms (bacteria and fungi) and natural processes can change the Hg in the environment from one form to another. The most common organic Hg compound that microorganisms and natural processes generate from other forms is methyl mercury. Methyl mercury is of particular concern because it can build up in certain edible freshwater and saltwater fish and marine mammals to levels that are many times greater than levels in the surrounding water. Some inorganic Hg compounds are used as fungicides.

Table 11.2 gives a summary of the classification of the different chemicals as toxicants or carcinogens and the potential health effects that can be expected when exposed to these chemicals.

Table 11.2: Health effects – both carcinogenic and toxic effects associated with the different chemicals.

Contaminant of potential concern	Documented health effects	
	Toxic effects	Carcinogenic effects
Lindane or gamma-HCH	Lindane is toxic via the oral route as mildly toxic via the dermal route. Lindane has been found to liver damage as well as neurological, kidney, pancreas testes and nasal mucous membrane damage.	The EPA has classified gamma-HCH (lindane) into the category "suggestive evidence of carcinogenicity, but not sufficient to assess human carcinogenic potential" (US EPA 2001a, 2002b). No cancer potency factors have been estimated for gamma-HCH (US EPA 2001a, 2002b; IRIS 2005). The International Agency for Research on Cancer (IARC) has classified lindane as a Class 2B carcinogen (possible human carcinogen).
BHC or benzene hexachloride or gamma-HCH	Exposure to high levels of HCH can cause blood disorders, dizziness, headaches, seizures, and changes in the levels of sex hormones.	HCH has caused cancer in animals.
Aldrin or 1,2,3,4,10,10, hexachloro-hexahydro-dimethano-naphthalene	Animals exposed to high amounts of aldrin or dieldrin also had nervous system effects. In animals, oral exposure to lower levels for a long period also affected the liver and decreased their ability to fight infections. Studies in animals have given conflicting results about whether aldrin and dieldrin affect reproduction in male animals and whether these chemicals may damage the sperm. We do not know whether aldrin or dieldrin affect reproduction in humans.	There is no conclusive evidence that aldrin or dieldrin cause cancer in humans. Aldrin and dieldrin have shown to cause liver cancer in mice. The International Agency for Research on Cancer (IARC, 1982) has determined that aldrin and dieldrin are not classifiable as to human carcinogenicity. The EPA has determined that aldrin and dieldrin are probable human carcinogens.
Endrin	Studies in animals confirm that endrin's main target is the nervous system. Birth defects, especially abnormal bone formation, have been seen in some animal studies. The central nervous system is the primary target site for endrin toxicity. Convulsions and death have occurred within a few hours of ingestion. Less severe symptoms include headache, convulsions, dizziness, nausea, vomiting, nervousness, and confusion.	The EPA has determined that endrin is not classifiable as to its human carcinogenicity because there is not enough information to allow classification.
DDT, DDD and DDE	High levels of DDT can affect the nervous system causing excitability, tremors and seizures. In women, DDE can cause a reduction in the duration of lactation and an increased chance of having a premature baby. In animals, short-term exposure to large amounts of DDT in food affected the nervous system, while long-term exposure to smaller amounts affected the liver. DDE has been shown to be both estrogenic and anti-androgenic.	The International Agency for Research on Cancer (IARC) determined that DDT may possibly cause cancer in humans. The EPA determined that DDT, DDE, and DDD are probable human carcinogens.

Table 11.2 Continued.

DEHP	Harmful effects in animals generally occurred only with high amounts of DEHP or with prolonged exposures. Moreover, absorption and breakdown of DEHP in humans is different than in rats or mice, so the effects seen in rats and mice may not occur in humans. High amounts of DEHP damaged the liver of rats and mice. Whether or not DEHP contributes to human kidney damage is unclear. Skin contact with products containing DEHP will probably cause no harmful effects because it cannot be taken up easily through the skin.	The Department of Health and Human Services (DHHS) has determined that DEHP may reasonably be anticipated to be a human carcinogen. The EPA has determined that DEHP is a probable human carcinogen. These determinations were based entirely on liver cancer in rats and mice.
DBP	Di-*n*-butyl phthalate appears to have relatively low toxicity. Adverse effects have not been reported in humans as a result of exposure to di-*n*-butyl phthalate. In laboratory animals, studies show that eating large amounts of di-*n*-butyl phthalate can affect their ability to reproduce. Sperm production can decrease, but returns to near normal levels when exposure stops. Large amounts of di-*n*-butyl phthalate repeatedly applied to the skin for a long time can cause mild irritation.	There have been no cancer studies in humans and the one study in laboratory animals is inadequate. The EPA has determined that di-*n*-butyl phthalate is not classifiable as to human carcinogenicity based on inadequate evidence in both humans and animals.
Cd	Eating food or drinking water with very high cadmium levels severely irritates the stomach, leading to vomiting and diarrhoea, and sometimes death. Eating lower levels of cadmium over a long period of time can lead to a build-up of cadmium in the kidneys. If the levels reach a high enough level, the cadmium in the kidney will cause kidney damage, and also causes bones to become fragile and break easily.	The International Agency for Research on Cancer (IARC) has determined that cadmium is carcinogenic to humans. The EPA has determined that cadmium is a probable human carcinogen (B1) by inhalation. Skin contact with cadmium is not known to affect the health of people or animals because virtually no cadmium can enter the body through the skin under normal circumstances.
Hg	Chronic neurotoxic effects, damage to nervous system. Neurological (organic Hg) and renal disturbances (inorganic Hg). *Organically-bound mercury is more toxic than inorganic.* Elemental mercury is volatile and exposure by inhalation may also occur.	Class D: Not classifiable as to human carcinogenicity.
As	Chronic effects: skin lesions and hyper-pigmentation (Black Foot Disease). Arsenic can accumulate in the body. Acute effects: death from upper respiratory, pulmonary, gastrointestinal and cardiovascular failure. Nerve damage and sensory loss in the peripheral nervous system is a primary symptom of arsenic poisoning.	Class A: Known human carcinogen. Increases the risk of cancer of the skin and internal organs e.g. lung, liver, kidney, bladder and prostate cancer.

11.3.2 Dose calculation (Exposure concentration)

Exposure assessment is a key phase in health risk assessment, because without exposure, even the most toxic or carcinogenic compound does not pose a risk to human health. Exposure assessment involves the identification of potential receptors, exposure pathways, exposure media and receptor behaviour, which affects exposure duration.

A numerical estimate of dose (expressed as mg/kg/d) was calculated to determine the exposure concentration (US EPA, 1998).

The total dose is calculated using the following formula.

$$TotalDose_{medium} = C_{medium} \times IR \times ED$$

For **non-cancer risks**, the average daily dose (ADD) received during the period of exposure was used calculated as follows:

$$ADD = \frac{TotalDose_{w}}{Bw \times ED}$$

For the **risk of carcinogens** for exposures that last less than a lifetime, the dose is adjusted using the following formula:

$$LADD = ADD \times \left(\frac{ED}{Lft} \right)$$

The following table (Table 11.3) lists the chemicals that were identified and the concentrations found in water and fish from the UNR – used in the risk calculations

Table 11.3: Chemical concentrations measured in water from D1, D2 and fish fat.

Chemical	Concentration µg/L WATER		Concentration µg/kg FISH FAT	
	D2	D1	D2	D1
Lindane	14	0.58	15	88
Aldrin	ND	ND	ND	24
Endrin	0.57	ND	ND	ND
DDT	2.3	0.6	14	52
DDD	1.1	1.1	6	38
DDE	ND	0.02	22	215
BHC	ND	15	ND	ND
DEHP	700	600	ND	ND
DBP (84-74-2)	5000	6310	ND	ND
DEP *	3200	3200	ND	ND
PCB	ND	ND	5	25
Heptachlor	ND	0.68	ND	ND
Cd	500	ND	ND	ND
As	5000	ND	ND	ND
Hg	16000	ND	ND	ND
Pb *	1700	ND	ND	ND

ND = Not detected

* **Risks could not be calculated as no slope factor or reference doses are available**

11.3.3 Trans-media transfer calculations

For some exposure scenarios a chemical concentration in one medium must be converted to a concentration in another medium to which a person can be exposed. An example of this is a chemical concentration in air may be calculated from a chemical concentration in water. This would take into account that some chemical compounds are volatile and a person may be exposed to contaminated air while showering based on the chemical being present in the water.

To calculate the concentrations in vegetables the following two calculations were used.

11.3.3a Home-grown vegetables

Water to root uptake equations

$$C(r) = RCF(w) \times C(w)$$

$$\log(RCF(w)-0.82)=(0.77\times\log(Kow))-1.52$$

C(r) Concentration in root

C(w) concentration in water

Proportion in Root_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ 100.00%

RCF = Root Concentration Factor

Kow = Octanol-water partition coefficient

Water to transpiration stream (root uptake)

$$C(st)=TSCF(w)\times C(w)$$

$$TSCF(w)=0.784^{-((Log(Kow)-1.78)^2)/2.44)}$$

C(st) Concentration in stem

C(w) Concentration in water

Proportion in stem_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ 100.00%

TSCF = Transpiration stream concentration factor

Kow = Octanol-water partition coefficient of the compound

11.3.3b Dermal Absorption Calculations (Source: US EPA, 1992)

The following equations are used to calculate the absorbed dose of chemical per square centimeter of skin per event.

