A PILOT STUDY ON THE OCCURENCE OF ENDOCRINE DISRUPTIVE CHEMICALS IN A DDT-SPRAYED AREA

Report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

BACKGROUND

The Luvuvhu river catchment near Thohoyandou, Vhembe District in Limpopo Province, is a tropical, high-risk malaria area where 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) has been used annually since 1945 for controlling malaria. The Albasini dam was built in the river close to Louis Trichardt and outside (west) of the DDT-sprayed area. The Nandoni dam was recently constructed in the middle section of the Luvuvhu river, east of the town Thohoyandou. The dam supplies water to the urban areas of Louis Trichardt and Thohoyandou and the rural communities in the northern part of the Limpopo (Department of Water Affairs and Forestry (DWAF), 2005). A large new fish weir was built near the Xikundu village as part of the Nandoni water supply scheme.

DDT is a broad-spectrum insecticide, very popular due its low cost, effectiveness, long residual persistence and low acute toxicity in mammals (Metcalfe, 1989). Although DDT has been banned from international use, it is still sprayed onto the interior surfaces of homes to decrease the incidence and spread of the malaria by controlling mosquitoes (Attaran et al., 2000; Roberts et al., 1997). Technical-grade DDT as applied has estrogen-like properties (Bishop et al., 1991; Fry and Toone, 1981; Fry et al., 1987; Guillette et al., 1994) due to the *o,p'*-isomer of DDT (Metcalf, 1995). The persistent metabolite 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p*'-DDE) inhibits androgen binding to the androgen receptor, androgen-induced transcriptional activity, and normal male prepubertal development (Kelce et al., 1995). Abnormalities in the reproductive system are associated with *in utero* DDT- or DDE-exposure of male animals and include amongst others, abnormal development of ovarian tissue (Fry and Toone, 1981), reduced penis size in alligators (Guillette et al., 1999), hypospadias (Gray et al., 2001) and undescended testis (Facemire et al., 1995; Gray et al., 2001).

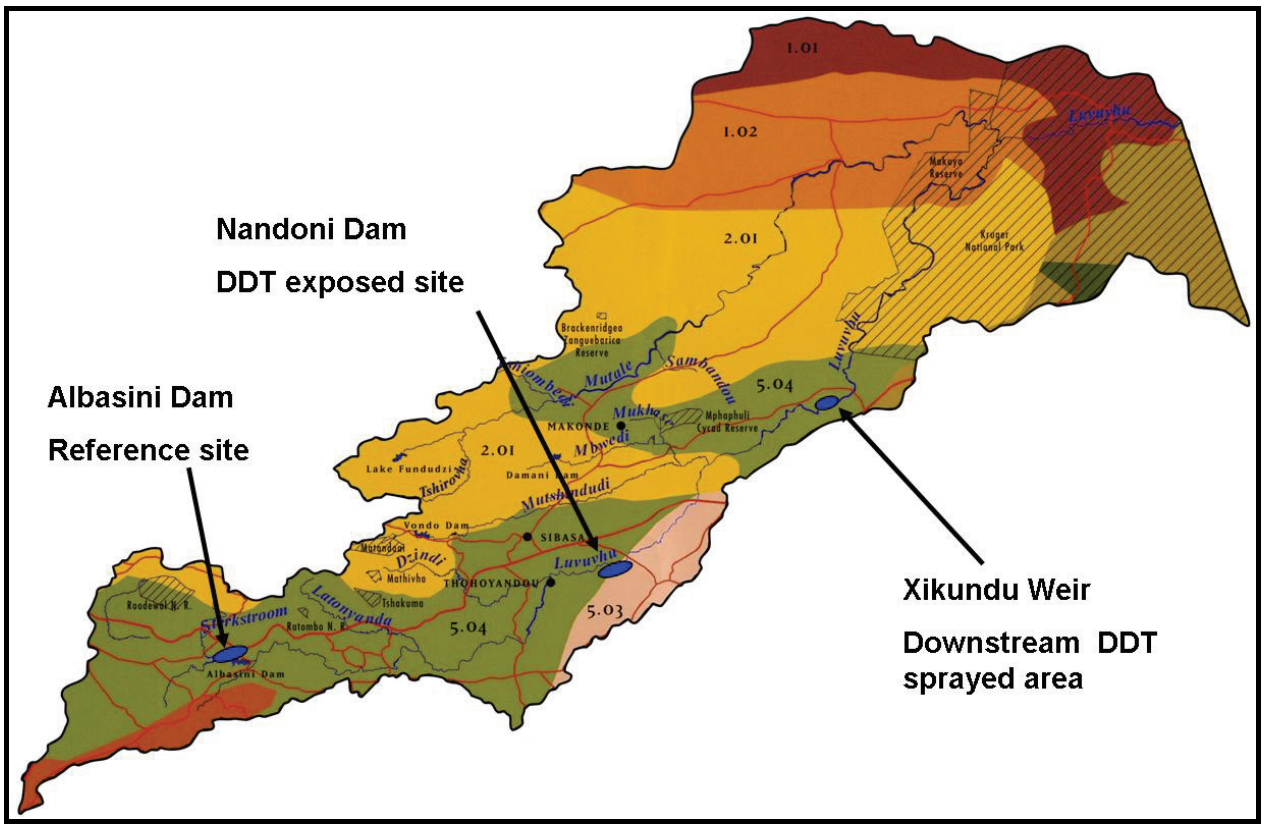
The acute toxicity of DDT in mammals is low, but no information is available on the impact of chronic, low-dose exposure (endocrine disruptive) of DDT and the effects on aquatic and human health in South Africa, Africa or other countries where DDT is being used. Recently Aneck-Hahn et al. (2007) demonstrated in a cross-sectional study on healthy male subjects (n=311) aged between 18 and 40 years (23±5) living in a currently DDT-sprayed area in Limpopo Province, South Africa that the ejaculate volume, sperm count, motility and morphology were adversely affected. The authors concluded that the high exposure levels of *p,p'*-DDT and *p,p'*-DDE in these men are reasons for concern and may have far reaching implications for reproductive and general health.

OBJECTIVE

The objective was to analyse water, sediment and fish tissues for DDT residues and estrogenicity of water as a pilot for a more comprehensive study.

METHODOLOGY

Water, sediment and fish were sampled at the Albasini- and Nandoni dams as well as the Xikundu weir in the Luvuvhu river system, Limpopo Province.



A map indicating the three sites where sampling was done in the Luvuvhu river catchment (Map adapted from State of the Rivers Report, WRC, 2001).

Collection of water and sediment was done in July (low flow 1) and October 2005 (low flow 2) and January 2006 (high flow) for target chemical analyses. Male *O. mossambicus* and *C. gariepinus* were collected during low flow 2 using gill nets. Macroscopic evaluation of the fish and testicular microscopy were done and available fat collected for analytical chemistry.

RESULTS

The water temperature ranged from 20.1°C to 24.3°C during the low flow and from 25°C to 33.7°C during the high flow period. The pHs measured from 5 of 9 (55.5%) samples were higher than the suggested range for South African inland waters of 6.0 and 8.0 pH units.

The percentage oxygen saturation ranged between 64.9% (sublethal range) and 124.1%. The lowest value measured at ND during the high flow season.

Cytotoxicity was found at Albasini Dam on the YES assay, while estrogenicity was demonstrated at Nandoni Dam on the YES and ER-Calux® assays. No pesticide residues were found in water or sediment, but bio-concentrated levels were present in both *O. mossambicus* and *C. gariepinus.* The Pb, Al, Cr, Fe and Zn levels in water were high compared to guidelines. Sufficient numbers of *O. mossambicus* were found but the number of *C. gariepinus* was limited. Seventeen of 30 (57%) of male *O. mossambicus* had intersex with testicular oocytes observed in the testes.

DISCUSSION OF RESULTS

pH provides an indication of acidity or alkalinity of a water body/course and ranges between 6.0 and 8.0 in most natural waters of South Africa (DWAF, 1996), but in this study pH varied between 7.29 and 8.9 at different times and localities. Previous studies also noted higher pH in the Luvuvhu river system (Heath and Claassen, 1999; Fouché, 2005) and the State of the Rivers Report: Letaba and Luvuvhu River Systems (State of the Rivers Report, WRC, 2001) pointed out that the predominant water quality problem across the catchment has a tendency towards eutrophication. In the current study the higher pH levels measured at all localities during low and high flow seasons may create optimal conditions for algal blooms and increased aquatic weed growth.

Dissolved oxygen (DO) concentration was measured during the high flow season at Nandoni Dam when the percentage of the saturation concentration was only 64.9% but within the guidelines (DWAF, 1996) using the 1 day Minimum Allowable Values (MAV). According to DWAF (1996) adequate DO concentrations is vital for the survival and performance of the aquatic biota because it is necessary for the respiration of all aerobic organisms. However, too low DO levels can cause sublethal hypoxia in fish from temperate areas and could result in behavioural changes, altered metabolism and physiology, deterioration of major organs, reduction in reproductive output, lowered endurance and swimming capacity and reduced growth (Breitburg, 2002).

The YES screen indicated a quantifiable amount of estrogenic activity (0.14-0.3 ng/L EE) at Nandoni Dam during the low flow season. Of specific concern is that all the samples collected from the Albasini Dam were cytotoxic on the YES. The metal concentrations found could contribute to the cytotoxicity, but other chemicals unaccounted for in this study, could also add to the effect. In those samples that exhibited only cytotoxicity, the estrogenic

activity could be masked and therefore give a false negative result, as the estrogenic response may lie in the toxic range. The breakdown product, DDE, is known to have anti-androgenic activity and thus could also account for the cytotoxic and submaximal response in the YES. 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) (Bornman et al., 2007*).* The ER-Calux® confirmed the hypothesis that the toxic response in the YES could well be underestimating estrogenic activity or giving a false negative. The estrogenicity values between 0.14 and 0.31 ng/L ER-Calux® at Nandoni Dam.

The high water levels of lead (Pb) possibly indicate recent Pb contamination in the Luvuvhu river system. Pb is known to be toxic at very low concentrations. However, the toxicity of Pb depends on variables such as pH, salinity, temperature, dissolved oxygen and most important hardness of the water. The effect of pH was not of concern in this study as the background pH of the Luvuvhu river seemed to be more alkaline than acidic (pH<6) where Pb tends to be toxic.

Although this study only focussed on the four endocrine disrupting metals (EDM) arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg), the high levels of aluminium (Al), Chromium (Cr), iron (Fe), and zinc (Zn) in water and sediment cannot be ignored. However, this study was not designed to revise the link of endocrine effects to metal concentrations. Such links are known to exist (Johnson et al., 2003) and the presence of these metals retains them among the possible causes for the observed effects, but this needs a more detailed study.

None of the chemicals including the DDTs tested were present above the detection limit in either water or sediment samples. This was contrary to the previous findings of Burger (2005) where various agrochemical and industrial residues were found. However, *p,p'*-DDT, -DDD and DDE, lindane, endosulfan and endrin residues were found in fat tissue from *O. mossambicus*. *C. gariepinus* fat samples contained *p,p'*-DDT and *o,p'- and p,p'*-DDD. This implied that those fish were exposed at some time during their lifecycle and subsequently bio-concentrated the chemicals in lipid tissue.

