DDT for Malaria Control: Effects in Indicators and Health Risk

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

Dichloro-diphenyl-trichloroethane (DDT) is a pesticide used in control of the malaria-carrying mosquito. While banned internationally, some countries, including South Africa, have been granted restricted use for indoor residual spraying (IRS) under the Stockholm Convention (SC), which South Africa agreed to on 4 September 2002. The SC seeks the elimination of twelve chemicals or classes of chemicals, the Persistent Organic Pollutants or POPs, one of which is DDT. This allows that DDT may be produced and used for the purpose of controlling disease vectors in accordance with World Health Organization (WHO) recommendations and guidelines and when locally safe, effective, and affordable alternatives are not available. One such area is the Vhembe District Municipality of Limpopo Province, in the north-eastern corner of South Africa bordering Zimbabwe and Mozambique where the highest incidence rate of malaria cases in Limpopo Province occurred between 1998 and 2007 (Gerritsen et al., 2008). DDT spraying was introduced in 1945 for malaria vector control, and since 1966 DDT is being sprayed annually in this area (pers. comm., P. Kruger, Limpopo Malaria Control Programme).

However, DDT is not only one of the POPs, but is also an endocrine disrupting chemical (EDC). EDCs represent a diverse range of man-made chemicals discharged into the environment that mimic or antagonize the function of hormones. These EDCs may interact with physiological systems and cause alterations in development, growth and reproduction in wildlife and particularly in exposed fish (Jobling and Tyler, 2003). The current literature shows growing evidence that exposure to DDT and the major breakdown product DDE (1,1-(dichloro-2,2-bis(*p*-chlorophenyl) ethane) may be associated with adverse health outcomes such as breast cancer, diabetes, decreased semen quality, spontaneous abortion, and impaired neurodevelopment in children (Eskenazi et al., 2009).

Technical-grade DDT substance applied during indoor residual spraying (IRS) is composed of 65-80% of the active insecticidal ingredient, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDT) (Bouwman et al., 2006). The other components include 15-21% of the less insecticidal 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-ethane (*o,p'*-DDT), and about 4% of 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDD) (Metcalf, 1995). 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). The persistent metabolite of DDT, *p,p*'-DDE inhibits androgen binding to the androgen receptor, androgen-induced transcriptional activity, and normal male prepubertal development (Kelce et al., 1995; Rogan and Chen, 2005). Danzo (1997) reported that not only was *p,p*'-DDE the most potent of the xenobiotics tested at inhibiting 5-α-dihydro-testosterone binding to the androgen receptor, but both *o,p*'-DDT and *p,p*'-DDT are also potent androgen inhibitors.

Exposure of male embryos to EDCs during the early stages of fetal development has been linked to the increased incidences of male reproductive health disorders including hypospadias, undescended testes, intersex, subfertility and testicular cancer (Paulozzi 1999; Sharpe and Skakkebaek, 1993; Toppari et al., 1996). Dalvie et al. (2004) found high levels of DDT and metabolites in occupationally DDT exposed spray workers. Recently Aneck-Hahn et al. (2007) demonstrated that healthy males living in this currently DDT-sprayed area had impaired semen quality. These findings confirmed the observations by De Jager et al. (2006), in participants from rural communities in the malaria region of Chiapas, Mexico with a history of high use of DDT until 1997.

De Jager et al. (2009) also demonstrated a weak association between DDT/DDE plasma concentrations and the incidence of sperm with chromatin defects in young men from this part of Limpopo Province. Newborn males in this area have a higher prevalence of cryptorchidism (testis not descended into scrotum) and hypospadias (urethral opening not at tip of penis, but along the underside of the penis) (Bornman et al., 2009) than other South African studies reported in newborns from non-malaria areas (Delport et al., 1995; Venter et al., 1995). Bornman et al. (2009) also found that the incidence of hypospadias was 5.2% of newborn babies, compared to the "normal" incidence of 0.4% (i.e. ± 1 in 250). The study concluded that maternal exposure to home spraying of DDT during the mothers' life, may during fetal development increase the risk of urogenital defects by 33%. High levels of DDT were found in plasma of mothers and cord blood of their babies (Bornman et al., 2005). In malaria areas pyrethroids are also used and some of those were present in water and breast milk samples in a DDT-sprayed area from KwaZulu-Natal (Sereda et al., 2009), emphasizing the possibility of concomitant exposure to multiple chemicals.

Pilot studies examining environmental contamination with DDT in the Limpopo Province raised concerns about the water quality and estrogenic activity in an impoundment where DDT spraying takes place. Animals were tested that were either sharing the domestic environment or could be used as an indicator of environmental contamination. Chickens, terrestrial and aquatic birds, as well as two fish species were contaminated (Burger, 2008;

Barnhoorn et al., 2009). In addition testicular oocytes (intersex) were observed in male *Oreochromis mossambicus* (Bornman et al., 2009a).

OBJECTIVES AND AIMS

The overall aim of this study was to determine whether environmental levels of DDT and DDE may contribute to adverse health effects in aquatic animals. Chemical residue analysis was done in water, soil, sediment and biota and biological testing for estrogenicity was performed on water samples. The aquatic health was determined in and around water sources in the area. The information was integrated into a scenario-based health risk analysis and the EDC assessment techniques compared and suggestions made for a South African relevant toolkit manual of tests for wider application in other spraying areas.

However, as the study area and the fish populations offered several more opportunities for detailed research, the following aspects were also addressed:

- hormone analyses of estrogenic substances namely the natural estrogens estradiol, estrone and estriol and also ethinyl-estradiol (from “the Pill”);
- a histomorphological study of the heart of *Clarias gariepinus* and *Oreochromis mossambicus;*
- testicular apoptosis as a molecular investigation to understand the process of testicular damage in two bio-sentinel fish species, inhabiting the Luvuvhu River;
- sperm motility assessment of the fish *O. mossambicus* and *C. gariepinus;*
- a detailed Histology-based Fish Health Assessment (HBFHA) in a DDT sprayed area;
- the use of *O. mossambicus* as a sentinel species to assess the possible effects of DDE exposure *in vivo* on health and reproduction.

METHODOLOGY

This study was conducted in the Vhembe District Municipality in the Limpopo Province. All sample sites were along the Luvuvhu River, which flows through an area where indoor residual DDT spraying occurs, with the major sampling sites for fish being the Albasini (AD) and Nandoni (ND) Dams and the Xikundu Weir (XW). Sampling for the purpose of assessing the status of fish communities occurred at five riverine sites in the Luvuvhu River, namely Lotanyanda, Hasana, Tshikonelo, Xikundu Weir and Mhinga. Leafy vegetables and chicken samples were collected from the village of Tshakhuma as the reference site to the sprayed village Dididi around the Nandoni Dam. Tshakhuma village is about 35 km to the west in an area where DDT was never sprayed, upstream in the Luvuvhu river.

Analytical chemistry and bioassays

Water and sediment samples were analysed for organochlorine pesticides (OCs), alkylphenols, phthalates (surveys 3 and 4, second high and low flow seasons), and hormones in water samples (Surveys 3 and 4). The OCs included: alpha-Hexachlorocyclohexane (HCH), gamma-HCH (lindane), heptachlor, aldrin, dieldrin, beta-HCH, delta-HCH, 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 and polychlorinated biphenyls (PCB). The alkylphenols included nonylphenol (*p*-NP) and octylphenol (OcP) while the phthalates included dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP) benzyl butyl phthalate (BBP), and di-(2-ethylhexyl) phthalate (DEHP). The hormone analyses consisted of ethinylestradiol, estradiol, estrone and estriol. The metal analysis included the four endocrine disruptive chemicals cadmium (Cd), arsenic (As), lead (Pb) and mercury (Hg).

DDT and metabolites were measured in fish fat and brain tissue as well as in vegetable leaves (pumpkin, cowpea and lugu/spinach) and chicken muscle and fat in the study areas.

In vitro bio-assays for estrogenicity or anti-estrogenicity in water samples were performed by the recombinant yeast estrogen screen (YES) and the T47D-KBluc reporter gene assay. The MDA-kb2 reporter gene assay was used to measure possible androgenic or anti-androgenic activity.