For **inorganic chemicals**, the absorbed dose is calculated as follows:

$$DA_e = Kp\times Cw\times t_e$$

For **organic chemicals**, DA is calculated as follows:

$$DA_e = 2\times Kp\times Cw\times((6\times\tau\times t_e)/p)^{\frac{1}{2}}$$

for $t_e < t^*$

or

$$DA_e = Kp\times Cw\times((t_e/(1+B))+(2\times\tau\times((1+3B)/(1+B))))$$

for $t_e > t^*$

where

DA is dose absorbed per unit area of exposed skin per event.

Kp is the dermal permeability coefficient of the chemical from water.

Cw is the concentration of the chemical in water.

t_e is the duration of the event.

τ is the lag time for diffusion through the skin, based on the diffusion path through the skin and the diffusivity coefficient of the chemical.

π is the mathematical constant, 3.14159

t^* is the time required for the transport of organic compounds to reach steady-state; a function of lag time and lipophilicity (a function of τ and B).

B represents the relative contribution of permeability coefficients for the chemical in the stratum corneum and the viable epidermis ; proportional to lipophilicity.

The dermal permeability coefficient, Kp, is derived from a chemical's octanol-water partition coefficient and molecular weight:

$$Log(K) = 2.72 + (0.71 \times \log(Kow)) - (0.0061 \times MW)$$

where

Kow is the octanol-water partition coefficient

MW is the molecular weight

The lag time for diffusion, τ, is derived from the molecular weight of the chemical and the path length through the skin:

$$t = Lsc / \left(6 \times 10^{-2.72 - 0.0061 \times MW}\right)$$

where

Lsc is the path length through the skin.

The relative permeability coefficient for the stratum corneum and viable epidermis, B, is derived from the chemical's octanol-water partition coefficient:

$$B = Kow / 10{,}000$$

The time required for the transport of organic compounds to reach steady-state, t^*, is defined by the following functions of and B:

If $B < 0.1$, then $t^* = 2.4$

If $0.1 < B < 1.17$, then $t^* = (8.4 + 6 \log B)\,\tau$

If $B > 1.17$, then $t^* = 6\ (b - (b\text{-}c))\,\tau$

where

$$b = \left(2(1+B)^2\right)/\pi - c$$

$$c = (1+3B)/3$$

The dose (or exposure concentration) presented in this assessment reflect the exposure from measured concentrations of contaminants in water and fish only and the exposure pathways, as well as the specific numerical parameters applied to each exposure scenario.

The following table (Table 11.4) summarises the exposure parameters used in this assessment.

Table 11.4: Exposure Parameters Used in risk assessment and risk calculations.

EXPOSURE INFORMATION	UNITS	VALUES
Body weight (Bw)	Kilogram (kg)	70
Life expectancy (Lft)	Days (d)	70 (70 x 365)
Exposure duration (ED)	Days (d)	350 per year
Drinking water intake rate (IR)	Litres (L)	2
Dermal exposure rate	Hours (h)	0.2 h per day based on 23000cm^2 skin surface area
Vegetable exposure rate	kg	Daily 40% of 0.2kg per event
Fish ingestion rate	kg	Daily 0.005kg per event per 350 days per year #

Based on an averaging factor of 0.05kg fish eaten per day but with conversion of chemical content in fat to amount of fat eaten. This is based on freshwater fish having a MAXIMUM 10% fat content – whereas they have on average only 1% fat content

11.3.4 Risk Characterisation

Risks calculated for the various assumptions for D2 and D1 water and for fish fat using the measured concentrations of organic chemicals and metals are presented in the following tables (Table 11.5-7) and figures (Figure 11.5-8).

Table 11.5: Cancer risks and toxic effects resulting from metal exposure.

Metals	Cd	As	Hg
Hazard quotient	**27.4**	**456**	155
Carcinogenic risk	Not carcinogenic	**0.088 ~ 1 in 100**	Not carcinogenic

Table 11.6: Cancer Risks and Toxic effects as a result of exposure to Dam 2 water and fish.

Dam 2 Area										
CANCER RISK	**Lindane**	**DDD**	**DDT**	**Endrin**	**BHC**	**DEHP**	**DDE**	**DBP**	**PCB**	**Total**
Cancer risk drinking water 2L	**2.0E-04**	3.0E-06	9.0E-06			**1.0E-04**				3.1E-04
Cancer risk accidental ingestion 10ml.	1.0E-06	2.0E-08	5.0E-08			6.0E-07				1.7E-06
Cancer risk fish ingestion	6.0E-07	4.0E-08	1.0E-07			0.0E+00	2.0E-07		6.0E-06	6.9E-06
Cancer risk vegetable watering	**2.0E-04**	**2.0E-04**	**4.0E-04**			**1.0E-03**				1.8E-03
Cancer risk dermal -12 min	**1.0E-04**	3.0E-05	**2.0E-04**			**2.0E-04**				5.3E-04
Cancer risk 80% removal of contaminants (used for drinking)	5.0E-05	6.0E-07	2.0E-06			2.0E-05				7.3E-05
Total	5.5E-04	2.3E-04	6.1E-04			1.3E-03	2.0E-07		6.0E-06	2.7E-03
TOXIC RISK	**Lindane**	**DDD**	**DDT**	**Endrin**	**BHC**	**DEHP**	**DDE**	**DBP**	**PCB**	**Total**
HQ drinking water 2L	**1.300**		0.130	0.050		**1.000**		**1.400**		3.9
HQ accidental ingestion 10ml.	0.006		0.001	0.000		0.005		0.007		0.0
HQ fish ingestion	0.003		0.002							0.0
HQ vegetable watering	**1.000**		**6.000**	0.210		**10.000**		**6.000**		23.2
HQ dermal - 12 min	0.600		**2.700**	0.050		**2.100**		**1.300**		6.8
HQ 80% removal of contaminants (used for drinking)	0.270		0.030	0.009		0.200		0.300		0.8
Total	3.179		8.863	0.319		13.305		9.007		34.7

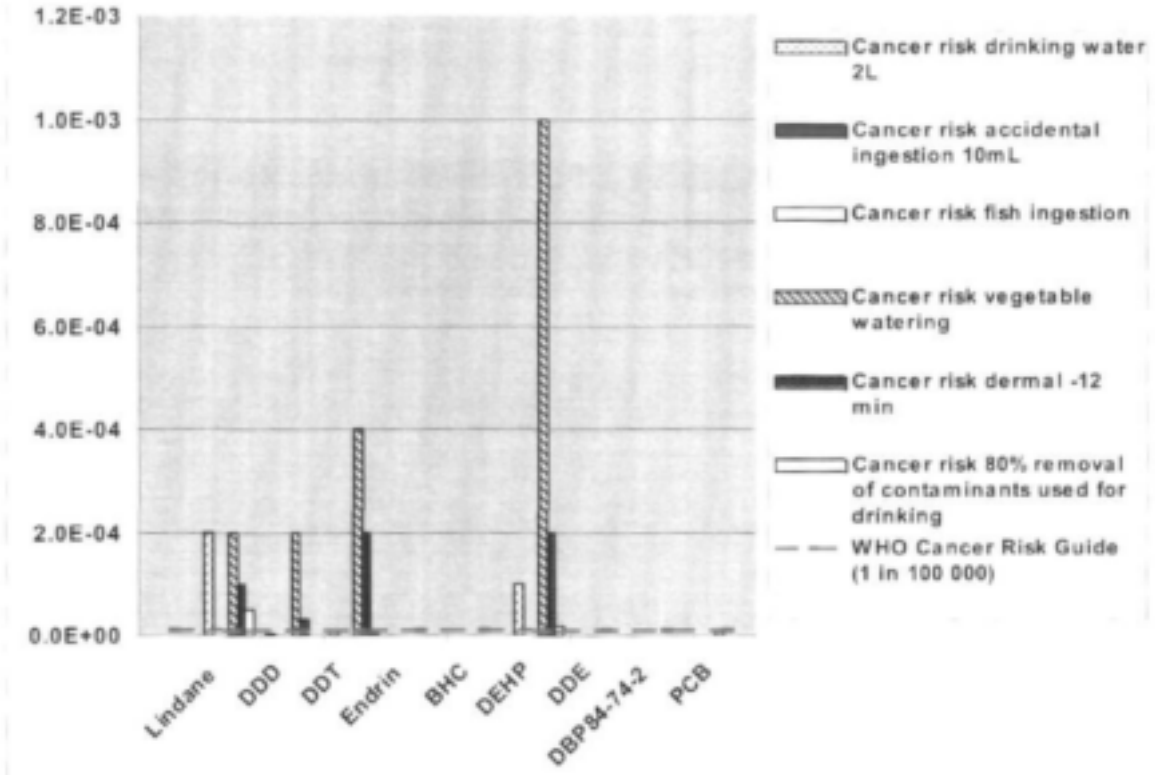

Fig. 11.5: Risk of carcinogenic effects from exposure to Dam 2 water and fish.