Only a few *C. gariepinus* were found for evaluation and no intersex stigmata were found. However, during this study intersex was observed for the first time in *O. mossambicus* and it is now the second species in South African waters with the reported intersex condition.

Primary oocytes scattered through out the testicular tissue were observed similar to the previous findings in *C. gariepinus* (Barnhoorn et al., 2004). In total 57% of the *O. mossambicus* caught in the Luvuvhu had intersex. Intersex in fish is a well documented and accepted phenomenon in fish from waters possibly polluted with xeno-estrogens. The specific agent(s) causing the intersex in *O. mossambicus* was not clear.

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LIST OF ABBREVIATIONS

AEV	Acute effect value
Al	Aluminium
ARC	Agriculture Research Council
As	Arsenic
ATSDR	Agency for Toxic Substances and Disease Registry
BC	Breeding colors
Bdl	Below detection limit
BHC	Benzene hexachloride or gamma-HCH
Cd	Cadmium
CEV	Chronic effect value
CPRG	Chlorophenol red-β-galactopyranoside
Cr	Chromium
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
DO	Dissolved oxygen
DWAF	Department of Water Affairs and Forestry
EC	Electrical conductivity
EC_{50}	Effective concentration in 50% of exposed samples
EDC	Endocrine disrupting chemical
EDM	Endocrine disrupting metal
EDTA	Ethylenediaminetetraacetic acid
EE	estradiol equivalents
EE max	Estradiol equivalent maximum
ER	Estrogen receptor
Fe	Iron
GCMS	Gas chromatography-mass spectrometry
GPS	Global positioning system
GSI	Gonadal somatic index
Hg	Mercury
HIS	Hepatic somatic index
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
L	Lumen

MAP	Mean annual precipitation
MAR	Mean annual runoff
MAV	Minimum allowable values
OcP	Octylphenol
OCs	organochlorine pesticides
Pb	Lead
PCB	Polychlorinated biphenyl
p-NP	Nonylphenol
PO	Primary oocytes
RNON	Reverse phase nonylphenol
S	Spermatozoa
SA	South African
SD	Standard deviation
TDS	Total dissolved salts/solids
t-NP	Technical nonylphenol
TWQR	Target water quality range
UGPI	Urogenital papilla index
UGPLI	Urogenital papilla length index
UL	Upper lip
VTG	Vitellogenin
XW	Xikundu weir
YES	Recombinant Yeast Screen
Zn	Zinc

1 INTRODUCTION AND OBJECTIVES

1.1 Literature Study

DDT is a broad-spectrum insecticide, very popular due its low cost, effectiveness, long residual persistence and low acute toxicity in mammals (Metcalfe, 1989), and was first used in 1939 (Van Metre et al., 1997). In 1973 the use of DDT in the United States was limited to the control of emergency public health problems. However, in selected regions of the world where malaria is endemic, such as South Africa, Swaziland, and Madagascar, DDT is sprayed onto the interior surfaces of homes to decrease the incidence and spread of the disease by controlling mosquitoes (Attaran et al., 2000; Roberts et al., 1997). In South Africa DDT is being used in three provinces namely Limpopo, Mpumalanga and KwaZulu-Natal. In Limpopo Province DDT was introduced for malaria mosquito control in 1943 and since 1966 is being sprayed annually to control the disease.

Although DDT has been banned for international use, countries like South Africa have restricted use for malaria vector control. South Africa is a signatory to the Stockholm Convention on the control of Persistent Organic Pollutants (POPs) ratified during the World Summit on Sustainable Development in August 2002. Parties to the Convention undertake to limit and control the release of persistent organic pollutants into the environment.

Technical-grade DDT as applied is composed of 65–80% of the active ingredient, *p,p'*-DDT. The other components include 15–21% of the nearly inactive *o,p'*-DDT, up to 4% of *p,p'*-DDD, and up to 1.5% of 1-(*p*-chlorophenyl)-2,2,2-trichloroethanol (Metcalf, 1995). Technical grade DDT may also contain DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) and DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane) as contaminants. Both DDD and DDE are breakdown products of DDT (ATSDR, 2002).

Technical DDT has estrogen-like properties (Bishop et al., 1991; Fry and Toone, 1981; Fry et al., 1987; Guillette et al., 1994), which is largely due to the *o,p'*-isomer of DDT, (Metcalf, 1995) which mimics the action of the natural ligand, 17β-estradiol. Results from numerous studies using a wide range of experimental approaches suggest that binding to the estrogen receptor and subsequent events are the predominant mechanism by which estrogenic effects are expressed (Bulger and Kupfer, 1983). Using the *in vitro* E-screen test, Soto et al. (1997) found that *o,p'*-DDT, *o,p'*-DDD, and *p,p'*-DDT were full estrogenic agonists, *p,p'*-DDE and *p,p'*-DDD were partial agonists and technical DDT as a mixture was a full agonist . The estrogenic potency of these substances was several orders of

magnitude weaker than 17β-estradiol and diethylstilbestrol. Therefore 10^7 times more *o,p'*-DDT, *o,p'*-DDD, *p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, and technical DDT was needed to produce maximal cell yields than 17β-estradiol (Soto et al., 1998).

The persistent metabolite of *p*,*p´*-dichlorodiphenyl-trichloroethane (DDT) 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p*'-DDE) inhibits androgen binding to the androgen receptor, androgen-induced transcriptional activity, and normal male prepubertal development (Kelce et al., 1995). Danzo (1997) reported not only was *p,p*'-DDE the most potent of the xenobiotics tested at inhibiting 5α-dihydrotestosterone binding to the androgen receptor, but both *o,p*'-DDT and *p,p*'-DDT were potent inhibitors. *p,p'*-DDE also showed partial antagonistic effects on MCF-7 cell proliferation (Soto et al., 1998). Abnormalities in the reproductive system are associated with *in utero* DDT- or DDE-exposure of male animals and include amongst others, abnormal development of ovarian tissue (Fry and Toone 1981), reduced penis size in alligators (Guillette et al., 1999), hypospadias (Gray et al., 2001) and cryptorchidism (Facemire et al., 1995; Gray et al., 2001).

In a pilot study on catfish, chickens and game birds as sentinels of environmental chemical exposure Burger (2005) found various chemical residues. Both surface water dams monitored during this study contained quantifiable levels of alpha-endosulfan, beta-endosulfan, lambda-cyhalothrin, PCB-20, gamma-BHC, dieldrin, *p-p*'-DDD and very high levels of p-nonylphenol. In sediment some of the chemicals were present but at levels lower than the detection limit and therefore not quantifiable. *p*-Nonylphenol (*p*-NP) was detected in the sediment from one pool. Composite fat samples from chicken collected from three currently DDT-sprayed villages namely Dididi, Tshiulongoma and Tshikudini contained traces of PCB-180, *p-p*'-DDD, *p-p*'-DDT, *p-p*'-DDE and *p*-NP all at ppm (µg/kg) levels. Although only a small number of birds were found, liver samples of all the terrestrial birds contained *p-p*'-DDE residues, two of seven aquatic birds, and very high levels in the detritus feeder bird. Blood samples from a few catfish contained *p-p*'-DDE, *p-p*'-DDD and *p*-NP levels.

The acute toxicity of DDT in mammals is low, but no information is available on the impact of chronic, low-dose exposure (endocrine disruptive) of DDT and the effects on aquatic and human health in South Africa, Africa or other countries where DDT is being used. In a recent WRC study by Bornman et al. (2007) in an urban nature reserve, various estrogenic compounds such as *p,p'*-DDT, lindane, alkylphenols and phthalate residues were present in water samples. The estrogenicity of which was sufficient to

pose a risk of reproductive damage in fish (Matthiessen et al., 2006) and this was confirmed by the occurrence of intersex 50% of male *C. gariepinus* sampled. Recently Aneck-Hahn et al. (2007) demonstrated in a cross sectional study on healthy male subjects (n=311) aged between 18 and 40 years (23±5) living in a currently DDT-sprayed area in Limpopo Province, South Africa that the ejaculate volume, sperm count, motility and morphology were adversely affected. The authors concluded that the high exposure levels of *p,p'*-DDT and *p,p'*-DDE in these men are reasons for concern and may have far reaching implications for reproductive and general health.

1.2 Luvuvhu river

The Luvuvhu river catchment (Fig 1.1) near Thohoyandou, Vhembe District in Limpopo Province, is a tropical, high-risk malaria area where 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) has been used annually since 1945 for controlling malaria. The Luvuvhu river and some of its tributaries (including the Mutshindudi and Mutale rivers) are perennial rivers that rise in the Soutpansberg Mountains and run for about 200 km through a diverse range of landscapes before joining the Limpopo river near Pafuri in the Kruger National Park (KNP). The Luvuvhu Catchment is part of the larger Limpopo system, which extends into Mozambique. It covers 5 941 km^2, with a mean annual precipitation (MAP) of 608 mm, mean annual evaporation of 1 678 mm and mean annual runoff (MAR) of 519 million cubic metres (ranging from 85 to 1 900 million cubic metres) (Department of Water Affairs and Forestry (DWAF), 2003).

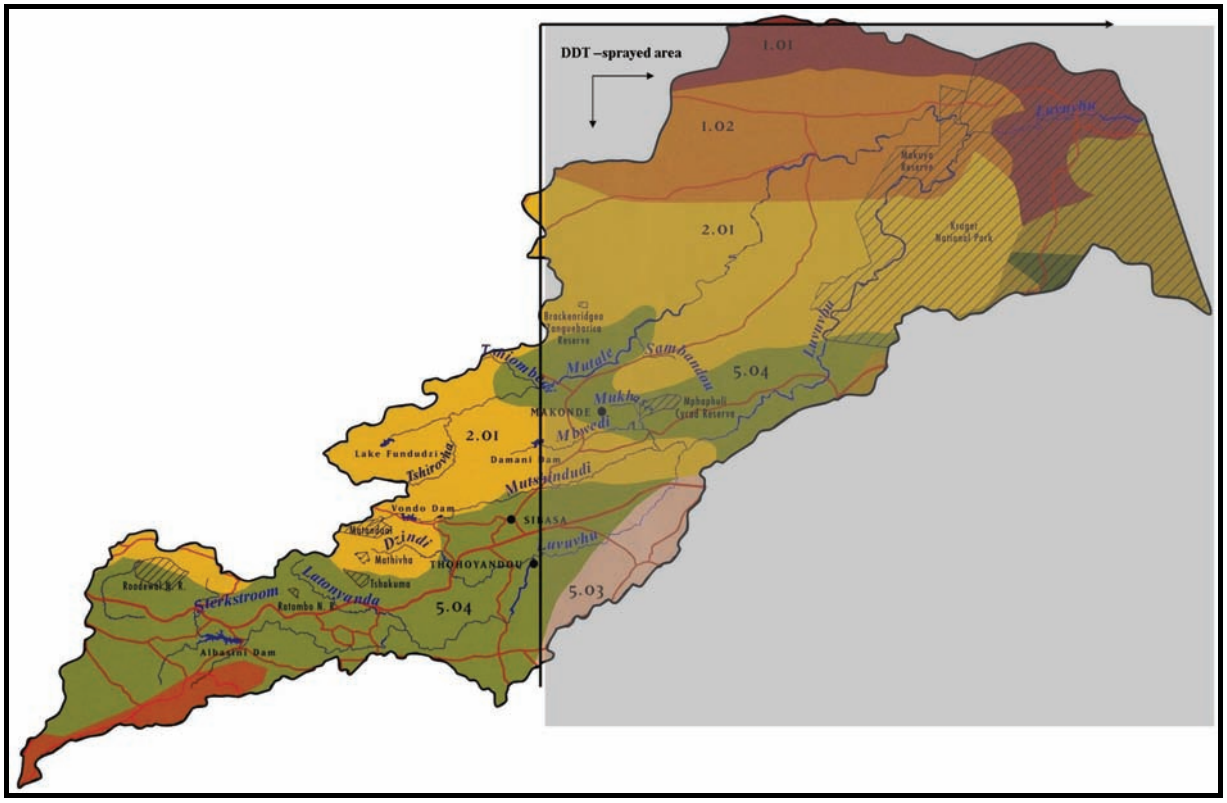

Figure 1.1: The Luvuvhu river catchment and the DDT-sprayed area in Limpopo Province. (Map adapted from State of the Rivers Report, WRC, 2001).