Biota

C. gariepinus and *O. mossambicus* were collected during four surveys from the major sites attempting to cover two high and low flow seasons. Various indices of fish health were determined including the use of biomarkers such as histomorphology of the heart, testicular apoptosis, computer assisted sperm analysis (CASA) and histology based fish health assessment.

In addition to indices of fish health, *C. gariepinus* and *O. mossambicus* were exposed under controlled conditions in the aquarium to various environmentally relevant concentrations of DDT (*C. gariepinus*: 0.659 µg/L, 1.36 µg/L, 2 µg/L and 2.724 µg/L and DDE (*O. mossambicus*: 0.1 µg/L, 0.4 µg/L, 0.7 µg/L) and the reproductive effects assessed.

Possible effects of DDT and metabolites on vertebrate and macro-invertebrate populations were studied at seven sites representing the Luvuvhu river system.

Human health risk assessment

The data for water and biological samples were used to conduct both a quantitative and qualitative scenario-based human health risk assessment. Human data of the epidemiological studies performed in this area were obtained for the health risk assessment. These included human data on semen quality and plasma *o,p'*- and *p,p'*-DDT, -DDD, and -DDE levels from exposed and unexposed men and the epidemiological data on urogenital birth defects in newborns. The United States Environmental Protection Agency (US EPA) and World Health Organization (WHO) risk assessment methods were adopted, making use of relevant South African values when available for toxins.

RESULTS AND DISCUSSION

All the values of the different macro variables of water samples measured over the four surveys were within the guidelines values recommended for drinking water by the Federal-Provincial-Territorial Committee on Drinking Water (2008), World Health Organization (WHO, 2006) and the Department of Water Affairs and Forestry (DWAF, 1996a) of South Africa as well as SANS 241 (2006).

Potable water from the community taps had no detectable DDT residues. In water samples from Survey 1 *p,p*-DDT was found at ND (0.12 µg/L) and no other OC residues were found. During the next Survey the following levels at ND and XW: 0.3 and 0.4 µg/L *p,p*-DDE respectively and no other OCs were found. During Survey 3, detectable levels were found in AD and ND of *p,p*-DDE, and ND of *p,p'*-DDD and *p,p'*-DDT at AD. In Survey 4 all the sites with the exception of ND contained *p,p*-DDE residues. At AD during Survey 3 and at XW in Survey 4 the ΣDDT were 1.75 and 1.8 µg/L respectively, which is higher than the WHO limit of 1 µg/L in drinking water. The presence of *p,p'*-DDT and -DDE in water from ND was unexpected considering the high dilution factor of the newly constructed dam. Furthermore, the occurrence could have been missed as water is not necessarily the ideal matrix for *p,p'*-DDT and -DDE determinations. The higher levels downstream from ND were anticipated, as these sites are located within the area where indoor DDT-spraying occurs.

In Survey 4 PCB153 was present at 2.8 µg/L at Hasana, and dieldrin at AD (1.6 µg/L), ND (1.1 µg/L), XW (3.5 µg/L) as well as at Mhinga (1.9 µg/L). The EPA limit (EPA guidelines, 1986) for drinking water of dieldrin is 2.0 µg/L and the water at both ND and XW could pose a problem to people collecting for drinking purposes. No nonyl- or octylphenol residues,

ethinyl-estradiol, estradiol, estrone or estriol or any of the phthalates were found in any water samples.

In the sediment samples collected during Surveys 1 to 3 no DDT residues were detected. In Survey 4 ND contained 1.4 µg/kg, Tshikonelo 1.8 µg/kg and XW 4.3 µg/kg *p,p*'-DDT and at the same sites 3.7, 3.8, 12.8 µg/kg *p,p*'-DDE respectively were found. No octylphenol residues, any of the phthalates (DMP, DEP, DBP, BBP, DEHP and DOP) or any of the hormones were detected. During Survey 3, *p*-NP was present ranging from 29-851 µg/kg at sites downstream from AD and during Survey 4 at all sites ranging between 52.7-309 µg/kg. *p*-NP, a known estrogenic, was the most prevalent chemical and in the highest concentrations in sediment of all the sites.

The majority (92%) of the vegetables from the exposed area contained *p,p'*-DDD (ranging from 10-80 µg/kg), with 16.7 % (n=2) homesteads also having residues of 60 and 80 µg/kg *p,p'*-DDT, and 10 µg/kg *p,p'*-DDE respectively. No residues were detected in vegetables from the reference area.

Chicken livers from the exposed area contained *p,p'*-DDT in 75% of samples ranging from 50.6 to 604.8 µg/kg; *p,p'*-DDD in 83% ranging from 128.2 to 541 µg/kg, and 100% had *p,p'*-DDE at levels of 50.6 to 604.8 µg/kg. Moreover, 50% of the liver samples also contained *o,p'*-DDT ranging from 52.3 to 77.9 µg/kg. In muscle tissue from chickens in Dididi *p,p'*-DDT and *p,p'*-DDE levels ranged from 52.5-326.3 µg/kg and 68.6-838.6 µg/kg respectively. Single chicken liver and meat samples in the reference group also contained *p,p'*-DDT and *p,p'*-DDE albeit at much lower levels.

The table below summarizes the different tests and assays and the findings.

Summary of findings used for integration in the health risk assessments.

Test or substrate	Outcome
water chemistry	DDT residues at AD and ND, DDE at AD, ND and XW
	ΣDDT AD and XW, levels > WHO limit for drinking water
	dieldrin levels at ND and XW > EPA guidelines drinking water
sediment chemistry	DDT and metabolites at AD, ND and XW
	p-NP major chemical
Soil	all isomers, *p-p'*-DDT 0.91 ± 1 µg/kg, *p-p'*-DDE 15.8 ± 16.1 µg/kg
	reference site all isomers, fewer samples, lower levels except one 72.2 µg/kg DDE)
vegetables	92% samples DDD residues in exposed village, 16.7% (n=2) also DDT and DDE
	no residues in reference village
Chicken - liver	*p-p*'-DDT (75% samples, range 50.6-604.8 µg/kg), DDD (83%, 188.2-541.0 µg/kg) *p-p'*-DDE (100% samples, 50.6-604.8 µg/kg) single positive sample in reference area 50% samples also estrogenic *o-p'*-DDT (range 52.3-77.9 µg/kg)
- muscle	*p-p'*-DDT and -DDE 52.5 to 326.3 and 68.6 to 838.6 µg/kg respectively single positive sample in reference area
- fat	100% *p-p'*-DDT 45094±2579.5 µg/kg; 192024.2 ± 35892.3 µg/kg *p-p'*-DDE 409.4 ± 165.7 µg/kg *o-p'*-DDT (estrogenic isomer)
estrogenicity YES	cytotoxicity
kBluc	estrogenicity AD, ND and XW
fish communities	decline species diversity, not DDT or chemical related
	possibly affected by physical habitat, erosion, agriculture practices
	worst at Tshikonelo and Mhinga
	possible negative effect of fishing practices
invertebrates	changes not DDT or metabolite related
	possible cases are organic contamination (sewage), increased nutrients,
	washing clothes and bathing
Heart	chronic inflammatory changes all three layers of the heart
	C. gariepinus more affected
apoptosis	increased apoptosis both fish species and in both caspase-3 and TUNEL assays when compared to laboratory reared fish changes not DDT or metabolite related
sperm motility	motility and velocity impaired in both fish species
histology-based evaluation	adverse effects on gills, liver and gonads intersex in *O. mossambicus*
Laboratory exposure	
C. gariepinus	adverse effects on early life stages at environmentally relevant concentrations
O. mossambicus	EROD (7-ethoxy resorufin O-deethylase) and CEA (Cellular Energy Allocation) identified biological effects of environmental stress

All the DDT isomers were detected in chicken fat samples from Dididi with *p, p'*-DDT and *p,p'*-DDE at extremely high levels of 45094.4 ± 2579.5 µg/kg and192024.2 ± 35892.3 µg/kg respectively. The concentration of 409.4 ± 165.7 µg/kg of *o,p'*-DDT is also reason for concern as this isomer is estrogenic. Since chicken fat would form part of cooked chicken, this could be an important route of DDT exposure. At the reference site 440 ± 760 µg/kg *p,p'*-DDT and 190 ± 170 µg/kg *p,p'*-DDE were found. In soil samples from Dididi all the DDT isomers were detected and the *p,p'*-DDT level was 0.9 ± 1 µg/kg and *p,p'*-DDE 15.78 ± 16.1 µg/kg. At the reference site all isomers were also detected, but in fewer samples (n = 6), 66.7%) at lower levels except for *p,p'*-DDE at one homestead (72.2 µg/kg). Since chicken could not have ranged 35 km to the DDT sprayed area, it seems fair to assume that some DDT was being illegally used in the reference area and caused the subsequent contamination and detection. The high soil values at one homestead further supports the possibility of illegal use of DDT outside the official DDT-sprayed area.