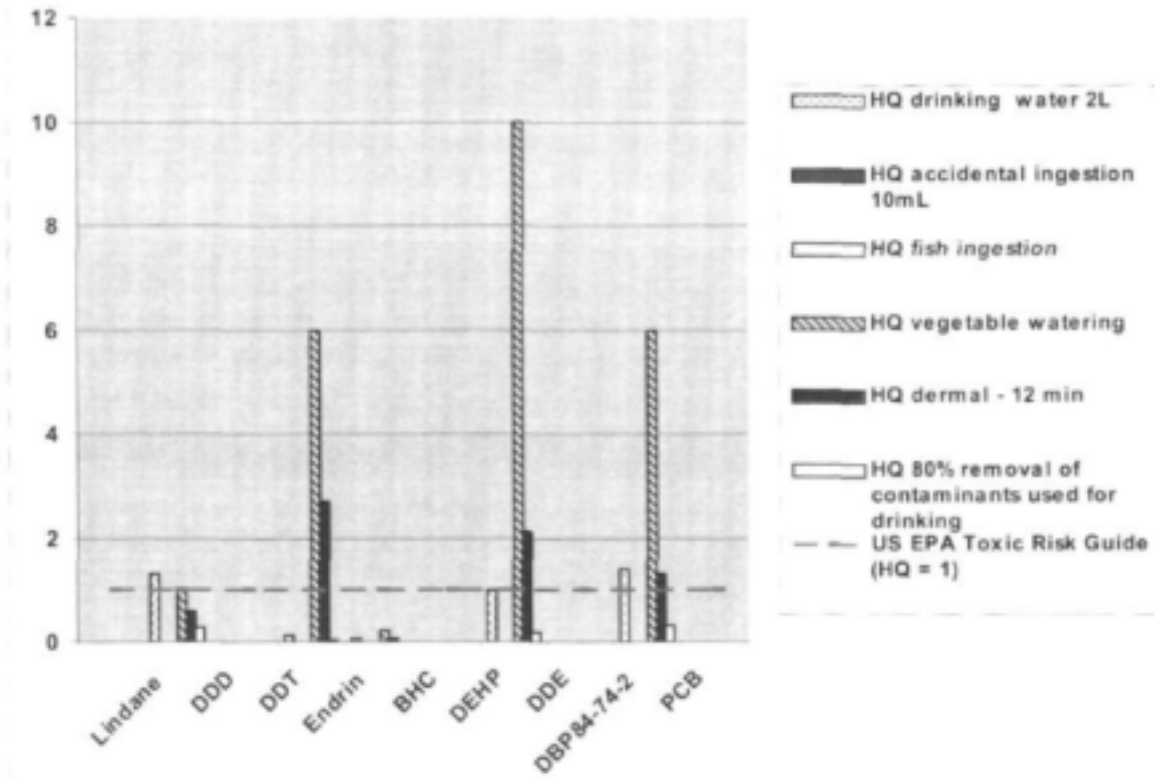

Fig. 11.6: Risk of toxic effects from exposure to water and fish from Dam 2.

Table 11.7: Cancer Risks and toxic effects as a result of exposure to Dam 1 water and fish.

Dam 1 Area											
CANCER RISK	**Lindane**	**DDD**	**DDT**	**Aldrin**	**BHC**	**DEHP**	**DDE**	**Heptachlor**	**DBP 84-74-2**	**PCB**	**Total**
Cancer risk drinking water 2L	9.00E-06	3.00E-06	2.00E-06		**3.00E-04**	**1.00E-04**	8.00E-08	4.00E-05			4.5E-04
Cancer risk accidental ingestion 10mL	4.00E-08	2.00E-08	1.00E-08		2.00E-06	5.00E-07	4.00E-10	2.00E-07			2.8E-06
Cancer risk fish ingestion	1.00E-05	8.00E-07	2.00E-06	4.00E-05			6.00E-06			2.00E-05	7.9E-05
Cancer risk vegetable watering	7.00E-06	**2.00E-04**	**1.00E-04**			**1.00E-03**	3.00E-06	9.00E-05			1.4E-03
Cancer risk dermal -12 min	4.00E-06	3.00E-05	5.00E-05		7.00E-07	**2.00E-04**	8.00E-07	2.00E-05			3.1E-04
Cancer risk 80% removal of contaminants (used for drinking)	2.00E-06	6.00E-07	5.00E-07		6.00E-05	2.00E-05	2.00E-08	7.00E-06			9.0E-05
Total	3.2E-05	2.3E-04	1.5E-04	4.0E-05	3.6E-04	1.3E-03	9.9E-06	1.6E-04		2.0E-05	2.3E-03

TOXIC RISK	**Lindane**	**DDD**	**DDT**	**Aldrin**	**BHC**	**DEHP**	**DDE**	**Heptachlor**	**DBP 84-74-2**	**PCB**	**Total**
HQ drinking water 2L	0.050		0.030			0.820		0.040	**1.700**		2.6
HQ accidental ingestion 10mL	0.000		0.000			0.004		0.000	0.009		0.0
HQ fish ingestion	0.060		0.020	0.160							0.2
HQ vegetable watering	0.040		**1.600**			**8.600**		0.090	**7.000**		17.3
HQ dermal - 12 min	0.020		0.720			**1.800**		0.020	**1.700**		4.3
HQ 80% removal of contaminants (used for drinking)	0.010		0.007			0.160		0.008	0.350		0.5
Total	0.1803		2.3772	0.16		11.384		0.1582	10.759		25.0

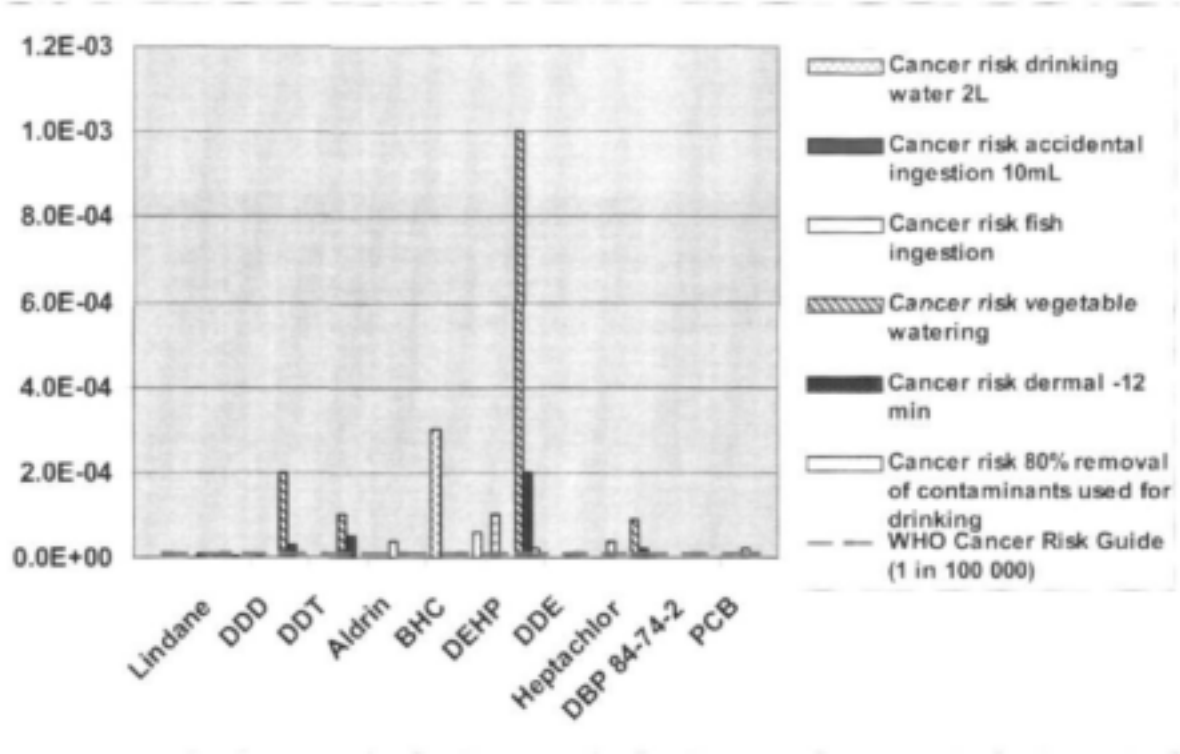

Fig. 11.7: Risk of carcinogenic effects from exposure to Dam 1 water and fish.