Dams in the Luvuvhu river catchment include the Albasini and Nandoni dams and the smaller Mambedi, Tshakhuma, Damani, Vondo, and Phiphidi dams, of which the latter two lie in the Mutshindudi river (Fig 2.1). The Albasini dam was built close to Louis Trichardt and outside (west) of the DDT-sprayed area. The area around the Albasini Dam is a private conservancy. Riparian vegetation consists of dense stands of large trees, shrubs and reeds. Land-use activities include forestry (11%) and agriculture (20%). Plantations cover 44% of the upper reaches of the Luvuvhu and Lotanyanda rivers, decreasing to less than 10% towards the Albasini Dam, while subsistence farming is only about a third of the total agricultural component (DWAF, 2003). The Nandoni dam was recently constructed in the middle section of the Luvuvhu river east of the confluence with the Dzindi tributary and to the east of the town Thohoyandou. The dam supplies water to the urban areas of Louis Trichardt and Thohoyandou and the rural communities in the northern part of the Limpopo (www.vhembe.co.za/places.html). A large new fish weir was built near the Xikundu village as part of the Nandoni Scheme. Near the western Kruger National Park border, in the steep Lanner Gorge, the Mutale river joins the Luvuvhu river from where it traverses through the Kruger National Park and joins the Limpopo River at Crook's Corner on the Mozambique border.

Although in some villages water is available in individual homes, the majority has to rely on the community water tap, on the “water trucks”, or else the people have to collect water from the river in plastic containers. The river is used for various purposes including bathing, washing cars and clothing.

1.3 Sentinel species used in study

The sharptooth catfish, *Clarias gariepinus* and the Mozambique tilapia *Oreochromis mossambicus,* both indigenous species, are present in large numbers across the country. *C. gariepinus* inhabit calm waters from lakes, streams, rivers, swamps to floodplains, some of which are subjected to seasonal drying, but the presence of the accessory air breathing organs allow the catfish to survive (Skelton, 1993). *O. mossambicus* occurs in all but fast-flowing waters, thriving in standing waters. It is quite a hardy fish being tolerant of fresh, brackish or marine waters and can survive temperature ranges from 15˚C to 40˚C. *O.mossambicus* feed on algae, especially diatoms, and detritus (the organic matter in which DDT and its metabolites accumulate) and larger individuals might even take insects or other invertebrates (Skelton, 1995). *O. mossambicus* is common in the study area.

The males of both species also have distinct uro-genital sexual papillae, located behind the anus, which are absent in females (de Graaf and Janssen, 1996) to determine sex allocation. The normal ultra structure and histology of the gonads of male *C. gariepinus* and *O. mossambicus* have been studied in detail at the University of Johannesburg formerly Rand Afrikaans University (Steyn, 1984; Pieterse, 2004). Sentinel species such as *C. gariepinus* and *O. mossambicus* should provide useful models to screen the Luvuvhu river system for DDT and metabolite pollution.

1.4 Hypothesis

The hypothesis for this study was that DDT and metabolites are present in water bodies, bio-concentrated in aquatic species and those levels are low at the Albasini Dam (references site), and intermediary levels in the Nandoni Dam with the highest levels at the Xikundu Weir.

2 MATERIALS AND METHODS

2.1 Sampling sites

Water, sediment and fish were sampled at the Albasini- and Nandoni dams as well as the Xikundu weir in the Luvuvhu river system, Limpopo Province (Fig 2.1).

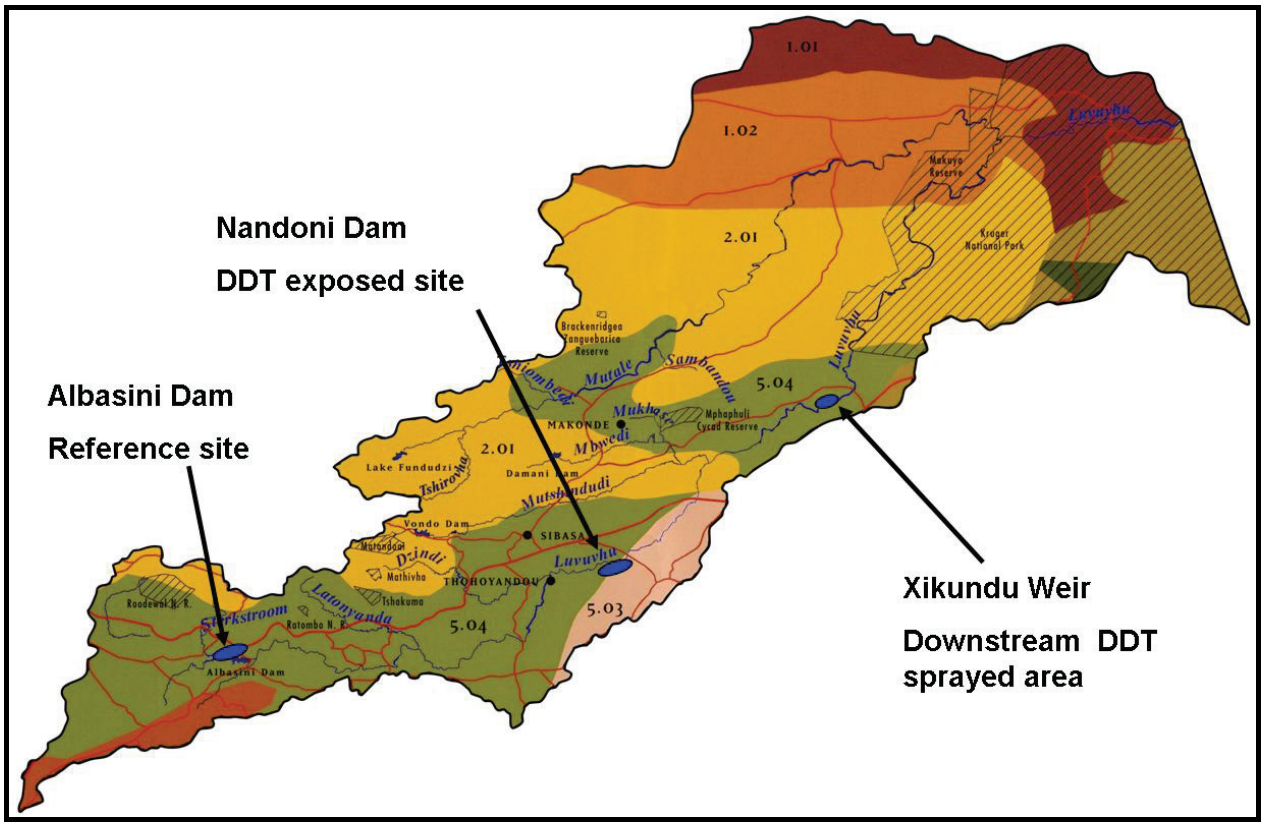

Figure 2.1: A map indicating the three sites where sampling was done in the Luvuvhu river catchment (Map adapted from State of the Rivers Report, WRC, 2001).

Albasini dam GPS; (758 m; S 23º05.974'; E 30º05.998'):

This dam is situated outside the DDT-sprayed area and has been designated the "control/reference" site throughout this project.

Nandoni dam GPS; (490m,S 22º88.945'; E 30º35.745'):

The newly constructed Nandoni Dam is situated in the DDT-sprayed area.

Xikundu weir GPS; (450m; S 22º48.506'; E 30º47.924'):

This site lies to the east of Thohoyandou, towards the Kruger National Park after the Luvuvhu river has run through about 70km of DDT-sprayed area.

2.2 Sample collection

2.2.1 Water and sediment

Collection of water and sediment was done in July (low flow 1) and October 2005 (low flow2) and January 2006 (high flow). Water samples were collected at a depth of 5-15cm under the water surface in clean (methanol pre-washed) glass bottles and stored in a

refrigerator until further analysis. Sediment was collected from the bottom of the locality in methanol pre-washed glass bottles and stored at 4ºC until analysed.

Surface water and sediment samples for metal analyses were collected in 250ml plastic jars. Samples were collected on the same days and at the same locality as samples for other target analyses. 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 frozen in the laboratory until analysed.

During each sampling the water temperature (ºC), pH, conductivity (µS/cm), total dissolved salts (ppm) as well as oxygen percentage (%) and mg/l of each locality were measured. This was done using a PH Scan 2 pH meter (Eutech Instruments), a Cyberscan CON 110 conductivity/TDS/°C meter RS 232 (Eutech Instruments) and a Cyberscan DO 100 meter for dissolved oxygen.

2.2.2 Sampling of water and sediment

Collection of water and sediment was done in July (low flow 1) and October 2005 (low flow 2) and January 2006 (high flow). Water samples were collected at a depth of 5-15cm under the water surface in clean (methanol pre-washed) glass bottles and stored in a refrigerator until further analysis. Sediment was collected from the bottom of the locality in methanol pre-washed glass bottles and stored at 4ºC until analysed.

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2.2.3 Sampling of selected fish species

Male *O. mossambicus* and *C. gariepinus* were collected during low flow 2 using gill nets. All fish were weighed and measured. Blood was taken immediately from the caudal aorta, using cold aprotinin EDTA/heparin-vacutainers for catfish and tilapia respectively.

A set of blood was drawn using vacutainers with no additive. The samples were kept on ice until centrifuged at 4°C, 1300 *g* for 15 minutes using a Mistral 3000i centrifuge. Plasma and serum samples were then stored in Eppendorf tubes and glass tubes respectively at –20°C for target chemical analyses and vitellogenin (VTG) analysis once the assay becomes available.