The high levels detected in fish fat and brain tissue emphasizes the bio-concentration of DDT in various tissues of exposed fish. In this regard bioaccumulated levels in fat tissue provide an indication as to what the fish might have been exposed to over a life time. It also raises the possibility that fish behaviour may be adversely affected as fat tissue levels are in equilibrium with blood levels.

The bioassays showed that all water samples collected from the dams had a toxic response in the YES assay. The toxic response in the YES may be an underestimation of true concentrations or false negatives as found in previous studies (Bornman et al., 2007). The cause of the cytotoxicity is not known but may indicate the presence of non-estrogenic compound(s). Estrogenic activity was detected using the T47D-KBLuc assay during Survey 3 with AD having the highest EEq (0.57 ng/L) followed by ND (0.11 ng/L) and then XW. Although these values did not exceed the Predicted-No-Effect-Concentration (1 ng/L) for 17β-estradiol in water, technical problems such as no pH adjustment, could underestimate the real situation. The estrogenic activity in the T47D-KBLuc assay while the YES showed cytotoxicity highlight the need for the use of a battery of screening bioassays which can complement each other and provide a more comprehensive assessment on the estrogenic activity in environmental samples. There was no androgenic activity detected using the MDA assay. This may be due to the complexity of the samples, or the assay itself. The US EPA experiences similar difficulties and this assay may not be the ideal choice for screening environmental samples.

The fish communities showed a decline in the species distributions within the Luvuvhu River, but the decline was not due to DDT or its isomers, or any other chemical contaminant measured in this study. The fish communities were however affected by changes in the physical habitat, both natural and anthropogenically induced such as reduced vegetation and increased erosion in the riparian zone from cattle and human activity, as well as changed habitat availability due to the changes in flow as a result of the presence of impoundments (XW and ND upstream from Tshikonelo). As for the spatial changes, Tshikonelo and Mhinga showed the most altered fish community structures, whilst XW, and to a lesser extent Hasana, had less modified community structures. Evaluation of temporal trends showed a number of fluctuations but with no seasonal changes evident. At all of the sites within the Luvuvhu River extensive fishing using gill nets, seine nets, cast nets and traps was observed and therefore it is recommended that illegal fishing should be addressed.

The macro-invertebrate community structures were influenced by seasonal changes, but also identified negative impacts within the Luvuvhu River. As was shown by the fish communities, there were no significant effects on invertebrate communities due to DDT contamination. The changes that were apparent at most of the sites within the Luvuvhu River were hypothesised as being due to organic (sewage) pollution and increased nutrient levels as a result of the locals washing and bathing within the Luvuvhu River, on a daily basis. The Lotanyanda invertebrate community was the most dissimilar compared to all the other sites in the Luvuvhu River, however the site that showed the most change from natural conditions was Hasana. It would seem possible however, that other chemicals not tested for in this study could also play a role.

In the case of each of the biomarkers used for *O. mossambicus*, field results, when compared to the relevant controlled aquarium exposures, showed that if exposure is prolonged, the induced response may decline, or return to background levels (adaptation). Adaptation processes are generally species-specific and/or contaminant-specific and specific biomarker levels are each uniquely affected. Thus, it can be assumed that the possibility does indeed exist that because of the prolonged exposure of *O. mossambicus* to the polluted selected environments, that any induced responses with regard to exposure to pollutants may have declined to nearly background levels and that the variations noted in the measurements obtained are as a result of natural environmental variations at each of the selected study sites. *O. mossambicus* were successfully utilised to identify contamination within the surrounding environment, but this species was not sensitive enough to assess small variations in organochlorine concentrations within the environment.

Three types of chronic cardiac inflammation were identified: myocarditis, epicarditis and to a lesser extent, endocarditis. *C. gariepinus* were more affected than *O. mossambicus* and fish from the control site were worse affected than fish in the two test sites. Future studies, focusing on associated skeletal muscle lesions of fish, with the inclusion of bacteriology and virology, are essential to further investigate the cause of the inflammatory response in the fish population in the Luvuvhu River.

The process of apoptosis is one form of programmed cell death (PCD), in which the cell has the ability to activate an inherent suicide mechanism that ultimately eliminates the damaged or malformed cell/s in an ordered fashion (Bortner et al., 1995; Bellamy et al., 1995). One tool which is used to quantify the extent of apoptotic activity is immunohistochemistry, using assays (Labat-Moleur et al., 1998). The use of caspase-3 and Terminal deoxynecleotidyl transferase-mediated dUTP Nick end-Labeling (TUNEL) assays for apoptosis, due to a host of insults has been investigated in various species (Frigo et al., 2005; McClusky, 2005; McClusky et al., 2008). The extent of apoptosis is upregulated in both *O. mossambicus* and *C. gariepinus*, when compared to the laboratory control testicular sections. Although an increase in positive caspase-3 and TUNEL cells could be identified, no direct correlation could be made between the observed positive cells and DDT levels in the fat tissue. However, this does not completely rule out the possibility of indirect exposure having a long-term endocrine disrupting effect, or the possibility of a mixture of environmental compounds increasing the extent of observed apoptosis

In fish, sperm motility and velocity are indicators of reproductive success (Alavi and Cosson, 2005). A computer aided sperm assessment, or CASA, is the most objective and comprehensive quantification available for the analysis of these indicators of sperm quality (Wilson-Leedy and Ingermann, 2007). Both fish species showed a decrease in motility and velocity in the exposed sites compared to the control site. It is likely that, if not solely attributable to DDT, a combination of pollutants was responsible for the damaging effects in terms of sperm motility parameters. It is also clear that CASA parameters may serve as a more sensitive marker of reproductive toxicity. Although on a cellular level the effects on the testis were slightly higher in the DDT-polluted area, it could not be correlated to DDT levels.

Histopathological alterations of *C. gariepinus* and *O. mossambicus* were found in the gills, liver and gonads. There was no proof that fish from ND and XW was more severely affected than AD and although the chemical mixtures in the various dams will be different, fish health was adversely affected in all three dams. It should be noted that intersex was present in the

testes of *O. mossambicus* during all surveys in ND, XW and AD, but the exact cause was not clear.

Chronic partial life-cycle exposures of *C. gariepinus* to environmentally relevant water concentrations of DDT over 21 days showed no severe effects in adult *C. gariepinus* exposed. However, a weak dose-response relationship suggests that prolonged duration and increased concentration of DDT exposure may lead to irreversible long term adverse effects in *C. gariepinus*. In all the exposures, the only parents that yielded a successful F1 generation were those exposed to the lowest level of DDT. However, the hatching success was not good and after 72 hours there were no survivors, illustrating that the early life stages of *C. gariepinus* are more sensitive to toxicants than adult fish.

In vivo toxicological exposure of *O. mossambicus* and biomarker responses were used to characterize the extent of the impact of environmental DDE contamination. The biomarker assay EROD (7-ethoxy resorufin O-deethylase), appeared to be the most rapid and sensitive indicator of the physiological status of the organism. The CEA (cellular energy allocation) biomarker was also a relatively rapid and sensitive indicator. It reflected the health of the organism and probably compensated for the energetic costs required by the other biomarker responses for combating environmental stresses. The results also indicate the need to understand the biochemical changes induced/inhibited in organisms, by factors that are independent of environmental chemicals. Good dose-related responses were noted in each of the controlled aquarium exposures and *O. mossambicus* successfully identified biological effects of environmental stress.