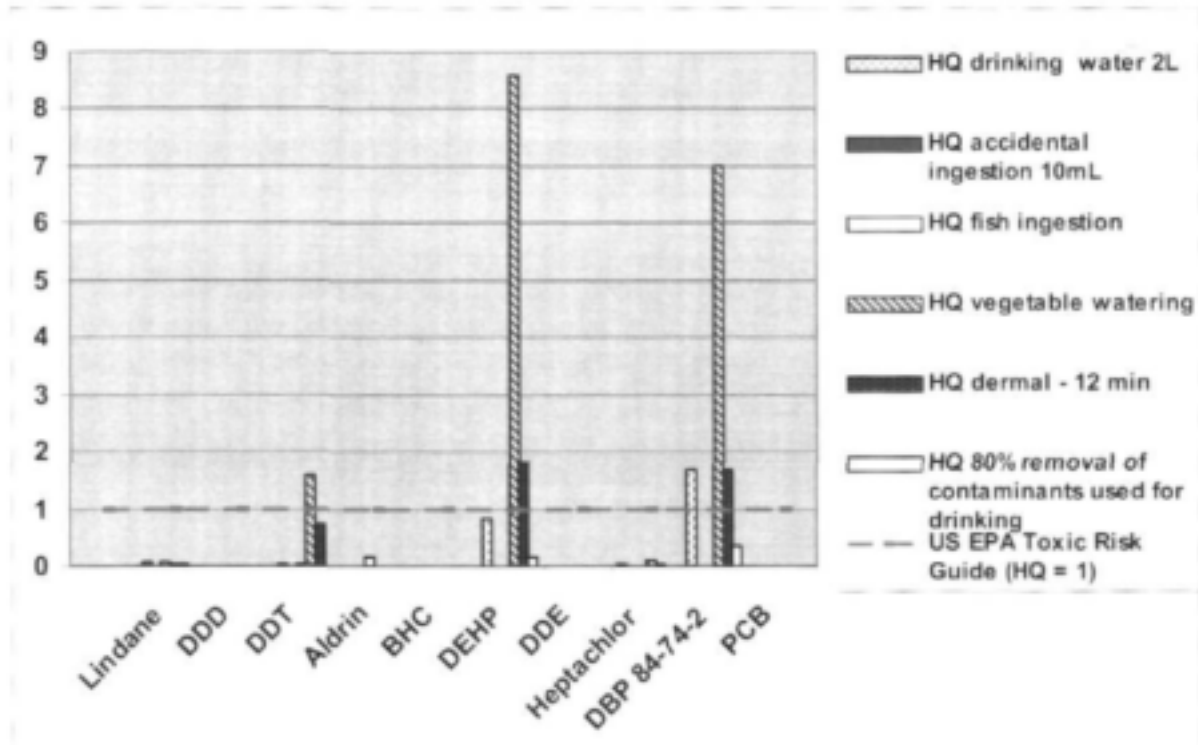

Fig. 11.8: Risk of toxic effects from exposure to water and fish from Dam 1.

11.4 Discussion

11.4.1 Quantitative Human Health Risk Assessment

In general, the health risk assessment indicates that the potential for serious health effects exists if people are exposed to the water from the UNR as described in the scenarios. Many chemicals were found in the study area which posed an unacceptable risk for people developing either carcinogenic or experiencing toxic effects using the water for domestic, agricultural or recreational purposes. Even using an 80% reduction scenario based on the percentage removal associated with a wastewater treatment plant using activated carbon (Snyder et al., 2003) the water posed a risk to human health.

People will be exposed to an unacceptable risk to develop cancer or toxic effects if they make use of D2 or D1 water. Interestingly, using untreated water from both these areas for vegetable watering would expose people to the highest risk of developing cancer and the highest chance of experiencing toxic effects. Dermal exposure to the water (assuming one is exposed on a daily basis for 12 minutes) also results in very high risks as a result of dermal absorption of the chemicals in the water.

Both the organic chemicals and metals were present in concentrations individually resulting in an unacceptably high health risk. Any possible synergistic effects of metals in combination with organic chemicals or any other combination could not be taken into account in the health risk assessment. The health risk assessment process can only consider additive effects. It is known that synergistic effects occur with many mixtures of chemicals.

The risks calculated showed D1 and D2 to have the same possible total risk of developing cancer (2,3 in 1000 vs 2,7 in 1000) even though different chemicals and different concentrations were encountered. Toxic effects are expected to be slightly higher due to exposure via D2 compared to D1 with hazard indices of 35 and 25 respectively. This indicates that the water contains 35 times and 25 times the chemical concentrations considered safe for a lifetime exposure.

The chemicals responsible for the worst of the adverse health effects expected in D2 were lindane, DDT and metabolites, and DEHP, whereas the chemicals responsible for the adverse effects in D1 were DDT and metabolites, BHC, DEHP and DBP.

11.4.2 Qualitative Risk Assessment

Although endocrine disrupting effects are of serious concern, the data necessary to apply a quantitative risk assessment is unavailable at this stage. One can however, make use of qualitative data.

A qualitative health risk assessment was conducted making use of the ecological data collected from the study area. Endocrine disrupting effects were observed at the cellular level with the YES assay detecting estrogenicity in one third of samples analysed. The aquatic animals also displayed endocrine disrupting effects in catfish, with intersex fish, increased apoptosis and macroscopic differences observed. Snails from the Urban Nature Reserve displayed statistically significant differences in penile sheath length to those from control sites. Data from the frog studies was not of high quality so are not considered.

Terrestrial animals' having less exposure to the water environment than aquatic animals allows for higher quality comparison to humans. Almost half of the striped mice from the UNR were found to have low sperm counts with 12% having no sperm at all. The original study leading to this project found culled Eland to have severely calcified testes (Bornman et al., submitted). This shows that small and large mammals exhibited endocrine disrupting effects.

The hormones found in D2 in 2002-2003 just before this study started (Burger et al., 2006) were at concentrations in the order of 25 – 350ng/L with estrogenic activity measuring at 0.16ng/L estradiol equivalents. These levels are 10 000 x higher than those known to cause initiate activity in breast cancer cells.

The abundant effects measured and high quality data of the effects seen in some of the species, indicates that the risk for endocrine disruption with regards to reproductive effects is high. This assessment is based on the effects observed in the many different levels of animals. These include the fish and snails, and more significantly, the small and large mammals. Also considered is the presence of estrogens in the water at concentrations known to initiate effects in human breast cancer cells. Even with the cells being more sensitive at inducing estrogen mimicking effects, the concentration was 1 000 times, which would suggest that this may occur in a human population.

Based on the estrogen concentrations and ecological effects there is a 'likely probability' that endocrine effects can be expected in a human population exposed to this water. One must also consider that the probability of endocrine disruption is in addition to the carcinogenic and toxic effects predicted in the quantitative risk assessment.

These risks predicted are an under-representation of health risks as they only consider water and fish as media of exposure. People are also exposed to chemicals via air and other food and therefore the true health risk will in fact be greater than what is presented in this report.

11.4.3 Uncertainty

There are many uncertainties within the health risk assessment process. All risk estimates involve some degree of uncertainty. Uncertainty in the health risk assessment (HRA) process exists at numerous levels. Uncertainty regarding exposure has two primary sources: uncertainty about contamination including concentrations of chemicals to which the potential population may be exposed over the duration of the exposure period, and uncertainty about the exposed population. In this study both of these were significant.

The exposed population could not be studied and the health risk assessment is based solely on hypothetical exposure scenarios. Therefore, uncertainty in the results will be substantial.

Uncertainty in the dose-response (carcinogenic and toxicological) data also exists. Many of the chemicals in this analysis did not have available dose response information. In addition, the reference doses that were used in this risk assessment have uncertainty factors of one or two orders of magnitude.

The results provided in this study are merely an indication of the potential health risks associated with potential exposure to D2 and D1 waters if used for domestic and other purposes.

These risks predicted are an under-representation of health risks as they only portray contact via water and ingestion of fish. These chemicals may be present in another medium such as air and therefore the true health risk will in fact be greater than what is presented in this report.

CHAPTER 12

COMPREHENSIVE DISCUSSION

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12.1 Objective

The objective of this study was to determine whether sufficiently high levels of EDCs exist in the general environment to exert adverse health effects on aquatic or terrestrial animals and also humans. At all four sampling sites and in various samples matrices several compounds were present not at the low levels, but at concentrations of concern for human consumption.

12.2 Control sites

The findings at "control" sites Lapalala and Suikerbosrand (SBR) confirmed the worldwide concern that there is nothing like a "clean, pristine" uncontaminated site. Lindane (11, 31.4% samples), 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), 25,7% samples) and the phthalate esters were the most prevalent chemicals at the UNR. The most probable sources of lindane were the chicken farm and landfill site (Figure 3.3), but since lindane is used as an insecticide on animals and animal premises, the other farming activities should be seriously considered. The slope from the farm favours the run-off of water towards the Channel and Dam 2. The water from Dam 1 was almost free of lindane residues (except 0.58ug/L in May 2005) and the possibility that lindane was coming from the inflowing stream into the UNR, seems negligible. The slope from the landfill site and run-off water will also only affect Dam 2 values. On the other hand, contamination of ground water and underground connections with D2 seems a possibility, but this does not apply to the Channel because of the building construction.

12.3 Chemical levels measured

Lindane in surface waters and soils is taken up and bioconcentrated by terrestrial and aquatic organisms (Just et al., 1990; Matsumura and Benezet, 1973; Ramamoorthy, 1985; Verma and Pillai, 1991; Viswanathan et al., 1988) and accumulates in the food chain (Szokolay et al., 1977). This process also applied to the UNR because of the high lindane levels in fish (Table 7.5) collected from all sites and sampling events.