Once the blood was drawn, fish were sexed according to the sexual papilla in the case of *C. gariepinus* and according to the upper-lip and breeding colours in the case of *O. mossambicus.* Fish were then sacrificed and the gonads were dissected after macroscopic evaluation, measured and weighed for gonadosomatic index (GSI) calculations. The gonads were fixed in Bouins fixative and after further dehydration in graded ethanol and embedding in paraffin wax; sections (5 µm) were cut and stained with Haematoxylin and Eosin. The gonadal slides were histologically examined using light microscopy using a range of magnifications. The liver was also taken out and weighed in order to determine the hepatosomatic index (HIS) (Goede and Barton, 1990).

In the case of *C. gariepinus* sharptooth catfish the secondary sexual papilla was measured and the urogenital papilla index (UGPI) determined.

Available fat was collected from each fish, wrapped in foil and stored at –20°C for target chemical analyses

2.3 Analyses

2.3.1 Determination of estrogenic activity in water samples

2.3.1.1 Recombinant Yeast Screen (YES)

The yeast was obtained from Prof. J.P. Sumpter's laboratory, in the Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex in the United Kingdom. The assay was performed according to the method described by Routledge and Sumpter (1996) and was described in detail in the report on K5/1505 (Bornman et al., 2007).

The assay was carried out according to the standard assay procedure (Routledge and Sumpter, 1996) in a Type II laminar flow air cabinet, to minimise aerosol formation. Serial dilutions of 17β-estradiol (Cat. No. E8875, Sigma) ranging from $1x10^{-8}$ M to $4.8 x 10^{-12}$ M (2.274 µg/L to 1.3 ng/L) were made in ethanol (Cat. No. 27, 0741, Sigma-Aldrich) and transferred to a 96 well micro-titre plate (Cat. No. 95029780, Labsystems). After allowing the ethanol to evaporate to dryness on the assay plate, aliquots (200 µL)

of the assay medium containing the yeast and CPRG were then dispensed into each sample well. 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 4.8×10^{-12} M (2.274 µg/L to 1.3 ng/L) which was extended to a concentration of 1.19 x 10-15 M (3.24 x 10-13 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 d. After 3 d incubation the colour development of the medium was checked daily at an absorbance (abs) of 540 nm for colour change and 620nm for turbidity of the yeast culture. The absorbance was measured on a Titertek Multiskan MCC/340 (Labsystems) 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 from yellow to orange. All experiments were performed in duplicate. The following equation was applied to correct for turbidity:

Corrected-value = test abs (540nm) - [test abs (650nm) - 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 (EE-max), slope, effective concentration at 50% (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 (Aneck-Hahn et al., 2005).

2.3.1.2 ER-Calux®reporter gene assay:

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®).

2.3.2 Analytical chemistry

For target chemical residue analysis, the compounds selected were the organochlorine pesticides (OCs) (Alpha-HCH, gamma-HCH (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), 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**.**

In the analytical laboratory water samples were filtered through a glass fibre filter before analysis commenced. The samples were analysed in the Residue Laboratory of the Agriculture Research Commission (ARC) at Onderstepoort Veterinary Institute, using standard methods, reverse phase nonyl phenol (RNON) 055 (Cacho et al., 1995) and gas chromatography - mass spectrometry (GCMS) 003 (Cacho et al., 1995). OcP, *p*-NP and OCs were extracted from water samples using solid phase extraction. Clean up was performed on a C_{18} cartridge and the analytes eluted with hexane-diethyl ether. Quantification was accomplished via a fortified calibration curve. The alkylphenols (APs) were detected using fluorescence detection at a detection limit of 0.5 μg/L. OCs were detected using Gas Chromatography coupled to a quadruple Mass Spectrometry detector and a detection limit of 0.5 μg/L.

The sediment samples were analysed using standard methods RNON 059 and GCMS 010 (Croce et al., 2003). OCs was extracted from sediment samples with dichloromethane-acetone (1:1, v/v) using a soxhlet extraction procedure. Sample clean up was performed on a C_{18} 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 C_{18} 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 were detected using GC coupled to a quadruple MS detector at a detection limit of 0.05mg/kg.

Metals were measured in water and sediment using the ICP-MS (Inductively Coupled Plasma – Mass Spectrometry) methodology. 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. A full ICP-MS scan was done with specific attention to the endocrine disrupting metals (EDM) in water and sediment. The EDM included Cadmium (Cd), Arsenic (As), Lead (Pb) and Mercury (Hg).

Water and sediment samples were analysed in duplicate for 70 different elements, including the four known endocrine disrupting metals (EDMs) Cd, As, Pb, Hg. 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. Not all elements are bio-available due to speciation therefore a second total extraction was done to ensure all the elements bound to the sediment are extracted.

The fish fat samples were analysed using standard methods in the Residue Laboratory, Onderstepoort, RNON: 057 (Tsuda et al., 1999) and GCMS: 008 (Bordet et al., 2002). The 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 C_{18} cartridge. Analytes were eluted with methanol from C_{18} and quantification is accomplished via a fortified calibration curve. The APs were detected using fluorescence detection at a quantification limit of 0.05mg/kg. OCs in fat samples were extracted and clean up was performed on a C_{18} 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 a fortified calibration curve. The OCs were detected using gas chromatography (GC) coupled to a quadruple mass spectrometry (MS) detector at a detection limit of 0.010mg/kg.

2.4 Statistical analyses

For the purpose of this study differences in selected groups of samples were calculated using the Microsoft software package Excel and Graphpad Prism (version 2.01).

3 RESULTS

3.1 Water and sediment

The physico-chemical parameters are shown in Table 3.1. The water temperature ranged from 20.1°C to 24.3°C during the low flow and from 25°C to 33.7°C during the high flow period. The pHs measured from 5 of 9 (55.5%) samples were higher than the suggested range for South African inland waters of 6.0 and 8.0 pH units. The percentage oxygen saturation ranged between 64.9% and 124.1%. The total dissolved solids/salts (TDS) ranged from 66.9 Mg/L (high flow, Nandoni Dam) and 528 mg/L (low flow 2, Nandoni Dam) and the electrical conductivity (EC) from 133.5 µS/cm (high flow, Nandoni Dam) and 840 µS/cm (low flow 2, Albasini Dam) (Table 3.1).

3.2 Estrogenicity of water

3.2.1 YES results

In order to facilitate interpretation of the results for each sample site, the findings were summarised into Tables 3.2 – 3.7. The tables provide the type of response and the EC_{50} and maximum estradiol equivalents (EE-Max) (ng/L) as extrapolated from the 17 β-estradiol curve (Figs 3a – 5b) for that specific experiment. All samples were analysed in triplicate.

3.2.1.1 Sample site: Albasini Dam

Cytotoxicity was observed on all three sampling occasions. Low flow 2 and the high flow 1 although the estrogenic activity (one or two points above the detection limit of the assay) was unquantifiable.

Table 3.1: Physical water quality data from the three localities selected in the Luvuvhu river.

	Albasini Dam			Nandoni Dam			Xikundu Weir			DWAF, 1996
Date	**Low flow 1**	**Low flow 2**	**High flow**	**Low flow 1**	**Low flow 2**	**High flow**	**Low flow 1**	**Low flow2**	**High flow**	
Temperature °C	21.5	33.7	25	24.3	26.9	27.8	22.6	20.1	33.7	5-30°C*
pH	7.75	8.9	8.6	8.78	8.85	7.48	7.29	7.79	8.55	6.0-8.0*
Oxygen ($O_{2)}$ %	117.9	103.1	106.5	124.1	122	64.9	101.8	87.5	116	> 40 % (Lethal)***
Oxygen (O_2) mg/L	10	7.38	8.77	10.38	9.8	5.57	8.66	7.91	8.37	> 4 mg/L (Dallas & Day, 1993)
Total Dissolved Solids (TDS) mg/L	155	418	158	89.7	528	66.9	82.5	102	68.4	****
Electrical conductivity (EC) µS/cm	301	840	315	178.4	263	133.5	162.5	208	137.3	*****

* = Inland waters of SA generally range between 5 – 30°C; Should not be allowed to vary from background levels for a specific time and site by more than 2°C or 10%.

** = SA waters range between 6.0 and 8.0 pH units; should not vary from the range of background pH values by > 0.5 of a pH unit, or by > 5% for a specific site and time of day and should be assessed by whichever estimate is the more conservative.

*** = Criteria for dissolved oxygen concentrations (percentage saturation) are given in terms of the minimum allowable values (See guidelines DWAF, 1996).

**** = TDS concentrations should not change by >15% from the normal cycles of water body under unimpacted conditions at any time of the year. Refer to DWAF 1996.

***** = EC is proportional to TDS and a useful surrogate measure of the TDS content of waters with a low organic content. Refer to DWAF 1996.

Table 3.2: Estrogenic activity in the Albasini Dam 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)
Low flow 1	X			0		
Low flow 2	X			1		
High flow	X			2		

*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)

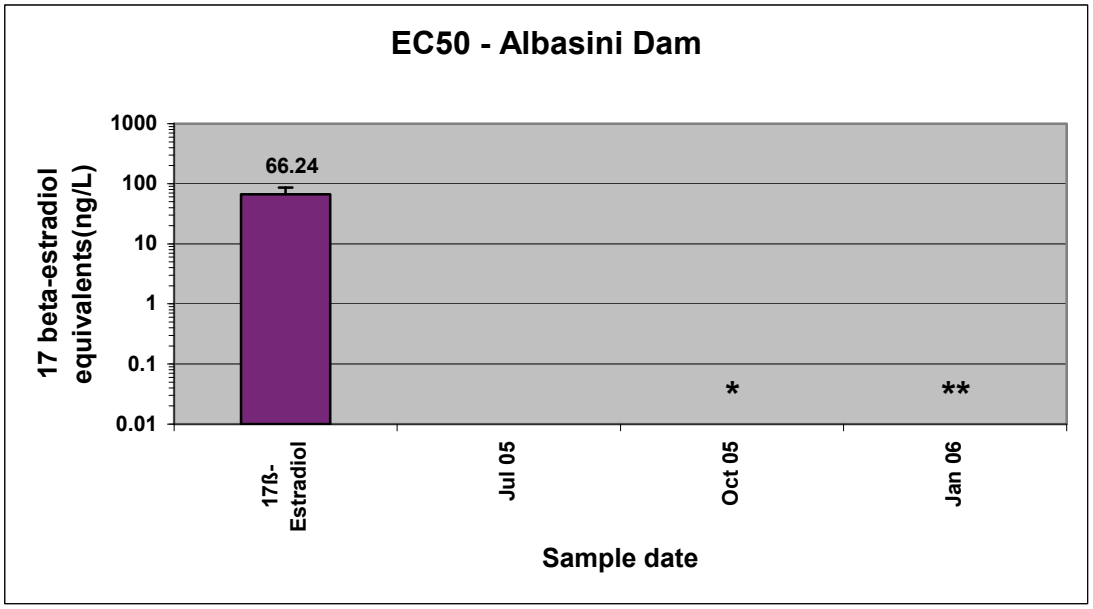

Figure 3.1: Albasini Dam water EC50 values expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit. (Jul 05 = low flow 1; Oct 05 = low flow2 and Jan 06 = high flow).