In general, assuming exposure as described in the main body of the report, the health risk assessment (cancer, toxins and EDC) indicates that the potential for serious health effects exists if people are exposed to affected water and selected food from the test areas as well as part of the control areas. These possible health effects include neurological, reproductive, and developmental effects. In addition DDT is known to be estrogen mimicking, and DDE anti-androgenic. People will be exposed to a risk of developing cancer as well as toxic effects if they make use of untreated dam water for various purposes. Ingestion of affected chicken and/or fish results in the highest risk of developing cancer and toxic effects. Carcinogenic risk from direct ingestion of drinking water is in the “acceptable” risk level according to the WHO definition of acceptable risk of 1 in 100,000. The same was found as a result of exposure to DDT through unintentional ingestion of soil. Dermal exposure to the water also results in high risks as a result of absorption of the chemicals that are the water. The risks calculated showed test and control dams to have similar DDT concentrations and therefore potential

health risks. Using dam water from both test and control areas for vegetable watering may expose people to a higher risk of developing cancer than the recommended 1 in 100,000 level as a result of ingestion of these vegetables. Toxic effects as described above may also be expected.

Chicken meat and fat were found to contain levels of total DDT, resulting in a predicted carcinogenic risk of 7 in 10,000 based on average fat concentrations in the test village. DDT concentrations in the chicken fat tested in the control village were within a range resulting in 2 in a million risk of developing cancer based on hypothetical assumptions described in the main report for carcinogenic risks. No toxic effects are anticipated as a result of the DDT levels in the chicken fat found in the control village. Literature has indicated that cooking reduces DDT in food by between 15-80% (Mes et al*.,* 1992; Khanna et al., 1997; Wilson et al., 1998). A hypothetical maximum value for removal was used to determine whether this would allow for a reduction in DDT to acceptable levels. This unfortunately did not occur, with risks remaining at 1 in 10,000 for average concentrations and 3 in 10,000 for the maximum concentration in the test village. The risks in the control village were far lower with a cancer risk of only 2 in 1,000,000 for average DDT concentrations assuming an 80% reduction/destruction of DDT and metabolite levels in the chicken.

In general the greatest anticipated risks of developing cancer resulting from exposure to DDT in the scenarios examined in this quantitative risk assessment is through ingestion of chicken, fish and vegetables. Ingestion of water does not contribute largely to the calculated risk of developing cancer.

A qualitative health risk assessment was conducted making use of the available data collected from the study area. Endocrine disrupting effects were observed at the cellular level with the T47D-KBluc assay detecting estrogenicity in three of nine (33.3%) samples analysed. The aquatic animals also displayed endocrine disrupting effects in *O. mossambicus*, with increased intersex fish, slightly increased apoptosis, reduced sperm motility, reduced sperm counts, and macroscopic differences observed. These effects will be attributable to the mixture of chemicals in the environment whereas the quantitative risk assessment assessed risks arising from exposure to DDT only.

In addition, a cross sectional study looked at DDT and DDE levels in blood and semen samples of males living in DDT sprayed villages compared to non-sprayed villages. The investigation looked at human semen quality associated with DDT exposure in the same study area (Aneck-Hahn et al., 2007) and showed that semen quality was impaired. The

mean lipid adjusted *p,p*-DDE values were 215 µg/g, while Kelce et al. (1995) observed potent anti-androgenic effects 63.6 µg/g lipid adjusted DDE concentrations. One can therefore anticipate the observed semen impairment where DDE concentrations were found to be between 2- 3 times higher and assume that other anti-androgenic effects can be anticipated.

Finally, based on the anti-androgenic effects of DDE and observed ecological effects, there is a 'likely probability' that endocrine effects can be expected in the exposed human population. One must also consider that the probability of endocrine disruption is an additional one 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 certain food as media of exposure. People are also exposed to chemicals via air and additional food and therefore the true health risk can in fact be far greater than what is presented in this report.

The last aim of the study was to compare EDC assessment techniques and develop an integrated, standardized SA relevant toolkit manual of tests for wider application in other spraying areas. Since another WRC funded study (K5-1816): *The compilation of a toolbox of bio-assays for detection of estrogenic activity in water)* is dedicated to the specific subject, only some recommendations relevant to this study will be made here.

This was the first study in South Africa to apply both the recombinant yeast estrogen screen (YES) assays and the T47D-KBluc reporter gene assay for estrogenic activity. The YES measures ERα activity, but the T47D-KBluc measures both ERα and ERß. Cytotoxicity was a major problem in this study, while the T47D-KBluc assay could detect estrogenicity. One of the reasons may be that the sample extract is more diluted in the T47D-KBluc assay, whereas with the YES assay the sample is more concentrated and therefore would show cytotoxicity more easily. The situation is further complicated by the fact that one cannot concentrate to the same extent for the T47D-KBluc assay and, if used in isolation, could result in not detecting low levels of estrogenic activity. Therefore, our findings indicate that both assays should be used when assessing estrogenic activity in water under South African conditions.

GENERAL

The aim was to collect 10 males and females each of both species at these sites over four surveys (n = 720 fish), but using 3 to 4 nets per site, we collected only 239 fish. There clearly seems to be a problem with fish numbers in this area.

After Survey 4 a letter was handed to Nature Conservation expressing the team's concerns on the **Illegal fishing and irresponsible/uncontrolled farming practices within the Luvuvhu River Catchment Management Area (CMA)** (see Appendix 1). No acknowledgement or response was received.

In view of the extensive environmental presence of DDT and residues, the high levels of *p*-NP are of particular concern as this is a rural area with very few industries. The current values were higher than the levels in a previous study at Rietvlei Dam (113 µg/kg) and Marais Dam (4.0 µg/kg) (Bornman et al., 2007), which are considered to be in an industrial area. In the Vhembe area, therefore not only should the impact of DDT be accounted for, but also other chemicals such as *p*-NP, which is an estrogenic chemical. Diet seems to be the major source of human alkylphenolethoxylates (APEs) intake and recently Ademollo et al. (2008) reported the presence of *p*-NP in breast milk samples from mothers in Italy. The concentrations in breast milk were close to the Tolerable Daily Intake (TDI) of 5 µg/kg body weight (bw) proposed by the Danish Institute of Safety and Toxicology. Dorea (2009) pointed out that not only breast milk, but also cow's milk and formula feeding abate exposure in nursing infants. Not only fish and water may be dietary sources of APEs, but they may also come from animals raised on fishmeal or recycled animal products.

In an area where breast milk will be inadvertently contaminated with DDT, it now seems that further studies including also industrial chemicals are warranted.

CONCLUSIONS

p,p'-DDT and -DDE levels were found in all matrices analyzed namely water, sediment, soil, chicken and fish tissue and vegetables. Water showed estrogenic activity, albeit low. Fish health effects including histo-morphological changes in the gills, liver, heart and gonads were demonstrated and sperm motility was impaired in higher DDT exposed waters. Community structures of fish and macro-invertebrates were influenced by DDT contamination within the Luvuvhu River. *In vivo* exposure of fish to environmentally relevant concentrations in the

aquarium showed no severe effects in adult *C. gariepinus* over a short-term period. Since DDT bioconcentrates in tissues, exposure for longer period as would occur in nature, may lead to irreversible long term adverse effects, especially in juveniles. Good dose-related responses, similar to what were observed in the field studies, occurred in each of the controlled aquarium exposures of *O. mossambicus.*

The *o,p'*-DDT levels (409.4 ± 165.7 µg/kg) in fat means an added estrogenic load to the insecticidal estrogenic *p,p'*-DDT. *o,p'*-DDT is a by product of DDT during production and if its presence in the final product used for IRS could be eliminated or at least lowered, a lot could be done to render the existing formulation safer while DDT is still being used.