The major component (85%) of technical grade DDT is the *p,p'*-DDT (85%) isomer, but it also contains *o,p*-DDT (15%), and *o,o'*-DDT (trace amounts). Technical grade DDT may also contain DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane) and DDE as contaminants. DDD was used previously as pesticide, but to a far lesser extent than DDT. Both DDD and DDE are breakdown products of DDT (ATSDR, 2002).

The DDT residues detected at the UNR could be a result of past use in farming activities. It may also still be released from the waste site, as observed in the USA (ATSDR, 2002) or it may also be released into the atmosphere from areas in South Africa where DDT is still being used for malaria vector control (Limpopo, Mpumalanga and KwaZulu-Natal). DDT and its metabolites also enter the atmosphere through the volatilization of residues in soil and surface water, albeit a result of past use. These chemicals will be deposited on land and in surface water as a result of dry and wet deposition. The process of volatilization and deposition may be repeated many times, and is referred to as a 'global distillation' from warm source areas to cold Polar Regions. Consequently, these chemicals have been found in snow, and animals in the Arctic and Antarctic regions where DDT was never used (ATSDR, 2002). It may also be the case with the UNR that the DDT residues were the result of environmental distillation. However, the possibility of illegal use and dumping of DDT into water could not be excluded. The finding of almost similar levels at all the sites, except the higher levels at D2 in Nov 2004, is in favor of precipitation from polluted air. For the reason of the extensive past use of DDT worldwide and the persistence of DDT and its metabolites, these chemicals are now virtually ubiquitous and are continually being transformed and redistributed in the environment.

In surface water, DDT will bind to particles in the water, settle, and be deposited in the sediment from where it is taken up by small organisms and fish in the water. DDT, DDD, and DDE accumulate in fatty tissues and from the aquatic food webs may enter higher trophic levels of the food chain. The impact of bioaccumulation on fish was emphasized by the very high DDT and metabolite concentration in fat (Table 7.5).

Of even greater concern is the possible impact of the high levels of *p,p'*-DDE, the persistent metabolite of DDT on the living organism as it is has antiandrogenic properties (Kelce et al., 1995). Adverse reproductive system effects associated with *in utero* DDT or DDE exposure in male animals include, amongst others, abnormal development of ovarian tissue (Fry and Toone, 1981), reduced penis size (Guillette and Guillette, 1996), hypospadias (Gray et al., 2001) and cryptorchidism (Facemire et al., 1995; Gray et al., 2001). Although the high values were found in male catfish there seems to be no reason why females should not have similar fat concentrations. If that is the case, the high DDT and metabolites will affect both oogenesis and spermatogenesis and may contribute to the defects seen in the sharptooth catfish. However, estrogenic and anti-androgenic chemicals in the aquatic environment water are most likely to affect the developing embryo after fertilization, especially if the free swimming larvae are male. This may contribute to the "feminization" of the male urogenital papilla (UGP) as well as the development of testicular oocytes and intersex. In this study altogether 28 of 97 (28.9%) intersex males were collected from the UNR over the two year period, which is extremely high. Although the chemical insult occurs during early embryogenesis, the effect will only become evident later in later life stages.

Although tissue concentrations of target chemicals were not measured in snails, frogs and small mammals, it is hypothesized that in D2 snails both the smaller penile sheath (containing the penis) and prepuce could result from feminizing compounds such as estrogenics and anti-androgenics. This possibility was supported by the novel finding of penile agenesis in six snails, which has not been reported in either *B. tropicus* or in South African waters before. Similar effects were reported by Tillmann et al. (2001) in *Marisa cornuarites*, a freshwater gastropod, after exposure to antiandrogens. Furthermore, the absence of mayflies and stoneflies and the abundance of highly tolerant organisms were, amongst others, signs of poor water quality in the system generally indicating industrial pollution (Graham and Dickens, 2002; Williams and Feltmate, 1992). The reduced biodiversity in the snail and macroinvertebrate population at the UNR is alarming and further supports the concern on the magnitude of environmental pollution. This aspect has to be studied in greater detail.

Although the frogs appeared to do fine in the UNR the skewed sex ratio observed in the main stream is of concern. The sample size is too small to come to a conclusive answer, but despite various extensive attempts, only a limited number of frogs could be found. This was attributed to the predatory sharptooth catfish, however, the possible impact of chemical pollution could not be excluded. The ratio of 4 females for each male is abnormal and of concern as the normal picture for *Xenopus* is a 50-50 ratio $\pm$ 10% (Du Preez et al., 2005). It is known that pollutants in water can cause demasculinization or complete sex reversal in frogs (Clark et al., 1999).

Not only aquatic animals at the UNR seemed to be affected, but also terrestrial animals. Barnhoorn et al. (2002) found macroscopic calcification in testes of eland (*Tragelaphus oryx)*. On microscopy epithelial hypertrophy and adenomatous proliferation of the rete testis were observed as well as degeneration of the seminiferous epithelium. The degenerative lesions included vacuolization of Sertoli cells, and death and desquamation of differentiating germ cells. Although full complement of spermatogenesis was observed in focal areas, spermatogenesis was generally impaired consequent to progression of degenerative changes in seminiferous epithelium. The presence of adenomatous lesions of the rete testis in the eland was similar to diethylstilbestrol-induced rete adenocarcinoma in laboratory animals (Newbold et al. 1985; Newbold 2000) and was, therefore, indicative of possible chronic estrogenic exposure. The findings in eland (Bornman et al., submitted) are similar to the testicular dysgenesis syndrome in humans attributed to developmental exposures to chemicals (Skakkebaek et al., 2001). The fat samples of the UNR eland contained high levels of the estrogenic EDC, *p*-NP.

The finding of similar macroscopic and microscopic appearances in eland testes collected from SBR was completely unexpected. Where the UNR eland fat samples contained *p*-NP, at SBR not only *p*-NP, but also OcP and high levels of PCB153 and DDT. Both alkylphenols and the persistent

organochlorine pesticide DDT (both isomers and their metabolites DDE and DDD) have estrogenic activity (Sonnenschein and Soto, 1998; Sonneveld et al., 2005). Thus, although the possibility of simultaneous exposure to estrogenic agents such as phytoestrogens and estrogenic mycotoxins cannot be ruled out, considering the spectrum of pollutants present in the body fat, the testicular lesions observed in eland could have been caused by chronic impingement of these chemicals. In experimental paradigms, it has been demonstrated that a variety of xenobiotics released from fat during fasting produce estrogenic effects (Bigsby et al, 1997). The magnitude of concentration of estrogenic pollutants in body fat indicates that EDCs bioaccumulate in terrestrial mammals as in aquatic life (Barnhoorn et al., 2004). The levels of *p*-NP were even higher than those found in fat collected from catfish inhabiting the dams in the UNR. Those male fish had signs of feminization as well as a high prevalence of intersex. Water and sediment samples had estrogenic activity on the yeast screen test (Aneck-Hahn, 2002). The eland were dependent on these water sources in the Reserve.

Exposure *in utero* to *p,p'-DDT* or its metabolite *p-p'-DDE* or OcP induced atypical germ cells resembling carcinoma in situ (CIS) in rabbits (Veeramachaneni 2000; Veeramachaneni 2006). Human testicular cancer arises from CIS cells, which are suspected to originate from primordial germ cells that escaped normal differentiation *in utero* (Skakkebæk et al. 1987; Rajpert-De Meyts et al. 1998). The first cases in animals of atypical germ cells resembling CIS cells of human testis were reported in a subfertile, unilaterally cryptorchid stallion (Veeramachaneni and Sawyer 1998) and an infertile rabbit (Veeramachaneni and VandeWoude 1999). Although a few atypical germ cells were encountered in eland testes, detailed morphological evaluation ascertaining CIS was not possible because of limitations of tissue fixation and processing. Interestingly, a variety of testicular tumours including rete adenocarcinoma and seminoma along with microlithiasis and CIS were found in Sitka black-tailed deer suspected to have been developmentally exposed to an environmental estrogenic agent(s) (Veeramachaneni et al., 2006). For these reasons, the novel findings in eland may be the first evidence that non-aquatic wildlife are also being impacted by environmental pollution of EDCs in South Africa.

The findings of Sertoli cell vacuolization and sloughing of the epithelium observed in eland were similar to those observed in rats following experimental exposure to *p*-NP (De Jager et al. 1999a), and also similar to findings in small mammals. In the striped mouse the testicular histopathology showed various degrees of degeneration including apical sloughing, degeneration of spermatogonia, vacuolization and shrinking of the seminiferous tubules. These findings are typical of exposure to chemicals such as DDT, PCBs and other EDCs (De Jager et al., 1999a). In another terrestrial species from the UNR, Bornman et al., (submitted) found in eland generally impaired spermatogenesis, Sertoli cell vacuolization and sloughing of the seminiferous epithelium. Moreover, adenomatous changes of the rete testis, reflective of possible chronic estrogenic exposure, were found. Although the number of

mice studied was small, the low sperm count and absence of any sperm in two mice are reasons for concern. A decrease in sperm count is the most common manifestation of testicular dysgenesis (Bay et al., 2006), and although tissue levels of target EDCs were not measured, it seems likely that these chemicals could have had an effect. The finding of impaired spermatogenesis in these terrestrial animals raises the question what there source and route of contamination would have been. This could imply a much wider and more serious level of environmental contamination in the UNR than previously anticipated. These aspects should be addressed in future projects.