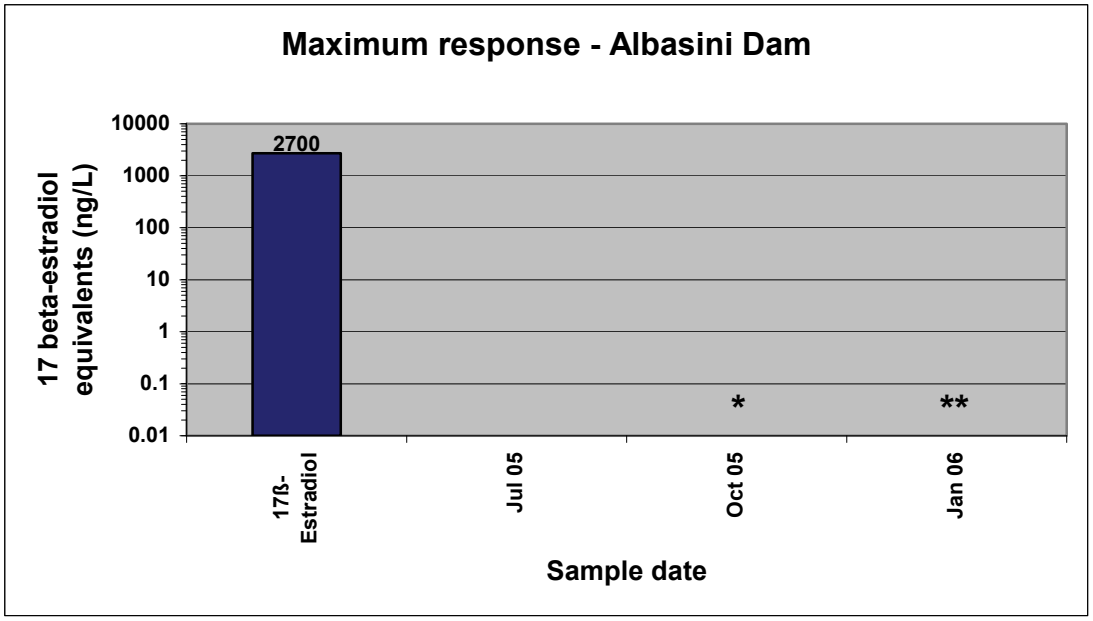

Figure 3.2: Albasini Dam water maximum estrogenic activity expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit. (Jul 05 = low flow 1; Oct 05 = low flow 2 and Jan 06 = high flow).

3.2.1.2 Sample site: Nandoni Dam

Two of the three sampling (Low flow 1 and Low flow 2) occasions were positive for estrogenic activity (one point above the detection limit of the assay) was observed during the high flow season.

Table 3.3: Estrogenic activity in the Nandoni Dam 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			
Low flow 1		X		3	0.15	0.27
Low flow 2		X		3	0.05	0.21
High flow	X			1		

*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)

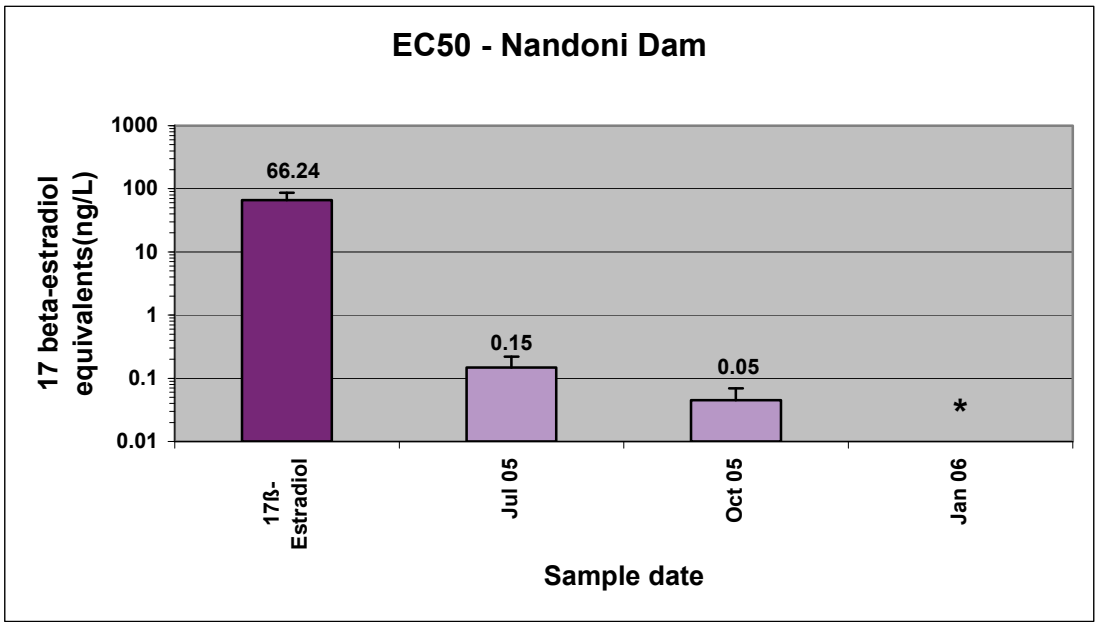

Figure 3.3: Nandoni Dam water EC50 values expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit. (Jul 05 = low flow 1; Oct 05 = low flow 2 and Jan 06 = high flow).

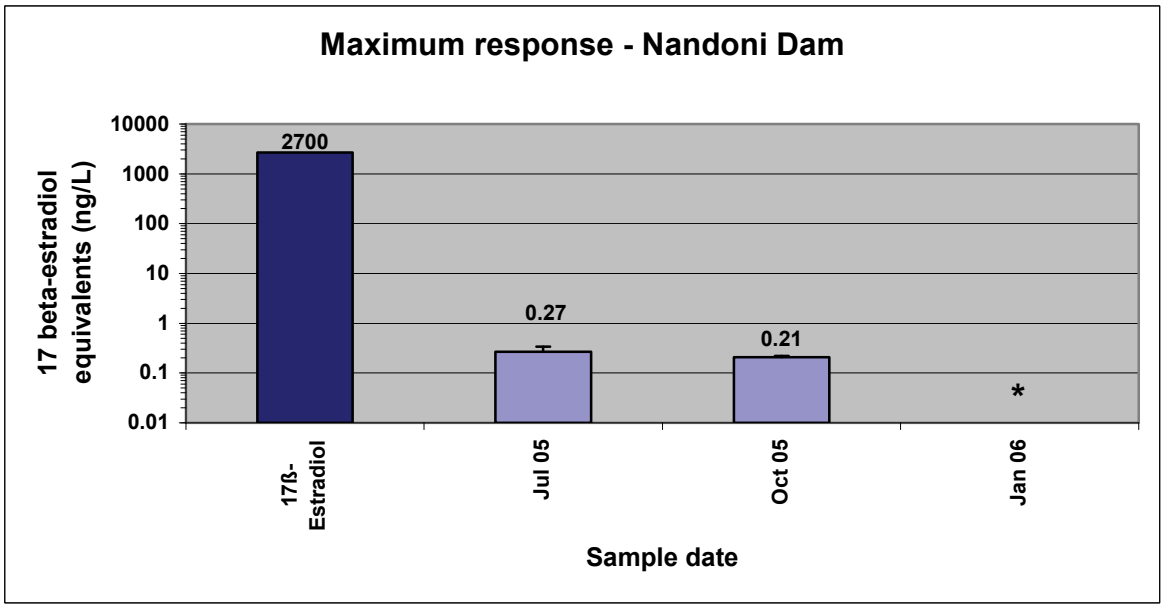

Figure 3.4: Nandoni Dam water maximum estrogenic activity expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit. (Jul 05 = low flow 1; Oct 05 = low flow 2 and Jan 06 = high flow).

3.2.1.3 Sample site: Xikundu Weir

Of the three sampling occasions only low flow 2 had an indication of estrogenic activity (one point above the detection limit of the assay) although it cannot be quantified. Cytoxicity alone was observed in the hf sample.

Table 3.4: Estrogenic activity in the Xikundu Weir 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			
Low flow 1				0		
Low flow 2				1		
High flow	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)

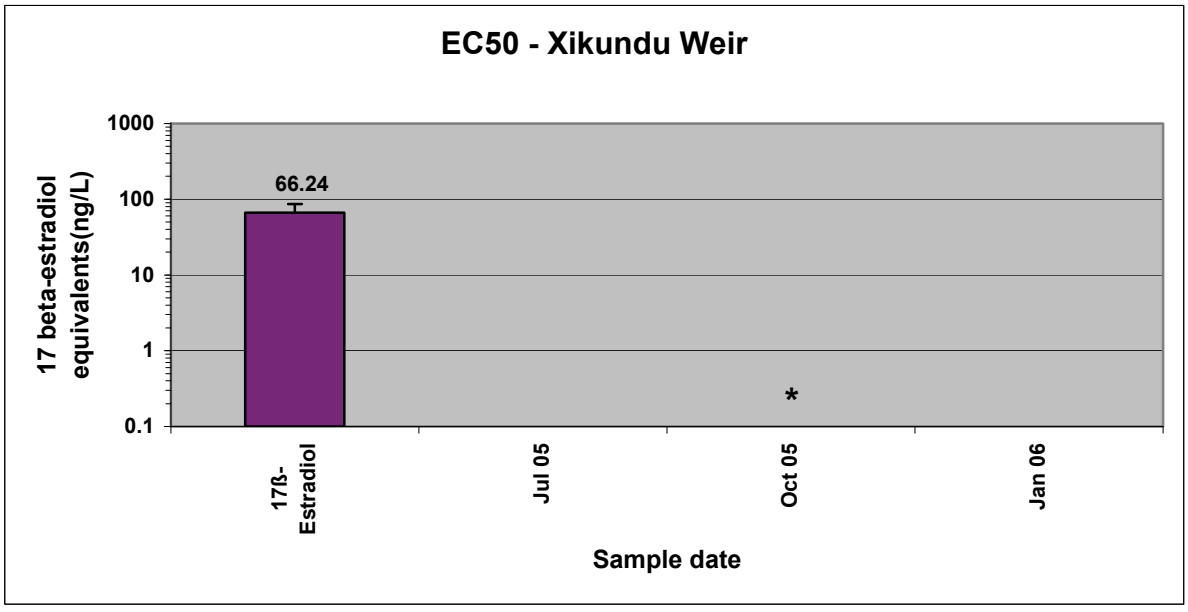

Figure 3.5: Xikundu Weir water EC50 values expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit. (Jul 05 = low flow 1; Oct 05 = low flow2 and Jan 06 = high flow).