The presence of *o,p'*-DDD in chicken liver and fat is also of concern. *o,p'*-DDD (Mitotane®) has been used to treat adrenocortical carcinoma and Cushing's disease in humans for almost four decades (Bergenstal et al., 1960; Wooten and King, 1993). *o,p'*-DDD selectively induces necrosis of the *zona fasciculata* and *zona reticularis* of the adrenal cortex and inhibition of cortisol synthesis.

Skinner-Adams et al. (2008) pointed out that the global epidemiology of HIV/AIDS and malaria overlap, as a considerable number of HIV-infected individuals live in regions with different levels of malaria transmission. The consequences of co-infection with HIV and malaria parasites are not fully understood, but it seems that the infections act synergistically and together produce devastating outcomes. DDT, DDE and DDD all induce differential degrees of humoral and cellular immune suppression, but the clear responses are by DDD and DDE. Suppression of immune responses by DDD and DDE is an important determinant of the toxicity of DDT (DDE > DDD > DDT) (Banerjee et al., 1996). To the best of our knowledge, the possible relationship between *o,p'*-DDD levels and the human immunodeficiency syndrome has not been studied, but it seems warranted in view of our findings

The predicted human health risks for both carcinogenic and toxic effects are high, with chicken and fish ingestion causing the highest risks, followed by vegetable ingestion, (where DDT levels result from DDT in water used to irrigate the vegetables). Dermal exposure also results in high risks. The lowest (and acceptable according to WHO recommendations) risk is from direct exposure to water through ingestion and highlights the importance of examining all possible exposure routes when conducting a risk assessment.

Endocrine disrupting effects were also observed and can be anticipated in the exposed population with effects observed in both the human and animal population and at the cellular level.

CONFERENCE PRESENTATIONS

1 Barnhoorn IEJ, Van Rensburg C and Bornman MS. Environmental levels of DDT in a currently sprayed area: consequences for health. Poster presentation SETAC Europe 17th Annual meeting, 20-24 May 2007, Porto, Portugal.

2 Marchand MJ, Pieterse GM and IEJ Barnhoorn, 2007. Sperm motility and testicular histology of two feral fish species from a currently DDT-sprayed area, South Africa. 1st International Workshop on the Biology of Fish Sperm, Vodnany, Czech Republic, 29-31 August 2007.

3 Barnhoorn IEJ, Bornman MS, Van Dyk JC. Levels of DDT and DDE in fish fat from currently DDT- sprayed and non-sprayed areas, South Africa. 18th SETAC Europe meeting, Warsaw, Poland 18-23 May 2008.

4 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.

5 GM Pieterse, JC van Dyk, MJ Marchand, JHJ van Vuren, IEJ Barnhoorn and MS Bornman, 2007. Quantitative and qualitative histological assessment as a tool to assess fish health. Poster presentation SETAC Europe, 17th Annual Meeting Congress, Porto, Portugal, 20-24 May 2007.

6 Marchand MJ, Pieterse GM, Van Dyk JC and Barnhoorn, IEJ. 2008. The effects of DDT on the reproductive health of two fresh water fish species, *Clarias gariepinus* and Oreochromis mossambicus, from South Africa. SETAC Europe, 18th Annual Meeting Congress, Warsaw, Poland, 25-29 May 2008.

7 Pieterse GM, Marchand MJ, Van Dyk JC and Barnhoorn, IEJ. 2008. A histo-morphological study of the normal testis of an indicator species of estrogen-polluted waters in South Africa. SETAC Europe, 18th Annual Meeting Congress, Warsaw, Poland, 25-29 May 2008.

8 Pieterse GM, Van Dyk JC, Marchand, MJ and Barnhoorn, IEJ. 2008. Quantitative fish health assessment and water pollution. 1st International Workshop on the Aquatic Toxicology and Biomonitoring, Vodnany, Czech Republic, 27-29 August 2008.

9 Marchand MJ, Pieterse GM and IEJ Barnhoorn, 2008. A field-based seasonal study on the effects of DDT on testicular histopathology and sperm motility of freshwater fish. 1st

International Workshop on the Aquatic Toxicology and Biomonitoring, Vodnany, Czech Republic, 27-29 August 2008.

10 Pieterse GM, Marchand MJ, Van Dyk JC and Barnhoorn IEJ. Can fish health tell anything about water pollution? Poster: SETAC Europe 18th Annual meeting, 59-29 May 2008, Warsaw, Poland.

11 van Dyk JC, Bornman MS, Barnhoorn IEJ and Pieterse GM. Epicarditis and myocarditis in feral catfish, *Clarias gariepinus*, from two impoundments in the Luvuvhu River, Limpopo, South Africa: A novel finding. Poster: SETAC Europe 18th Annual meeting, 59-29 May 2008, Warsaw, Poland.

12 Marchand MJ, Pieterse GM and Barnhoorn IEJ. The effects of DDT on the reproductive health of two fresh water fish species, *Clarias gariepinus* and *Oreochromis mossambicus*, from South Africa. Presentation: SETAC Europe 18th Annual meeting, 59-29 May 2008, Warsaw, Poland.

OTHER IMPORTANT INFORMATION

University of Johannesburg Zoology student attended the Pellston Workshop in the USA (27-31 January 2008). Marcelle Marchand was one of two students selected internationally to attend the Pellston Workshop for the Society for Environmental Toxicology and Chemistry (SETAC) on Science-based Guidance and Framework for the Evaluation and Identification of PBTs (Persistent Bioaccumulative Toxic substances) and POPs (Persistent Organic Pollutants), 27 January-1 February 2008, Pensacola, FL USA. All expenses were paid by SETAC.

The Pellston Workshop was an amazingly stimulating experience. 30-40 experts gathered in a remote location to conduct 3-4 days of focused discussions and produce a book of interest and importance to advancing environmental science and better regulatory decision making. Many of the 44 SETAC-sponsored “Pellstons” have had major influences on the scientific and regulatory communities.

The aim goals of the workshop were to improve the science of ongoing global activities on PBT and POP assessments and to provide scientific guidance to industry, regulators and international agencies for these assessments. The results of the workshop, in the form of workshop summary compilations and full length articles of which Marcelle will be one of the co-authors, are likely to be available in due course.

JUNIOR KAPTEIN SCOTT MEDALJE (25 SEPTEMBER 2008)

Marcelle Marchand received the Junior Kaptein Scott Medalje (Junior Captain Scott Medal) on 25 September 2008 in Potchefstroom from the SA Akademie vir Wetenskap en Kuns (SA Academy for Science and Arts). This medal is awarded for the best M.Sc thesis in Zoological Sciences at a South African University in 2006-2007.

EXCELLENCE IN WATER RESEARCH AWARD (16 OCTOBER 2008)

Marcelle Marchand, Ph.D student in Aquatic Health received an award as a Young Researcher at "The Excellence in Water Research Awards" evening that was held on 16 October 2008. WISA, Water Research Commission and the CSIR hosted this special awards evening in Honour of Dr Gerrie Stander, in acknowledgement of focused, productive, multidisciplinary research dedicated to the growth and intellect of the respective research disciplines, betterment of science and ultimately in service of our rainbow South Africa.

YOUNG SCIENTIST AWARD WINNER FROM SETAC (28 MAY 2008)

Marcelle Marchand also received the prestigious SETAC Europe Young Scientist Award (YSA) for her poster presentation at the 18th Annual meeting of the Society of Environmental Toxicology and Chemistry (SETAC) in Warsaw, Poland in 2008. The award is made annually and is intended to honour individual prominent performance in scientific work of a junior scientist under the age of 30.

The aim of her research was to use a standardized quantitative histological assessment tool to evaluate the effect of Endocrine disrupting chemicals (EDCs) on the health status of fish in two dams in an Urban Nature Reserve, Gauteng (Bornman et al., 2007). The research focused on the histopathological alterations in selected organs of the fish as an indicator of exposure to environmental toxicants.