The inappropriate occurrence of cell death in D2 catfish emphasized the effect of chemicals on the testis. The water (per L) contained 14.04ug lindane, 3.18mg DEP, 3.93mg DMP and 0.46mg DEHP respectively, and was toxic and inconclusive for estrogenicity. Moreover, the catfish fat samples bioconcentrated these chemicals and contained lindane, aldrin, PCB153 and DDT and metabolites. The concentrations in fat reflect the body burden of these chemicals, and therefore provide an indication of the exposure at a cellular level. The catfish had a significantly higher incidence of caspase 3-dependent germ cell apoptosis, compared to laboratory-reared catfish, both in terms of number of caspase-positive cells and the number of spermatogenic tubules containing at least one cluster of apoptotic cells, all of which may be the molecular basis of testicular regression and/or demasculinisation. The apoptotic events occured predominantly in primary and secondary spermatocytes, germ cell stages that are exquisitely androgen-dependent. The findings of this study will form the basis of future endeavours in which *C. gariepinus* will serve as a sentinel species to study the effects of endocrine disruptors on male reproduction.

Not only agrochemicals were present in the water and sediment samples, but also industrial chemicals including OcP, *p*-NP and the phthalate esters. The compounds were present at all four sites, albeit in higher concentrations in D1. Phthalates are a family of chemicals that are produced in the millions of tons annually worldwide, and are a principal component of many diverse products flexible polyvinyl chloride plastic (PVC), cosmetics and other personal care goods, pesticides, building materials, lubricants, adhesives, and film, among other items. Okubo et al. (2003) found that various phthalate esters suggested anti-estrogenic activities in vitro. Both DBP and DEHP affect particularly the developing male reproductive tract, although effects on the liver, kidneys, lungs, and blood clotting are also of concern (DiGangi et al., 2002). Phthalates are ubiquitous in the environment and it is possible that humans are continuously exposed to them. The most possible source is from the industrial activity higher up in the catchment, also because the levels in D1 were higher than at the other sites.

It is important to note that not all effects of EDCs are mediated via the cell receptor pathway (Pretorius and Bornman, 2005). Chemicals like *p*-NP, lindane and others may use non-receptor-mediated mechanisms and disrupt the cell ion pumps, such as Ca^{2+}. Disruption of Ca^{2+}-pumps will also disrupt

the endoplasmic reticulum Ca^{2+} pumps and function (Hughes et al., 2000), as well as inhibit the inophosphate $(IP)_3$-sensitive Ca^{2+} channels. These molecular events are crucial in particularly the Sertoli cell (Khan et al., 2003). Each Sertoli cells supports a specific number of spermatogonia and developing sperm during spermatogenesis. The Sertoli cells also forms the blood-testis barrier and controls the transport of substances to the developing sperm. Impairing Sertoli cell function will negatively impact on the development of especially germ cells that are sequestered behind the blood-testis barrier, such as spermatids and sperm (Bergmann et al., 1984).

12.4 Endocrine disrupting metals

Not only EDCs were present in the water and sediment, but the four endocrine disruptive metals (EDMs) were also detected in water from all four sites. Cadmium (Cd) levels were high in Dam 1 and Vlei, but, although lower at other sites, still above the chronic effect value (CEV). Lead (Pb) occurred in all samples at levels higher than the acute effect value (AEV) (DWAF, 1996). Mercury (Hg) was only once detected (>AEV) and arsenic (As) was high in Dam 2. These metals may be endocrine disruptive, or add to the disruption in a synergistic or additive way. Irrespective of the precise mechanism, the EDMs most probably add negatively to an already overloaded organic system.

12.5 The health risk assessment

The health risk assessment indicates that if untreated water is used for domestic purposes, unacceptable health risks (carcinogenic as well as toxic effects) could be anticipated. The greatest health concern could be if the water is used for irrigation of vegetables with a hypothetical risk of developing cancer calculated to be 2 in 1 000 for Dam 2 water and 1 in 1 000 for Dam 1! Any risk over 1 in 100 000 is considered by the World Health Organisation (1993) to be unacceptable.

This risk is followed closely by dermal absorption of the chemicals through direct contact, with risks of developing cancer in the order of 5 and 4 in 10 000 for D2 and D1, respectively. Lindane, DDT, and DEHP were the chemicals causing this risk in D2, and DEHP in D1.

Domestic use for consumption of water was also found to result in unacceptably high risks with an estimated 3 in 10 000 and 4 in 10 000 risk of developing cancer for D2 and D1 respectively. DEHP is the chemical responsible for this particular risk.

Risks of toxic effects were unacceptably high, particularly for the use of untreated water for vegetable watering (caused by DDT, DEHP and DBP). Risk of toxic effects was also high through dermal absorption for D2 and by DBP for D1.

Adding the possible impact of EDMs, hazard quotients were 27 to >450 times higher than that assumed to be safe for a life time consumption. The cancer risk for As was calculated to be close to 1 in a 100.

Risks were also calculated for the organic chemicals assuming 80% reduction as a result of going through a treatment process. This is the assumed removal of estrogen mimicking compounds going through a water treatment process using activated carbon (Snyder et al., 2003). It appears that the treated water is safe to use, provided that the process remains functional.

A qualitative health risk assessment making use of both ecological data and the concentration of natural and synthetic hormones in D2 was also undertaken. The results indicated that the risk for endocrine disruption, focussing on reproductive effects, is high. This assessment is based on the effects observed in many different levels of animals and includes the fish and snails, and more relevantly, the small and large mammals. The hormones found in D2 in 2002-2003 (Burger et al., 2006 *in press*) were at concentrations in the order of 25 – 350ng/L with estrogenic activity measuring at 0.16 ng/L estradiol equivalents. These levels are up to 10 000 ×higher than those known to cause initiate activity in breast cancer cells. Even assuming that the cells are far more sensitive at inducing estrogen mimicking effects, one can still assume that these effects can be anticipated in a human population based on both the hormone and ecological results.

12.6 Final conclusion

In summary, one can expect that untreated water from both D1 and D2 used for domestic, agricultural or recreational activities would result in unacceptably high human health risks. These risks include carcinogenic risks, toxic effects and endocrine disruption.

Although no method is available to calculate the long-term risk of EDCs and EDMs on the ecosystem of the UNR, the findings of high chemical residue levels in water, sediment and tissue, skewed sex ratios, reduced biodiversity, gonadal malformations in sharptooth catfish and freshwater snails, intersex in catfish, histological impacts on spermatogenesis in catfish and striped mouse are matters of serious concern. It is highly unlikely, if at all possible that such a diversity of effects in a range of biosentinel animals, could be coincidental.

CHAPTER 13

SUGGESTED TOOLKIT

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In view of the findings of this study, the following is suggested for endocrine disrupting chemicals (EDCs):

13.1 Chemical analyses

The selection of sites, appropriate methods of sample collection, transport, storage and extraction of samples are all crucial aspects that may affect the reliability of the target analysis. Strict adherence to standardized guidelines in accredited laboratories by expert technologists seems imperative if dependable and consistent results from various laboratories are expected.

In a Workshop held with Prof Bruno Le Bizec from France, it was agreed that **Sampling and sample handling** be done as follows to allow for chemical and pharmaceutical residue analysis:

1. Collect samples in methanol pre-washed transparent glass bottles
2. Cover glass with foil to avoid UV radiation
3. Add 0.02% sodium azide for preservation and refrigerate
4. Transfer to laboratory as soon as possible
5. Centrifuge 5000
6. Filter through Whatman No 5 paper
7. Pass through glass filter 0.22µm
8. Pass through SPE HLB cartridge (Waters ®, SA)
9. Elute with ethanol/methanol
10. Perform HPLC, GC-MS etc. analysis

13.2 Compounds of concern

The Water Research Commission (WRC) compiled a list of Priority Chemicals for South Africa (Burger, 2005). This list should now be revised using amongst others, the list of The European Commission on substances where evidence exists on endocrine disrupting activity in intact animals of at least one species (Category 1 chemicals) http://ec.europa.eu/environment/endocrine/strategy/substances_en.htm as guideline. Of the 66 chemicals in Category 1, humans were considered likely to be exposed to 60 (Table 13.1) and these may pose a particular risk to human health. The list however, only contains

anthropogenic chemicals and the natural and synthetic estrogens should be added because of their presence and potency (Burger et al., 2006 *in press*).

Table 13.1: List of 66 substances with classification high, medium or low exposure concern according to EU.