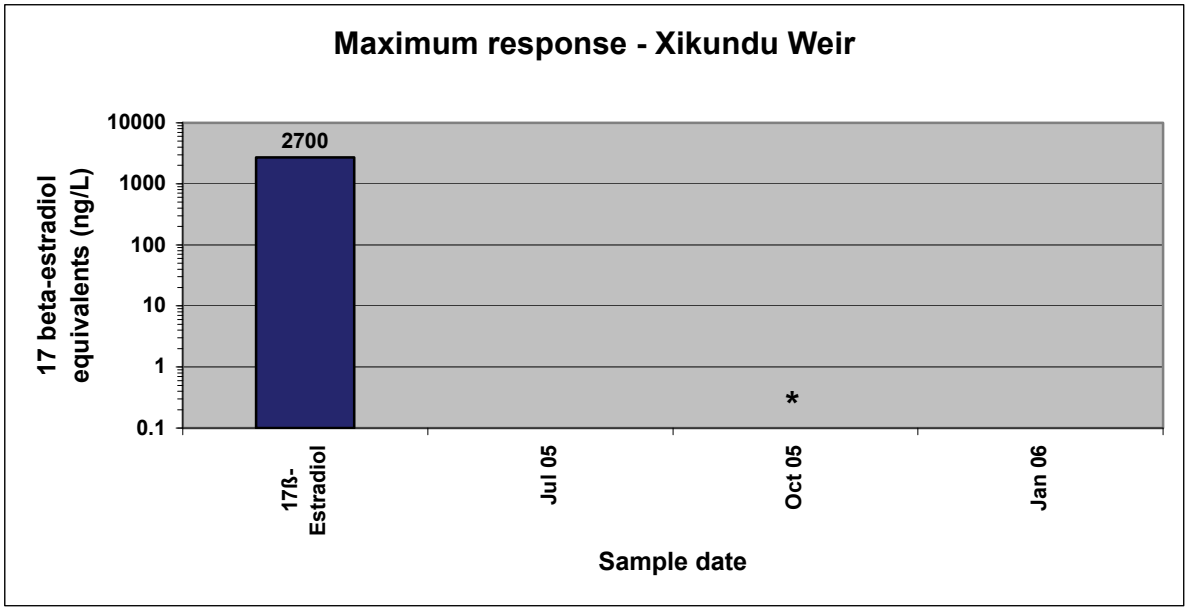

Figure 3.6: Xikundu Weir water maximum estrogenic activity expressed as 17β-estradiol equivalents, using the YES assay. * One point above detection limit. ** Two points above detection limit. (Jul 05 = low flow 1; Oct 05 = low flow2 and Jan 06 = high flow).

It is important to note that the above samples are not necessarily negative for any estrogenic activity, they are just below the detection limit of the assay. Likewise, samples with less than three points above the detection limit are not negative, but cannot be quantified for estrogenic equivalents in this assay.

Only Nandoni Dam had samples that were positive for estrogenic activity (3 or more points above detection limit, Table 3.3. None of the samples reached the maximum response obtained with 17 β-estradiol.

Table 3.5: Summary of the frequency of sampling of the sites that had a cytotoxic response.

Sample site	Date sampled	Frequency of sampling (%)
Albasini Dam	low flow1 Low flow 2 High flow	100
Nandoni Dam	High flow	33
Xikundu Weir	High flow	33

% Frequency is calculated as follows:

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

Cytotoxicity was found in 5 of the 9 samples (Table 3.5) in other words 56 % of the samples were cytotoxic. Due to the cytotoxicity found in the samples the estrogenic activity could be underestimated or completely missed, as the estrogenic response may lie in the toxic range.

These results emphasized the need to include the proposed estrogenic (T47D-kBluc) and androgenic (MDA-kb2) reporter gene assays (Bornman et al., 2007) 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).

3.2.2 *ER-Calux®*

Estrogenic activity was detected in all 8 samples sent for analysis. The EEs ranged from 0.138-0.578 ng/L (Table 3.6). The following table compares the results of the YES assay and the ER-Calux® assay.

Table 3.6: The EEs of all the localities using the ER-Calux® compared to the YES results.

Sample site	Sample date	ER-Calux® EE (ng/L)	YES EE (ng/L)
Albasini Dam	Low flow 1	0.162	Toxic (0 points above dl)
Albasini Dam	Low flow 2	0.138	Toxic (1 point above dl)
Albasini Dam	High flow	0.304	Toxic (2 points above dl)
Nandoni Dam	Low flow 1	0.578	0.27
Nandoni Dam	Low flow 2	0.153	0.21
Xikundu Weir	Low flow 1	0.209	0 points above dl
Xikundu Weir	Low flow 2	0.164	1 point above dl
Xikundu Weir	High flow	0.307	Toxic (0 points above dl)

3.3 Target analyses

All the selected target chemicals were below the detection limit of 0.05 µg/L in the water and 0.05mg/kg in sediment samples collected at the three sites during low flow 2.

3.4 Metal analyses of water and sediment

A full ICP-MS scan of all the metals including the endocrine disrupting metals (EDM) in water and sediment were executed and the results are presented in Tables 3.7 and 3.8. Although the endocrine disrupting metals will be focussed on during this study, which includes Cadmium (Cd), Arsenic (As), Lead (Pb) and Mercury (Hg), other metals were present at very high levels and is presented in Table 3.8.

3.4.1 Metals in water samples

Cd was not detected in water at any of the sites during sampling. As was detected at all the sites but values were below the target water quality range (<TWQR) of < 10 μg/L, with the highest level at AD (2.090 μg/L). Pb was detected at all the sites with concentrations higher than TWQR. Hg was not detected at in the water of any of the sites (Table 3.7).

3.4.2 Metals in sediment samples

Cd (0.082mg/kg) was found in the sediment of ND (Table 3.8) while there was no evidence of Cd at Albasini Dam the Xikundu Weir. As and Pb was found in the sediment at all the sites. 2.836 mg/kg Hg were found in the sediment from Albasini Dam and none in the sediment at the other two sites (Table 3.7). Although there are no available guidelines for metal levels in sediment, all the values measured were below the guidelines for water in DWAF (1996).

3.4.3 Other metal levels found in high concentrations in the water and sediment

Other metal levels (Table 9) in water above the TWQR that could impact negatively on the aquatic ecosystem. The TWQR according to the department of water affairs and forestry (DWAF, 1996):

Table 3.7: The EDM concentrations in water and sediment samples from Albasini Dam, Nandoni Dam and Xikundu Weir during October 2006 (low flow 2) and DWAF (1996) guidelines.

Metal	Cadmium (Cd)			Arsenic (As)			Lead (Pb)			Mercury (Hg)		
Matrix/Guideline	Water (µg/L)	Sediment (mg/kg)	DWAF (µg/L)	Water (µg/L)	Sediment (mg/kg)	DWAF (µg/L)	Water (µg/L)	Sediment (mg/kg)	DWAF (µg/L)	Water (µg/L)	Sediment (mg/kg)	DWAF (µg/L)
Albasini Dam	nd	Nd	*	2.090	16.969	10[#]	5.290	11.551	0.2-1.2[$]	nd	2.8	0.04[**]
Nandoni Dam	nd	0.082	*	1.360	14.737	10[#]	6.330	19.263	0.2-1.2[$]	nd	nd	0.04[**]
Xikundu Wwei	nd	Nd	*	1.670	12.354	10[#]	18.000	10.441	0.2-1.2[$]	nd	nd	0.04[**]

nd = not detected

* = Depend on the hardness of the water (mg $CaCO_3$/L) (Refer to DWAF, 1996).

= The chronic effect value (CEV) = 20 µg/L; and the acute effect value (AEV) = 130 µg/L (Refer to DWAF, 1996).

$ = Depend on the hardness of the water (mg $CaCO_3$L) (Refer to DWAF, 1996).

** = The chronic effect value (CEV) = 0.08 µg/L; and the acute effect value 1.7 µg/L (Refer to DWAF, 1996).

Table 3.8: Metal levels in water and sediment above the Target Water Quality Range (DWAF, 1996).

Metal	Aluminium (Al)			Chromium (Cr)			Iron (Fe)			Zinc (Zn)		
Matrix/Guideline	Water (µg/L)	Sediment (mg/kg)	DWAF (µg/L)	Water (µg/L)	Sediment (mg/kg)	DWAF (µg/L)	Water (µg/L)	Sediment (mg/kg)	DWAF (µg/L)	Water (µg/L)	Sediment (mg/kg)	DWAF (µg/L)
Albasini Dam	109	6266.3	≤5/10*	11.390	103.2	**	120.69	25665.6	10 % ***	85.210	243.2	≤ 2 ****
Nandoni Dam	69	17132.4	≤5/10*	14.540	138.2	**	248.35	42300.8	10 % ***	82.400	510.8	≤ 2 ****
Xikundu Weir	114	6963	≤5/10*	14.850	68.8	**	179.06	22623.7	10 % ***	96.290	326.10	≤ 2 ****

* = ≤ 5 (pH < 6.5), CEV = 10 µg/L and AEV = 100 µg/L; ≤ 10 (pH > 6.5), CEV = 20 µg/L and AEV = 150 µg/L (Refer to DWAF, 1996).

** = Cr (VI) ≤ 7 µg/L, CEV = 14 µg/L and AEV = 200 µg/L; Cr (III) ≤ 12, CEV = 24 µg/L and AEV = 340 µg/L (Refer to DWAF, 1996).

***= Not be allowed to vary by 10% of the background iron concentration for a site or case at a specific time (Refer to DWAF, 1996).

****= ≤ 2 µg/L, CEV = 3.6 µg/L and AEV = 36 µg/L (Refer to DWAF, 1996).

3.5 Analyses of fish

3.5.1 Fish species availability

Although *C. gariepinus* was the selected sentinel species, *O. mossambicus* was the major species caught.

3.5.1.1 Albasini Dam

During the first morning 10 male *O. mossambicus* were collected and processed, while 7 females were released from the nets. *C. gariepinus* were only found the next day when 3 male *C. gariepinus* were caught and 2 females released. The water level of this dam was very low, but the water quality readings were good (Table 3.1). Crocodiles were spotted in the vicinity of the nets.

3.5.1.2 Nandoni Dam

Ten male *O. mossambicus* were sampled in the first hour of netting, and a further 12 males and 8 females were released. Only 4 male *C. gariepinus* were caught and 2 females released. This part of the Luvuvhu river was also crocodile invested.

3.5.1.3 Xikundu Weir

Ten male *O. mossambicus* were collected and sampled within a day and the 8 excess males and 6 females were released. Only one male *C. gariepinus* was caught and two females released. The Xikundu Weir was very low two weeks before the sampling and surprisingly only three catfish were caught. Crocodiles were also observed in water and on the embankment.

3.5.2 Sex determination in fish

In total, only 8 male *C. gariepinus* were caught at the three sites. No stigmata of intersex were found on the external urogenital papilla evaluation, laparotomy or microscopic evaluation.

Thirty male *O. mossambicus* were sexed according to the upper lip (UL) and breeding colours (BC) and the findings summarised in Table 3.9. After microscopic evaluation it was evident that 17 of the 30 (57%) male *O. mossambicus* had intersex (Table 3.10).