PUBLICATIONS EMANATING FROM THIS PROJECT

1. Marchand MJ, Pieterse GM and IEJ Barnhoorn. 2008. Sperm motility and testicular histology of two feral fish species from a currently DDT-sprayed area, South Africa. J. Appl. Ichthyol., 24:423-429.
2. Marchand MJ, Van Dyk JC, Pieterse GM, Barnhoorn, IEJ and Bornman MS. 2009. Histopathological alterations in the liver of the sharptooth catfish *Clarias gariepinus* from polluted aquatic systems in South Africa. Environmental Toxicology., (Accepted for publication on June, 2008, Reference TOX-07-190, Available online April 2009).
3. Patrick SM, Van Dyk JC, Olurunju SAS, Bornman MS. Testicular apoptosis in two bio-sentinel fish species inhabiting the Luvuvhu river in the Limpopo Province, South Africa. Submitted at Environmental Toxicology.
4. Van Dyk JC, Barnhoorn IEJ, Bornman MS. Epicarditis and myocarditis in the African catfish, *Clarias gariepinus* inhabiting the Luvuvhu river, South Africa. Submitted.

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

CAPACITY BUILDING

Individuals

Completed

Mr. J. MacDonald (M, W) "**Brain histology and the use of electron-microscopy to determine neural damage in feral DDT-exposed sharptooth catfish and Mozambique tilapia.** BSc Honours study, Department of Anatomy, Faculty of Health Sciences, University of Pretoria.

Mr. Sean M. Patrick (M, C) "**Apoptotic markers in the testes of feral DDT-exposed *Clarias gariepinus* and *Oreochromis mossambicus* from the Luvuvhu River system".** Master of Science in Physiology, Faculty of Health, University of Pretoria.

Students

Mrs Kerry A Brink (F,W) "**The endocrine disruptive effects of DDT on reproduction, represented in individuals, populations and communities".** PhD in Zoology, Faculty of Science, University of Johannesburg.

Ms Kieren J Bremner (F, W) **"The use of the Mozambique Tilapia (*Oreochromis mossambicus*) as a sentinel species of the possible effects on health and reproduction of DDE in vivo exposure and from a DDT sprayed area".** Master of Science

in Zoology, Faculty of Science, University of Johannesburg

Ms. Marcelle J. Marchand (F, W) **"The use of CASA and stages of spermatogenesis as an indicator of the effect of DDT and DDE**. PhD in Aquatic Health (Zoology), Faculty of Science, University of Johannesburg.

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- Ms APM Moolman (Water Research Commission), Chairperson
- Prof H Bouwman (Northwest University)
- Ms AEC Burger (Water Research Commission Consultant EDC Programme)
- Dr R Heath (Pulles Howard & De Lange, Johannesburg)
- Dr S Jooste (Resource Quality Studies (RQS), Department of Water Affairs and Forestry)
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8. DWAF Xikundu Weir: Mr S Nethononda

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AD	Albasini Dam
Al	aluminium
Alp	alkali-labile phosphate
ANOSIM	analysis of similarities
ANOVA	one way analysis of variance
AR	androgen receptor
ARC	Agricultural Research Commission
As	arsenic
ASPT	average score per taxon
ATP	adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BBP	benzyl butyl phthalate
BCF	beat cross frequency
BEST	Biomonitoring of Environmental Status and Trends Program
BF	Bouin's fluid
BS	Broken stick model
BSA	bovine serum albumin
Ca	calcium
$CaCO_3$	total hardness
CASA	computer assisted sperm analysis
CAT	catalase
Cd	cadmium
CD	circulatory disturbances
CEA	cellular energy allocation
CF	Condition factor
Cl	chloride
COD	chemical oxygen demand
CPUE	catch per unit effort
CSI	cardiac somatic index
Cu	copper
CYP	cytochrome P450
DAB	3, 3´-diaminobenzidine
DBP	dibutyl phthalate
DDD	1,1-dihloro-2,2-bis(p-chlorophenyl)ethane

DDE	1,1-dihloro-2,2-bis(p-chlorophenyl)ethylene
DDT	1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane
DEHP	di-(2-ethylhexyl) phthalate
DEP	diethyl phthalate
Df	degrees of freedom
DHT	dihydrotestosterone
DHTEq	dihydrotestosterone equivalents
DMP	dimethyl phthalate
DNA	deoxyribonucleic acid
DoH	Department of Health
DV	Dididi village
DWAF	Department of Water Affairs and Forestry
Ea	total energy reserves
E_2	17ß-estradiol
EC	electrical conductivity
Ec	energy consumption
ECG	electro-cardiogram
EDC	endocrine disrupting chemicals
EDMs	endocrine disrupting metals
EDTA	ethylenediaminetetraacetic acid
EEq	estradiol equivalents
EHC 9	Environmental Health Criteria 9
ELISA	enzyme-linked immunosorbent assay
Er	available energy reserves
ER	human estrogen receptor
ERα	human estrogen receptor alpha
ERE	estrogen-responsive elements
EROD	7-ethoxy resorufin O-deethylase
ERß	human estrogen receptor beta
ETS	quantification and energy consumption
FBS	fetal bovine serum
Fe	iron
FO	frequency of occurrence
FSH	follicle-stimulating hormone
GC-ECD	Gas Chromatograph*(y)* – Electron Capture Detector
GC-FPD	Gas Chromatography with Flame Photometric Detection
GC-MS	Gas Chromatograph*(y)* – Mass Spectrometry

GnRH	releasing hormone gonadotropin
GPS	global positioning system
GPx	glutathione peroxidase
GR	glucocorticoid receptor
GSM	geometric series model
GSI	gonadosomatic index
H	Shannon diversity index
H&E	Haematoxylin and Eosin
H_2O_2	hydrogen peroxide
HAI	health assessment index
HBFHA	histology-based fish health assessment
HCH	hexachlorocyclohexane
HCR	habitat cover ratings
Hct	haematocrit
HF	high flow
Hg	mercury
HPG	hypothalamus-pituitary-gonadal
HSMI	heart and skeletal muscle inflammation
I	inflammation
I_{CII}	cardiac inflammatory index
ICP-MS	inductive coupled plasma mass spectrophotometer
IHC	immunohistochemistry
IHI	index of habitat integrity
I_{org}	organ index
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
IRS	indoor residual spraying
KCl	potassium chloride
KNP	Kruger National Park
Lct	Leukocrit
LF	low flow
LH	luteinizing hormone
LIN	linearity
LS	log series model
LSDI	The Lubombo Spatial Development Initiative
MAP	mean annual precipitation
MAR	mean annual runoff

MDS	multi-dimensional scaling
Mg	magnesium
MMC	melanomacrophage centres
Mn	manganese
MOT	motility
N	number of individuals
NaCl	sodium chloride
NBF	neutrally buffered formalin
ND	Nandoni Dam
NMDS	multidimensional scaling
O_2-	superoxide anion
OC	organochlorine
OcP	octylphenol
-OH	hydroxyl radical
OHF	hydroxyflutamide
P	probability
PAHs	polycyclic aromatic hydrocarbons
Pb	lead
PC	progressive changes
PCB	polychlorinated biphenyls
PCD	programmed cell death
PFA	paraformaldehyde
p-NP	*p*-nonylphenol
POP	persistent organic pollutant
PROG	progression
RA	rank abundance
RC	regressive changes
RIA	radio-immunoassays
ROS	reactive oxygen species
rp1	reaction pattern 1
rp2	reaction pattern 2
rp3	reaction pattern 3
rp4	reaction pattern 4
rp5	reaction pattern 5
S	number of species
SASS 5	South African Scoring System v5
SHI	site habitat integrity

Si	silica
SIMPER	similarity percentages
SO_4	sulphate
SOD	superoxide dismutase
SPE	solid phase extraction
Sr	strontium
SSI	splenosomatic index
StAR	steroidogenic acute regulatory protein
T	tumour
TBT	tributyltin
TCA	trichloroacetic acid
TDI	tolerable daily intake
TDS	total dissolved solids
TLN	truncated log normal model
Tot-I	total index
TUNEL	terminal deoxynecleotidyl transferase-mediated dUTP Nick end-Labeling
TV	Tshakhuma village
TVA	Tennessee Valley Authority
TWQR	target water quality range
V	vanadium
VAP	velocity average path
VCI	Verband der Chemischen Industrie
VCL	velocity
VSL	straight line velocity
VTG	vitellogenin
W	importance factor
WHO	World Health Organization
WOB	wobble
X^2	Chi square
XW	Xikundu Weir
YES	Recombinant yeast estrogen screen
Zn	zinc
% MOT	% motile

CHAPTER 1

ENVIRONMENTAL CONTAMINATION/EXPOSURE AND HEALTH RISK ASSESSMENT IN A CURRENTLY DDT-SPRAYED AREA

Bornman MS

1.1 Introduction and rationale

1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) is a pesticide that has been banned for international use, but countries like South Africa have restricted use of DDT for indoor residual spraying (IRS) for malaria mosquito control. The Vhembe area in Limpopo Province is such an area where DDT was introduced in 1945 and has since been sprayed annually. DDT plays a central role in successfully combating malaria and saving lives.