Annex 15. List of 66 substances with classification high, medium or low exposure concern

NR	CASNR	Name	HPV/pers.	ECO	HUM	Total	Concern
11	12789-03-6	Chlordane	Highly Pers	2	1	1	High
12	57-74-9	Chlordane (cis- and trans-)	Highly Pers	2	1	1	High
20	143-50-0	Kepone = Chlordecone	Highly Pers	2	1	1	High
21	2385-85-5	Mirex	Highly Pers	2	1	1	High
24	8001-35-2	Toxaphene = Camphechlor	Highly Pers	2	1	1	High
42	50-29-3	DDT (technical) = clofenotane	HPV	1	1	1	High
56	50-29-3	p,p'-DDT = clofenotane	HPV	1	1	1	High
57	3563-45-9	Tetrachloro DDT = 1,1,1,2-Tetrachloro-2,2-bis(4-chlorophenyl)ethane	Highly Pers	1	2	1	High
63	50471-44-8	Vinclozolin	HPV	3	1	1	High
69	12427-38-2	Maneb	HPV	3	1	1	High
70	137-42-8	Metam Natrium	HPV	3	1	1	High
73	137-26-8	Thiram	HPV	3	1	1	High
74	12122-67-7	Zineb	HPV	3	1	1	High
78	58-89-9	Gamma-HCH = Lindane	HPV	2	1	1	High
87	330-55-2	Linuron (Lorox)	HPV	3	1	1	High
142	1912-24-9	Atrazine	HPV	2	1	1	High
163	34256-82-1	Acetochlor	HPV	3	1	1	High
164	15972-60-8	Alachlor	HPV	2	1	1	High
191	100-42-5	Styrene	HPV	3	1	1	High
198	118-74-1	Hexachlorobenzene = HCB	HPV	3	1	1	High
270	85-68-7	Butylbenzylphthalate (BBP)	HPV	3	1	1	High
279	117-81-7	Di-(2-ethylhexyl)phthalate (DEHP) Dioctylphthalate (DOP)	HPV	3	1	1	High
286	84-74-2	Di-n-butylphthalate (DBP)	HPV	3	1	1	High
326	80-05-7	2,2-Bis(4-hydroxyphenyl)propan = 4,4'-isopropylidenediphenol = Bi-phenol A	HPV	1	1	1	High
396	1336-36-3	PCB	Pers.		1	1	High
408	35065-27-1	PCB153	Pers.		1	1	High
410	32774-16-6	PCB169	Pers.		1	1	High
417	2437-79-8	PCB47	Pers.		1	1	High
422	32598-13-3	PCB77	Pers.		1	1	High
427	53469-21-9	Aroclor 1242	Highly Pers		1	1	High
428	12672-29-6	Aroclor 1248	Pers.		1	1	High
429	11097-69-1	Aroclor 1254	Highly Pers		1	1	High
430	11096-82-5	Aroclor 1260	Pers.		1	1	High
438	59536-65-1	PBBs = Brominated Biphenyls (mixed group of 209 Congeners)	Pers.		1	1	High
467	40321-76-4	1,2,3,7,8 Pentachlorodibenzodioxin	Pers.		1	1	High
472	No CAS 140	2,3,7,8 Tetrachlorodibenzo-p-dioxin (TCDD)	Pers.		1	1	High
487	57117-31-4	2,3,4,7,8 Pentachlorodibenzofuran	Pers.		1	1	High
525	688-73-3	Tributyltin	Metal	1	2	1	High
526	No CAS 050	Tributyltin compounds	Metal	1	2	1	High
527	56-35-9	Tributyltin oxide = bis(tributyltin) oxide	HPV / Metal	1	2	1	High
504	26354-18-7	2-propenoic acid, 2-methyl-, methyl ester = Stannane, tributylmeacrylate	Metal	1	2	1	High
512	No CAS100	Methoxyetylacrylate tributyltin copolymer	Metal	1	2	1	High
514	4342-30-7	Phenol, 2-[[(tributylstannyl)oxy]carbonyl]	Metal	1	2	1	High
515	4342-36-3	Stannane, (benzoyloxy)tributyl-	Metal	1	2	1	High
516	4782-29-0	Stannane, [1,2-phenylenebis(carbonyloxy)]	Metal	1	2	1	High
517	36631-23-9	Stannane, tributyl- = Tributyltin naphtalate	Metal	1	2	1	High

Table 3.1 continued

NR	CASNR	Name	HPV/pers.	ECO	HUM	Total	Concern
518	85409-17-2	Stannane, tributyl-, monomaphthenoyloxy	Metal	1	2	1	High
519	24124-25-2	Stannane, tributyl[(1-oxo-9,12-octadecad	Metal	1	2	1	High
520	3068-35-5	Stannane, tributyl[(1-oxo-9-octadecenyl)i	Metal	1	2	1	High
521	26239-64-5	Stannane, tributyl[[1,2,3,4,4a,4b,5,6,1	Metal	1	2	1	High
522	1983-10-4	Stannane, tributyltfluoro-	Metal	1	2	1	High
524	2155-70-6	Tributyl[(2-methyl-1-oxo-2-propenyl)oxy]stannane	Metal	1	2	1	High
528	No CAS 099	Tributyltincarboxylate	Metal	1	2	1	High
529	26636-32-8	Tributyltinnaphthalate	Metal	1	2	1	High
530	No CAS 101	Tributyltinpolyethoxylate	Metal	1	2	1	High
531	2279-76-7	Tri-n-propyltin (TPrT)	Metal	1	3	1	High
532	No CAS 051	Triphenyltin	Metal	1	3	1	High
589	900-95-8	Fentin acetate	Metal	1	3	1	High
536	95-76-1	3,4-Dichloroaniline	HPV	1	2	1	High
560	108-46-3	Resorcinol	HPV	3	1	1	High
141	61-82-5	Amitrol = Aminotriazol	HPV	3	1	1	Medium
182	1836-75-5	Nitrofen	HPV	3	1	1	Medium
216	140-66-9	4-tert-Octylphenol 1,1,3,3-Tetramethyl-4-butylphenol	HPV	1	1	1	Medium
254	25154-52-3	Phenol, nonyl-	HPV	1	1	1	Medium
523	1461-25-2	Tetrabutyltin (TBT)	HPV/ Metal	1	2	1	Low
538	99-99-0	4-Nitrotoluene	HPV	3	1	1	Low

13.3 *In vitro* bioassays

The Global Water Research Coalition (GWRC) is currently addressing the vital question whether there is a correlation between *in vivo* effects and *in vitro* bioassays, and if *in vitro* bioassays could provide a more cost- and time-effective system to test endocrine effects. This project also aims to determine which bioassays are effective test platforms and will compare the use of chemical analysis with the bioassays. By doing this the GWRC will develop "a toolbox to detect the *presence of estrogenic activity* in environmental waters" (http://www.globalwaterresearchcoalition.net).

In line with the list selected by the GWRC for *in vitro* bioassays, namely the yeast estrogen screen (YES), MELN reporter gene assay, KBluc reporter gene assay, ER-CALUX reporter gene assay and the E-Screen cell proliferation assay, it is recommended that the use of the YES and KBluc reporter gene assays be continued. The outcome of the current GWRC project will also shed light on the need to further develop new technology on *in vitro* bioassays in South Africa, or if the existing technology should just be transferred to more laboratories.

As mentioned for the chemical analysis, all aspects of sampling and sample handling clearly apply to the bioassays and the reliability of the assays. The following is used in the laboratory at the University of Pretoria for the YES:

13.3.1 Sample preparation

Water collected in 1L – amber glass Schott Suprax bottles (Cat. No. 21 802 54 56) pre-washed with ethanol (Cat. No. 27,0741, Sigma-Aldrich). Contamination or contact with the plastic lid of the bottles must be avoided.

13.3.2 Water extraction procedure

All glassware for sample storage, filtration and extraction of water samples are washed in chromic acid and rinsed once in methanol (Cat No. 34860, Riedel-de Haën) and twice in ethanol (Cat.No. 27,0741, Sigma-Aldrich). For extraction and enrichment of potential estrogen-like compounds a solid phase extraction (SPE) is performed. A volume of 1l is passed in 2 subsequent steps, through a glass wool filter (Cat. No. 000904, Macherey-Nagel), through a 0.45 micron, 47mm sterile filter (Cat. No. E04WG047S1, Osmonics, MicronSep) to remove particulates. The sample is extracted onto a preconditioned Chromabond C18 ec, SPE cartridge (Cat. No. 730 014, Macherey-Nagel), at a flow rate of 10ml/min (Routledge *et al*., 1998). The samples are then eluted from the cartridge with 3.5ml methanol into sterile glass tubes (Cat No. 13099, Pierce, amber reacti-vials™, 5ml). The methanol is then evaporated under a gentle nitrogen stream (Beresford, personal communication, 1999) using a Pierce, Reacti-Vap and a Reacti-Therm heating and stirring module. The sample residue is reconstituted with 1ml ethanol and placed into sterile amber glass bottles (Cat. No.154515, Chromatography research supplies, 4ml), and stored at -20°C prior to analysis.

However, since the WRC is partner to the GWRC project, the standard operating procedure for extraction of water samples should be considered (see document below). Unless various laboratories use standardized guidelines the WRC will not be able to expect dependable and consistent results.

Standard Operating Protocol
Global Water Research Coalition

GWRC-TDE-02A - Rev 1 (2 Mar 2006)
Assay method: Solid-phase extraction (SPE) of aqueous samples

Author(s): Frederic DL Leusch (CRC Water Quality and Treatment, Brisbane, Australia)
Reviewed by: Siobhan Hermanussen (EnTox, Brisbane, Australia)

Overview: This protocol describes extraction of organics from water samples by solid-phase extraction.