3.5.3 Gonadal development

Tables 3.11 and 3.12 represented the mean length and weight of species as well as the gonadal somatic index (GSI) and the hepatic somatic index (HSI). The GSI was calculated according to the equation: gonadal weight/(body weight-gonadal weight) × 100

(Van Aerle et al., 2001) and the HIS liver weight/body weight × 100 (Goede and Barton, 1990).

3.5.4 Gonadal histology of Oreochromis mossambicus

The histology of the male testes of *O. mossambicus* from AD, ND as well as the XW showed normal testicular development (Figure 3.7). The tubules contained spermatozoa, with no indication of primary oocytes. Histological evaluation of the male gonadal slides indicated intersex in most of the fish (56%). Intersex was observed as primary oocytes scattered between the testicular tissues (Figure 3.7). Although some of the testes seemed morphologically normal, histological evaluations identified the presence of primary oocytes scattered among the testicular tissue

Only normal testicular histology was observed in *C. gariepinus* similar to the findings of Steyn (1984) and Van Dyk (2006).

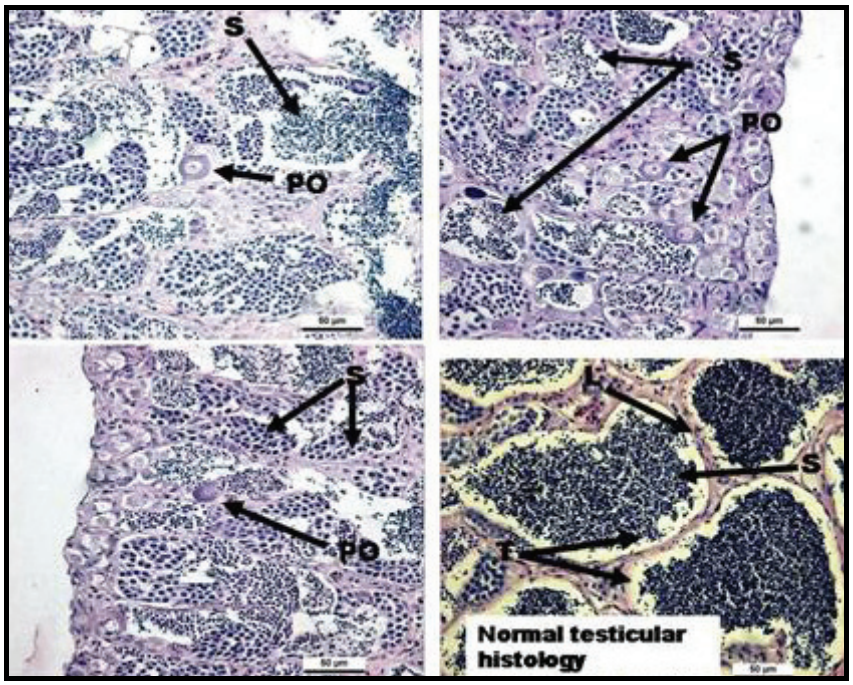

Figure 3.7: Transverse sections through the testes of mature *O. mossambicus* males from the Luvuvhu river sites showing normal testicular organisation and intersex gonads with primary oocytes (PO) scattered through out the testicular tissue. (S) Spermatozoa; (L) Lumen. Scale bar 50µm, 10-× magnification; Scale bar 100µm, 40-× magnification.

Table 3.9: Comparison of external phenotypic sexual characteristic findings (UL & BC), at laparotomy and microscopic evaluation of testes *O. mossambicus* caught at the three localities.

Locality	Albasini Dam			Nandoni Dam			Xikundu Weir		
Sex allocation	External (UL & BC)	Macroscopic (laparotomy)	Microscopic (histology)	External (papilla)	Macroscopic (laparotomy)	Microscopic (histology)	External (papilla)	Macroscopic (laparotomy)	Microscopic (histology)
Male	**10**	**10**	**2**	**10**	**10**	**3**	**10**	**10**	**4**
Uncertain	**0**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**2**
Intersex	**0**	**0**	**8**	**0**	**0**	**5**	**0**	**0**	**4**
Total	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**

Table 3.10: Total of intersex features in fish collected from the Luvuvhu river.

	Oreochromis mossambicus			***Clarias gariepinus***		
Locality	**Laparotomy**	**Laparotomy**	**Microscopic**	**Laparotomy**	**Laparotomy**	**Microscopic**
Albasini Dam	0	0	**8**	0	0	**0**
Nandoni Dam	0	0	**5**	0	0	**0**
Xikundu Weir	0	0	**4**	0	0	**0**
Total intersex fish			**17 of 30**	**Total intersex fish**		**0 of 8**

Table 3.11: Mean- weight, length, GSI and HSI of sexed *O. mossambicus*.

Locality	Mean weight ± SD (g)	Mean length ± SD (cm)	Mean GSI ± SD (%)	Mean HIS
Albasini Dam n = 10	530 ± 125	32.6 ± 3.2	0.236 ± 0.17	1.1050 ± 0.25
Nandoni Dam n = 10	725 ± 259	35.5 ± 2.5	0.116 ± 0.05	0.018 ± 0.009
Xikundu Weir n = 10	430 ± 177	29.65 ± 3.41	0.085 ± 0.063	0.956 ± 0.222

*** SD, standard deviation**

Table 3.12: Mean- weight, length, GSI and HSI of sexed *C. gariepinus*.

Locality	Mean weight ± SD (g)	Mean length ± SD (cm)	Mean GSI ± SD (%)	Mean HSI
Albasini Dam n = 3	2200 ± 984	64.67 ± 7.1	0.245 ± 0.15	0.322 ± 0.08
Nandoni Dam n = 4	1500 ± 577	64.75 ± 14.19	0.061 ± 0.05	0.762 ± 0.159
Xikundu Weir n = 1	1000	61	0.246	1.164

*** SD, standard deviation**

3.5.5 Urogenital papilla length index (UGPLI) of C. gariepinus

Due to the low numbers of catfish, the data was insufficient for any further analysis.

3.5.6 Fish fat EDC levels

The mean (± SD) of the selected EDCs measured in the fat of the two species are represented in Tables 3.13 and 13.14. No fat tissue was present in *O.mossambicus* collected from Albasini Dam. In samples from Nandoni Dam *p,p'*-DDE (0.281 ± 0.09 mg/kg) was the highest EDC measured, while *p,p'*-DDT (4.325 ± 2.392 mg/kg) was the highest in Xikundu Weir *O.mossambicus*. Apart from *p,p'*- DDT and *p,p'*-DDE, levels of lindane, *p,p'*- DDD, endosulfan, and endrin were found in the fat of *O. mossambicus*.

Although only 4 *C. gariepinus* were caught at Nandoni Dam (Table 3.15) a mean level of 2.125 ± 0.998 mg/kg *p,p'*-DDE was detected in the fat. *o,p'*-DDD (0.036 ± 0.022 and

p,p'-DDD (0.516 ± 0.314) were also measured in the fat of *C. gariepinus* from Nandoni Dam.

Table 3.13: The mean levels (± SD) of selected target EDCs in *O. mossambicus* fat.

Target EDC	Albasini Dam	Nandoni Dam (n=10)	Xikundu Weir (n=8)
Lindane (mg/kg)	n/a	0.048 ± 0.04	0.018 ± 0.007
Aldrin (mg/kg)	n/a	bdl	Bdl
Endosulfan 1 (mg/kg)	n/a	0.042 ± 0.02	Bdl
o,p'-DDE (mg/kg)	n/a	bdl	Bdl
p,p'-DDE (mg/kg)	n/a	0.281 ± 0.09	3.815 ± 0.337
o,p'-DDD (mg/kg)	n/a	bdl	0.06 ± 0.008
p,p'-DDD (mg/kg)	n/a	0.087 ± 0.0318	2.914 ± 0.433
o,p'-DDT (mg/kg)	n/a	bdl	0.264 ± 0.119
p,p'-DDT (mg/kg)	n/a	0.118 ± 0.036	4.351 ± 2.392
Endrin (mg/kg)	n/a	0.022 (n=1)	Bdl
PCB153 (mg/kg)	n/a	bdl	Bdl
Octylphenol (mg/kg)	n/a	bdl	Bdl
Nonylphenol (mg/kg)	n/a	bdl	Bdl

***bdl = below detection limit; n/a = no fat available**

Table 3.14: The average levels (± SD) of selected target EDCs in *C. gariepinus* fat.

Target EDC	Albasini Dam (n = 3)	Nandoni Dam (n = 4)	Xikundu Weir (n = 1)
Lindane (mg/kg)	n/a	bdl	n/a
Aldrin (mg/kg)	n/a	bdl	n/a
Endosulfan 1 (mg/kg)	n/a	bdl	n/a
o,p'-DDE (mg/kg)	n/a	bdl	n/a
p,p'-DDE (mg/kg)	n/a	2.125 ± 0.998	n/a
o,p'-DDD (mg/kg)	n/a	0.036 ± 0.022	n/a
p,p'-DDD (mg/kg)	n/a	0.516 ± 0.314	n/a
o,p'-DDT (mg/kg)	n/a	bdl	n/a
p,p'-DDT (mg/kg)	n/a	bdl	n/a
Endrin (mg/kg)	n/a	bdl	n/a
PCB153 (mg/kg)	n/a	bdl	n/a
Octylphenol (mg/kg)	n/a	bdl	n/a
Nonylphenol (mg/kg)	n/a	bdl	n/a

*** bdl = below detection limit; n/a = no fat available**

4 DISCUSSION

The findings of *O. mossambicus* and not *C. gariepinus* as major fish species in the Luvuvhu river system came as a surprise, since *C. gariepinus* can survive harsh conditions, including dry seasons. At all three collection sites crocodiles were spotted. This is in sharp contrast to studies performed at the urban Nature Reserve (Bornman et al., 2007*)* and at Hartebeespoort dam (unpublished data) where *C. gariepinus* was found in high numbers and no crocodiles were observed. The specific reason(s) for the low numbers of *C. gariepinus* is not clear, but the presence of crocodiles may provide some suggestion. *C. gariepinus* fills a niche in the food chain as a tertiary user and feed on small species when present. Crocodiles are classified as terrestrial and terrestrial animals as well and can feed on *C. gariepinus*, thereby decreasing their numbers. On the other hand, in the absence of crocodiles, such as in the urban Nature Reserve, *C. gariepinus* inhabits the waters in high numbers with few *O. mossambicus* present. However, up to date, the sampling methodology has only included the use of gill nets. The incorporation of angling (using traditional fishing tackle) as an additional sampling method, may prove to be more successful in obtaining the desired sample size of *C. gariepinus* as was found in a similar study in the Okavango river (GM Pieterse, personal communication). Angling should be included in the sampling protocol as it will also allow more efficient access to the shallow and highly vegetated waters of the river banks and dam shores (the optimal feeding habitat for both species) which are usually almost impossible to reach with gill nets.