However, DDT is not only one of the persistent organic pollutants (POPs), but also an endocrine disrupter chemical (EDC). EDCs represent a diverse range of man-made chemicals discharged into the environment that mimic or antagonize the action of hormones. These EDCs may interact with physiological systems and cause alterations in development, growth and reproduction in wildlife and particularly in exposed fish (Jobling and Tyler, 2003). Exposure of male embryos to EDC during the early stages of fetal development has been linked to the increased incidences of male reproductive health disorders including hypospadias, undescended testes, intersex, subfertility and testicular cancer (Paulozzi 1999; Sharpe and Skakkebaek, 1993; Toppari et al., 1996).

Two previous WRC reports containing pilot data on environmental contamination of DDT in the Limpopo Province raised concern about the water quality and estrogenic activity in an impoundment. Contamination of chickens, francolins and two fish species were also found (Burger, 2008) and testicular oocytes (intersex) was observed in male *Oreochromis mossambicus* (Bornman et al., 2009a). In a cross sectional study on healthy male subjects (n=311) aged between 18 and 40 years (23 ± 5) living in the currently DDT-sprayed area of the Vhembe District, Limpopo Province, Aneck-Hahn et al. (2007) demonstrated that semen quality was adversely affected and that men living in DDT IRS houses had extremely high plasma levels of DDT. Bornman et al. (2009b) showed that 11.5% of newborn boys had urogenital birth defects. Mothers and their babies also have high plasma levels of DDT (Bornman et al., 2005) raising the question as to how contamination occurred.

The research question was whether environmental levels of DDT and DDE may contribute to adverse health effects in aquatic animals and subsequently may pose a health risk for humans.

1.2 Aim

The objective of the study was to link possible health effects in aquatic biota from a DDT-sprayed area to adverse health effects in humans living in the same area. The study area also offered the opportunity to include invertebrates as the possible effect of endocrine disruptors on invertebrates is largely unknown.

The specific aims of the study focused on environmental exposure assessment and health risk of humans and aquatic wildlife in the Vhembe area, Limpopo Province where ongoing DDT spraying occurs:

1. to perform chemical residue analysis and biological testing for estrogenicity in water, soil, sediment and biota,
2. to determine aquatic health in and around water sources in the area,
3. to perform a scenario-based health risk analysis, and a human health risk assessment
4. to compare EDC assessment techniques and develop an integrated, standardized SA relevant toolkit manual of tests for wider application in other spraying areas.

Please note that the data from other studies were used to calculate human health impacts and this is different to what was mentioned in the application. Human health effects were not studied *per se* in this study.

1.3 Study area

This study was done in the Vhembe District Municipality near Thohoyandou in Limpopo Province (Figure 1.1). All the sites were in the Luvuvhu River, which flows through the currently DDT-sprayed area. The Luvuvhu river catchment is a tropical, high-risk malaria area where 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 200km 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 m^3 (ranging from 85 to 1 900 million cubic metres) (Department of Water Affairs and Forestry (DWAF, 2003).

The major sampling sites were the Albasini (AD) and Nandoni Dams (ND), and the Xikundu Weir (XW). Other sites in the Luvuvhu river where water quality, fish diversity and South African Scoring System v5 (SASS5) were investigated included Lotanyanda, Hasana, Tshikonelo, and Mhinga (Figure 1.1). Sampling was conducted during four surveys as a result of no distinctive high flow or low flow periods:

- Survey 1 = October 2006
- Survey 2 = March 2007
- Survey 3 = October 2007
- Survey 4 February 2008

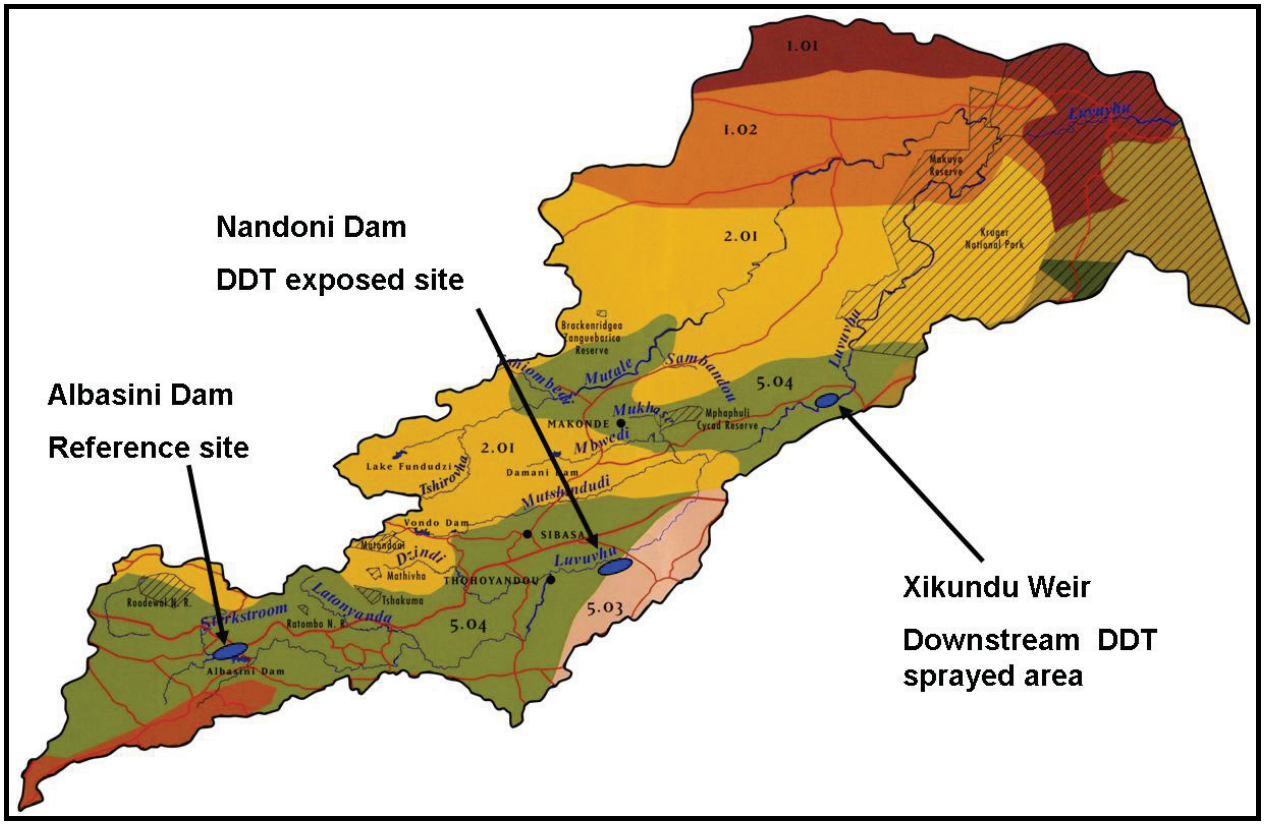

Figure 1.1: A map indicating the three sites where sampling was done in the Luvuvhu river catchment (Map adapted from State of the Rivers Report, WRC, 2001).