Source: Waters. (1999). Oasis sample extraction products for agrochemical and environmental analysis (Report WD003). Waters Corporation, Milford, MA, USA.

Hazards: Samples may contain hepatitis or other sewage-related diseases. Adequate vaccination and proper protective gear (gloves and standard lab-wear) are required in that case. Proper care should be taken with solvents (acetone, hexane, ethanol) and nitrogen and argon gas. Do not exceed maximum recommended vacuum (70 kPa, 20 mmHg) on the SPE manifold.

Laboratory equipment:
- 2.5 L amber glass bottles
- All glass filtration setup for 47 mm disc filter - Millipore XX15 047 30
- Visiprep SPE vacuum manifold, 24 ports - Supelco 57250-U
- Large volume sampler x 24 - Supelco 57275

Reagents:
- Concentrated HCl
- Distilled water
- Acetone HPLC grade
- Hexane HPLC grade
- Methanol HPLC grade
- Ethanol HPLC grade

Consumables:
- Glass pasteur pipette with rubber bulb
- pH 0-14 indicator strips - Ajax Finechem 2404-100
- Glass fiber filter with AP20 binding resin, 47 mm - Millipore AP20 047 00
- Silane-treated glass wool (only for raw sewage samples) - Alltech 4037
- Oasis HLB SPE cartridges, 6cc 500mg - Waters Corp 186000115
- 13x100 mm borosilicate test tubes
- MaxRecovery 12x32 mm tapered vials with PTFE/silicone septum - Waters Corp 186000326c

1) Collection and pre-treatment of samples

1.1. Collect 2.5 L of aqueous samples (such as sewage, surface water, groundwater, or tap water) in methanol-rinsed 2.5-L amber glass bottles.

1.2. Drop the pH of the sample to 2 by adding concentrated HCl dropwise with a glass pasteur pipette. Check pH using pH strips.

1.3. Bring sample back to the laboratory as soon as possible (extraction should be started within 4-6 h of collection). If absolutely necessary, samples may be stored overnight at 4°C.

1.4. *This step is necessary only if you are dealing with raw sewage samples (or similar samples). Otherwise, go immediately to step 1.5.* Raw sewage samples require pre-filtration. Assemble glass filtration unit (see Figure 1) but do NOT use a disc filter. Instead, load reservoir (no. 1 in Figure 1) with silane-treated glass wool. Connect the filtration unit to the vacuum inlet and pass the entire sample through the unit under vacuum. Once the entire sample has been pre-filtered, rinse the reservoir thoroughly with methanol and water and go to step 1.5.

1.5. Assemble glass filtration unit (see Figure 1) and load with 47 mm AP20 glass fiber filter.

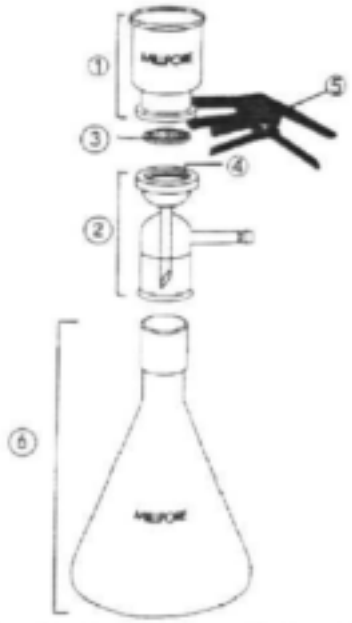

Figure 1. Glass filtration setup (from http://www.millipore.com/catalogue/nsf/docs/C170).

1.6. Connect filtration unit to vacuum inlet. Pass sample through filtration unit under vacuum, 300 mL at a time. You may need to replace the filter every once in a while, depending on the sample. Once all your sample has been filtered, you are ready to start solid-phase extraction.

2) Pre-conditioning of SPE cartridges

Important: *Do not allow the sorbent bed of the SPE cartridge to run dry during extraction, as this can significantly reduce retention efficiency.*

2.1. Load SPE cartridges onto SPE manifold and open the vacuum valves.

2.2. Add 5 mL of acetone:hexane (1:1) in the reservoir of each cartridge and allow the solvent to pass through the sorbent bed by gravity. Just before the solvent reaches the top frit (see Figure 2), add 5 mL of methanol and allow the methanol to pass by gravity. Again, just before the methanol has reached the top frit, add 5 mL of distilled water and allow it to pass by gravity. Finally, just before the water reaches the top frit, fill the reservoir with distilled water and close the vacuum valve at the bottom of the cartridge.

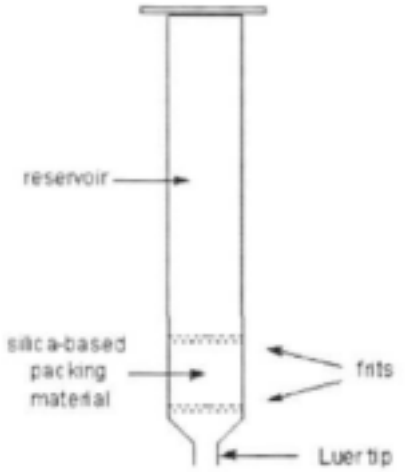

Figure 2. A solid-phase extraction column (from http://www.aquaculture.ugent.be/ATA/code/lip_extr.htm).

3) Extraction

3.1. Connect the SPE manifold to the vacuum trap and the vacuum trap to the vacuum inlet.

3.2. Connect the adapter of the large volume sampler to the top of the SPE cartridge (make sure the seal between the adapter and the SPE cartridge is tight) and drop the weight at the other end of the tube into the sample (in 2.5 L amber glass bottle).

3.3. Open the vacuum valve for all samples. Gently turn the vacuum on. Check that each sample is flowing from the sample bottle to the SPE cartridge. If it is not, the seal between the adapter and the SPE cartridge is not tight: close the vacuum valve for that sample and tweak the connection until you get a tight seal. Remember never to let the sorbent bed run dry while you are doing this. If necessary, top the cartridge up with distilled water.

3.4. Tweak the vacuum strength so that you achieve a flow rate of approximately 10 mL min (as a rule of thumb, that is equivalent to about 3 drops s). As time goes by, you may need to increase the vacuum, but do not exceed 70 kPa (20 mmHg).

3.5. When the vacuum trap is full, stop the vacuum, disconnect the trap, empty the contents down the drain, and reconnect the trap. Turn the vacuum back on, and set the flow rate to about 10 mL min.

3.6. After the entire 2.5 L sample has passed through the SPE cartridge, disconnect the large volume sampler and leave the cartridge on the manifold (with vacuum) to dry for 2-3 h.

3.7. Once the cartridge is dry, remove it from the manifold and wrap it in aluminium foil. The cartridge can be left at room temperature for up to 4 weeks without any loss if recovery efficiency. For a longer period of time, store at -20°C.

3.8. Disconnect the SPE manifold and thoroughly rinse all equipment with methanol and dHOH (deionised water).

4) Elution

4.1. Prepare the SPE manifold with the tube rack insert, and a 13x100 mm test tube for each sample.

4.2. Load the cartridges onto the SPE manifold (above the appropriate valves to elute into the test tubes).

4.3. Turn the vacuum valve of each cartridge on. Add 5 mL of methanol to the reservoir of each cartridge and allow the solvent to percolate through the sorbent bed and elute by gravity alone.

4.4. Once most of the methanol has eluted, connect the SPE manifold to the vacuum inlet and gently turn the vacuum on to remove most of the solvent from the sorbent bed (about 1-2 minutes).

4.5. Turn the vacuum off and add 5 mL of acetone hexane (1:1) to the reservoir of each cartridge. Again allow the solvent to percolate and elute by gravity, then turn the vacuum back on to remove the remaining solvent from the sorbent bed.

4.6. Once all solvent has eluted, the samples are ready to be blown down. The SPE manifold can be dismantled and thoroughly rinsed with methanol and dHOH.

5) Evaporate and reconstitute

5.1. Load the test tubes with the 10 mL eluted sample into the nitrogen blow-down unit. Lower the needles of the blow-down unit into the test tubes, and turn on the nitrogen flow to create a gentle flow on the surface of the samples (a small "dent" on the surface is ok, but do not allow the flow to cause the sample to "splash around").

5.2. Lower the needles of the blow-down unit every 30 min to keep a good (but gentle!) flow on the surface of the samples. It should take 1-2 h to blow the samples to dryness.

5.3. Once completely dry, remove the tubes from the blow-down unit. Reconstitute each sample by adding 500 µL of ethanol to each tube. Vortex the samples thoroughly.

5.4. For each sample, pipette 40 µL aliquots into 12 MaxRecovery vials.

5.5. Load the MaxRecovery vials in the nitrogen blow-down unit and evaporate all the ethanol.

5.6. Turn the nitrogen off, switch the line to the argon cylinder, and fill the MaxRecovery vials with argon. Screw the vials shut and store in an opaque box for transport.

Note about equivalent volumes:
Using this method, each aliquot of the sample contains **eV = 0.2 L** (2.5 * 40 / 500)

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