The temperature of water plays an important role in a water body as it affects the rate of chemical reactions and also the metabolic rate of the organisms inhabiting the water. The natural temperature fluctuations in water temperature, therefore, also determine the occurrence of different species and aspects of their lifecycle (DWAF, 1996). Fish are poikilothermic and respond to the temperature of the surrounding water. Fish are, therefore, very sensitive to sudden changes in water temperature, as a critical temperature for optimal development, reproduction and general health. Apart from effects on the aquatic biota, temperature fluctuations influence the solubility of different chemicals such as metals thereby converting them to toxic species in the ambient water (Dallas and Day, 2004).

pH provides as indication of acidity or alkalinity of a water body/course and ranges between 6.0 and 8.0 in most natural waters of South Africa (DWAF, 1996). In this study pH varied between 7.29 and 8.9 at different times and localities. pH is affected by temperature and an increase of 20°C causes the pH of freshwater to decrease by 0.1 of a unit (DWAF, 1996). It seems that in this study, changes in temperature could not contribute significantly to changes in pH. In a previous study in the Luvuvhu river (Heath and Claassen, 1999) the pH

mostly ranged between 7.4 and 8.7, possibly indicating a higher background pH range for the Luvuvhu river. In a study conducted at the Xikundu Weir (Fouche, unpublished) pH readings over a 12 month period ranged from 6.79 to 7.3, but during four surveys in 2004 the range was 7.6 to 8.7. One may, therefore, assume that the background (natural) pH range for the Luvuvhu river lies between 6.0 and 8.7 according to surrounding geological and atmospheric influences. Based on an assessment of the water quality from existing Department of Water Affairs and Forestry data and information from the State of the Rivers Report: Letaba and Luvuvhu River Systems (State of the Rivers Report, WRC, 2001), the water in the Luvuvhu/Mutale river catchments was of good quality and not adversely affected by the activities in the catchment. The water quality parameters measured at the time of the report generally did not exceed the South African Water Quality Guidelines. However, this report also pointed out that the predominant water quality problem across the catchment was a tendency towards eutrophication.

In the current study the higher pH levels measured at all localities during low and high flow seasons may create optimal conditions for algal blooms and increased aquatic weed growth (Van Vuren et al., 1999). Although the pH levels measured could possibly be within the background range for the area, the fluctuation at all three sites exceeded >0.5 pH units per measurement over the three samplings and this may be an early warning signal of an adverse impact on the water sources. An alkaline pH may result from certain industrial effluents and man-made eutrophication (Dallas and Day, 2004) and further supports the concern on water quality.

The total dissolved solids/salts (TDS) is an extent of the magnitude of all materials dissolved in water and is directly proportional to electrical conductivity (EC)(DWAF, 1993). EC estimates the amount of dissolved ions in water including CO_3^{2-}, HCO^-, Cl^-, SO_4^{2-}, NO_3^-, Na^+, K^+, Ca^{2+} and Mg^{2+}. Variations from the normal ranges of TDS and EC are general indicators of health as fish require constant concentrations of the major dissolved ions in water (www.duluthstreams.org). Sources that negatively affect EC and TDS are wastewater from sewage treatment plants, urban run-off, agricultural runoff, acid mine drainage and atmospheric inputs. The values measured in the Luvuvhu river fluctuated between the localities, but the ECs were always proportional to the TDS. The low TDS and EC values measured at all localities are typical after hard rains. When it rains, an amount of dissolved solids are washed into the watercourse and the actual TDS decreases as a result of dilution by the rainwater (www.duluthstreams.org). The very high EC at Albasini Dam during the one low flow period could be as a result of the very high ambient temperature or may be an indication of water seeping into the Albasini Dam. Very high dissolved salt concentrations

relates to direct effects of increased salinity on an aquatic organism such as fish. High levels of TDS have been shown to cause the extinction of fish populations (Cooper and Koch, 1984) as well as decrease survival and growth of Lahontan Cutthroat trout in Walker lake, Nevada (Dickerson and Vinyard, 1999).

Dissolved oxygen (DO) concentrations are generally close to saturation in clean surface waters (DWAF, 1996). The DO is reported in mg/L and as a percentage of the saturation concentration at the time of sampling. The only DO level of concern was measured during the high flow season at ND when the percentage of the saturation concentration was only 64.9%. This was lower than the TWQR of 80% and in the sublethal range. Low oxygen levels (hypoxia) in water is extremely dangerous to fish and other aquatic vertebrates. However according to DWAF (1996) two useful measures of the impact of dissolved oxygen depletion are the 7-day mean minimum concentration and the 1-day minimum concentration. The 1-day minimum allowable level is >40% (Lethal).

The YES screen indicated a quantifiable amount of estrogenic activity (0.14-0.3 ng/L EE) at Nandoni Dam during the low flow season. It is important to note that although 3 of the total of 9 samples analysed had levels below the detection limit of the assay, these are not necessarily negative for estrogenic activity. Likewise, samples with less than three points above the detection limit are not negative, but could not be quantified for estrogenic equivalents in the YES assay. .The submaximal estrogenic response of the samples could be attributed to the complexity of the sample mixture. As some endocrine disrupting chemicals such as the hydroxylated PCBs may have anti-estrogenic activity and this could inhibit the response (Moore et al., 1997).

Of specific concern is that all the samples collected from the Albasini Dam were cytotoxic on the YES and the high metal concentrations found could be responsible for the cytotoxicity. In those samples that exhibited only cytotoxicity, the estrogenic activity could be masked and therefore give a false negative result, as the estrogenic response may lie in the toxic range. It must also be noted that the study area is an endemic malaria area and DDT is still sprayed for vector control. The breakdown product of *p,p'*-DDE is known to have anti-androgenic activity and thus could also account for the cytotoxic and submaximal response in the YES. 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) (Bornman et al., 2007*).* 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 EEs were similar to those of the ER-Calux® (Nandoni Dam, low flow 2) and in others (Nandoni Dam, low flow 1) were very different. This difference could be due to the impermeability of the yeast membrane to some substances and therefore decreasing the estrogenic activity.

The estrogenicity values between 0.14 and 0.31 ng/L ER-Calux® corresponded with a study done by Matsui et al. (2000) in Japan where water contained 1 ng/L. Using the E-screen in water samples from Korea, Oh et al. (2000) found total estrogenic activity between 0.5 pg/L and 7.4 ng/L. In Flemish rivers >10 ng/L EE were present (Witters et al., 2001), while the estrogen like potential of Taihu water, ranged form 2.2 to 12.1 ng/L EE. The levels of estrogenic activity found in this study lie in the same range (0-10 ng/L EEs) 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 0-26.5 ng/L (Mean = 2.0 ng/L) EEs. 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 EE concentrations in excess of 1 ng/L, if bioavailable, are likely to cause ovotestis and other estrogen-induced intersexual abnormalities (e.g. vitellogenin induction) in some fish (Matthiessen et al., 2006).

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, it has become important that further research is done on the removal of EDC from water system.

The metal analyses showed low levels of Cd and Hg in sediment samples collected from Nandoni Dam and Albasini Dam respectively, and these metals do not seem to be a major concern in the aquatic environment. Pb and As on the other hand, were present in all water and sediment samples. The levels of As in water were below DWAF (1996) limits, in

contrast Pb levels were very high and ranged from 5.29 µg/L at Albasini Dam, to 6.33 µg/L at Nandoni Dam and 18.00 µg/L at Xikundu Weir respectively (0.2-1.2 µg/L, DWAF, 1996).

Although lead occurs naturally, it is also deposited through industrial activities, especially via past and present storage of battery recycling in populated areas, high automobile and truck traffic during construction, sewage sludge and spoil disposal areas, as well as sites where dredging has occurred (ATSDR, 1999). Another source could be the deposits of Pb containing dust particles from the atmosphere (ATSDR, 1999). Pb in the sediment is fortunately not readily available to aquatic organisms such as fish.

Although this study only focussed on the four EDM As, Cd, Pb and Hg, the high levels of aluminium (Al), Chromium (Cr), Iron (Fe), and zinc (Zn) in water and sediment cannot be ignored. However, this study was not designed to revise the link of endocrine effects to metal concentrations. Such links are known to exist (Johnson et al., 2003; Telisman et al., 2007) and the presence of these metals retains them among the possible causes for the observed effects, but this needs a more detailed study.

None of the chemicals including the DDTs tested were present above the detection limit in either water or sediment samples. This was contrary to the previous findings of Burger (2005) where various agrochemical and industrial residues were found. However, *p,p'*-DDT, -DDD and DDE, lindane, endosulfan and endrin residues were found in fat tissue from *O. mossambicus*. *C. gariepinus* fat samples contained *p,p'*-DDT and *o,p'- and p,p'*-DDD. This implied that those fish were exposed at some time during their lifecycle and subsequently bio-concentrated the chemicals in lipid tissue.

Only a few *C. gariepinus* were found for evaluation and no intersex stigmata were found. In a study conducted in an urban nature reserve intersex were found in more than 60% of catfish examined (Bornman et al., 2007) and it could simply be that the present study numbers were too low for any positive findings. However, during this study intersex was observed for the first time in *O. mossambicus* and it is now the second species in South African waters with the reported intersex condition. Primary oocytes scattered throughout the testicular tissue were observed similar to the previous findings in *C. gariepinus* (Barnhoorn et al., 2004). In total 57% of the *O. mossambicus* caught in the Luvuvhu river had intersex.

Intersex in fish is a well documented and accepted phenomenon in fish from waters possibly polluted with xeno-estrogens. Apart from estrogenic pollutants Matthiessen et al. (2006)

found that water with long term estrogenic activity (EE concentrations) above 1 ng/L causes conditions such as intersex in fish. In this study the estrogenic activity in the water at the specific intervals was below 1 ng/L. It is not possible to know if at some time it in fact exceeded this value and further studies are necessary.

The specific agent(s) causing the intersex in *O. mossambicus* was not clear. In the urban nature reserve study water, sediment and fat of specimens had high levels of the synthetic estrogen *p*-NP, which may nave been the cause of intersex at that locality. Although the present levels are not the direct cause of intersex, exposure at embryogenesis cannot be excluded as a possibility of the onset of an ovotestis showing in later stages of life. It also seems possible that species specific differences between *C. gariepinus* and *O. mossambicus* could account for the different intersex findings.

5 CONCLUSIONS AND RECOMMENDATIONS

Cytoxicity and estrogenic activity were demonstrated in water samples that contained very high levels of metals namely Pb, Al, Fe, Cr and Zn. The high pH of water raised concern on expanding eutrophication. DDT residues were present in fish fat samples. Intersex was demonstrated for the first time in *O. mossambicus* and the prevalence of 57% warrants further studies.

The following is recommended:

- Metal analysis must include the full range on the ICP-MS and not only the EDMs.
- Analyses of water hardness and other nutrients should be included in these studies.
- The effect of identified metals should be further investifated in the biological assays.
- Angling should be done in conjunction with the use of gill nets.
- Anti-androgenic activity must be measured in combination with the current estrogenicity assays.

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