1.4 Report layout

This report summarizes the scientific background relevant to the study with emphasis on the endocrine system and endocrine disrupting chemicals including DDT. It also reviews the use of two possible biosentinel fish species, as well as macro-invertebrates in terms of population and community effects. General information on the area is discussed.

This is followed by separate chapters (including Introduction, Materials and Methods, Results, Discussion and Conclusions) on analytical chemistry of water and sediment, and *in vitro* bio-assays for endocrine disruption. Chapters on the analytical chemistry of food, soil, potable water, fish fat and brain tissue then follow. The discussion of EDC effects on fish and macro-invertebrates is followed by the health findings in *O. mossambicus*. Histomorphology of the heart, testicular apoptosis, the histology based fish health assessment and sperm motility in both fish species are discussed. *In vivo* toxicological assessment of exposure of *C. gariepinus* and *O. mossambicus* are described. All the information gathered was integrated in a qualitative scenario-based health risk analysis.

CHAPTER 2

LITERATURE REVIEW

Bornman MS, Barnhoorn IEJ, Aneck-Hahn NH, Van Dyk JC, Marchand MM, Van Zijl MC, Brink KA, Patrick SM, Bremner KJ and Pieterse GM

2.1 The endocrine system

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 (Damstra et al., 2004). The endocrine system maintains a homeostatic environment, and is a regulator of growth, development, and reproduction in vertebrates. The hypothalamus-pituitary-gonadal (HPG) axis has evolved in vertebrates as the hormonal governor over spermatogenesis, oogenesis and reproduction in general (Bray et al., 2002). This axis is comprised of three components; gonadotropin-releasing hormone (GnRH) neurons, from the hypothalamus; secretion of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary; and the somatic cells of the testes and ovaries (Damstra et al., 2004).

In the male, hypothalamic and pituitary control of LH and FSH secretion is controlled by the feedback from the testis. Both the Leydig and Sertoli cells are involved in steroidogenesis within the teleost testis (Grier, 1981). LH targets the Leydig cells to stimulate testosterone, which activates androgen receptors in the seminiferous epithelium to control spermatogenesis. FSH targets receptors within the Sertoli cells to regulate spermatogenesis by stimulating Sertoli cell factors (O'Donnell et al., 2001). Teleosts have at least two pituitary gonadotropins that are structurally and functionally homologous to FSH and LH (Cheshenko et al., 2007).

The steroidal hormone estrogen is involved in the control of reproductory processes, including sexual differentiation, maturation, control of the cell cycle and proliferation (Chang et al., 2005). Estrogens have generally been associated with the regulation of female functions, however, they are produced in male vertebrates (Schultz and Miura, 2002) and estrogen receptor (ER) activity is expressed throughout the HPG axis (Grier, 1981) and in the testes of a variety of vertebrate species. This observation led to the suggestion that

estrogens take part in the regulation of testicular function (Damstra et al., 2004; Wu et al., 2002).

2.2 Endocrine disrupter chemicals (EDCs)

Some chemicals that are mostly anthropogenic and are used in our immediate and daily surroundings may be endocrine active substances or modulators and are called 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 will be 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).

There has been mounting concern over the potential adverse effects that xenobiotics, in particular xenoestrogens, have on the endocrine system (Aravindakshan et al., 2004). Reviewing all aspects of EDC is beyond the scope of this report and the reader is referred to recent publications on developments in endocrine disrupter research and human health (Phillips et al., 2008; Phillips and Foster 2008).

2.3 DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane)

DDT is a pesticide that was once widely used to control insects on agricultural crops and insects that carry diseases like malaria and typhus. DDD was also used to kill pests, but to a far lesser extent than DDT. One form of DDD (*o,p'*-DDD) has been used medically to treat cancer of the adrenal gland. Both DDE and DDD are breakdown products of DDT.

DDT is a broad-spectrum insecticide, very popular due its low cost, effectiveness, long residual persistence and low acute toxicity in mammals (Metcalf, 1989). DDT was first used in 1939 (Van Metre et al., 1997). In 1973 the use of DDT in the United States has been 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. DDT was introduced In Limpopo Province in 1945 and has been sprayed annually since.

Although DDT has been banned for international use, countries like South Africa have restricted use for malaria vector control. South Africa is a party 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 POPs into the environment.

DDT from past or current spraying during indoor residual spraying (IRS) may be released into the atmosphere and DDT, DDE and DDD may evaporate from contaminated water and soil. DDT, DDE, and DDD in the air will then be deposited on land or surface water and this cycle of evaporation and deposition may be repeated many times. As a result, DDT, DDE, and DDD can be carried long distances in the atmosphere and water, eventually end up in the Arctic and Antarctic regions, far from where they were ever used. In soil DDT and metabolites last very long, possibly hundreds of years (Agency for Toxic Substances and Disease Registry (ATSDR), 2002).

Technical-grade DDT (the substance applied during IRS) is composed of 65-80% of the active insecticidal ingredient, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDT) (Bouwman et al., 2006). The other components include 15-21% of the less insecticidal 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-ethane (*o,p'*-DDT), and about 4% of 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDD) (Metcalf, 1995). 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). The persistent metabolite of DDT, *p,p*'-DDE inhibits androgen binding to the androgen receptor, androgen-induced transcriptional activity, and normal male prepubertal development (Kelce et al., 1995; Rogan and Chen, 2005). Danzo (1997) reported that not only was *p,p*'-DDE the most potent of the xenobiotics tested at inhibiting 5-α-dihydro-testosterone binding to the androgen receptor, but both *o,p*'-DDT and *p,p*'-DDT are also potent inhibitors.

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).

2.4 Bio-indicators

2.4.1 *Clarias gariepinus*

The sharptooth catfish, *Clarias gariepinus* is an indigenous South African species and considered to be one of the most important tropical catfish species for aquaculture (De Graaf and Janssen, 1996; Skelton, 1993; Skelton, 1995). *C. gariepinus* is part of an ecologically diverse component of most river faunas throughout Africa, preferring the slow-flowing reaches of rivers and also lakes, 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 to changing environmental conditions.

2.4.2 *Oreochromis mossambicus*

The Mozambique tilapia, *Oreochromis mossambicus,* is a relatively large, deep-bodied, mouth brooding cichlid, endemic to the east coastal rivers from the lower Zambezi river system south to the Bushman's river system in the Eastern Cape. It is also found south of the Pongolo system and is often naturally confined to closed estuaries and coastal reaches of rivers. The fish is also widely dispersed beyond this range to inland regions and to the south-west and west coastal rivers including the lower Orange and rivers of Namibia (Skelton, 2001). *O. mossambicus* is common in the study area, the Luvuvhu river in the Limpopo Province.

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. The fish feeds on algae, especially diatoms, and detritus (the organic matter in which DDT and its metabolites accumulate). Larger individuals might even take insects or other invertebrates (Skelton, 2001). Breeding takes place in summer, with females raising multiple broods every 3-4 weeks during a season. Juveniles grow rapidly and may mature and breed within a year (Skelton, 2001).

2.5 Bioaccumulation

EDCs may be introduced into the atmosphere, sediment or water either through deliberate agricultural application of pesticides or by accidental releases by industry (Phillips and

Rainbow, 1994). Nevertheless, once in the environment, these chemicals may continue to persist, perhaps in different environmental phases, long after sources are discontinued, due to their characteristically high lipophilicity, combined with chemical stability and very slow biodegradation (Borga et al., 2001; Connell et al., 1999). The organochlorine DDT is a prime example of the persistence of high residue concentrations in biota.

When biota is exposed to the abovementioned features of EDCs in the water, sediment and/or food chain, uptake and bioaccumulation is almost spontaneous. According to Vallack et al. (1998), the lipophilic nature of EDCs reduces their solubility in water which promotes their accumulation into biota from the water (bioconcentration), whilst the resistance of EDCs to metabolic degradation in biota allows residues to persist within the food chains (biomagnification). The combination of uptake from these two environments (bioconcentration and biomagnification) will ultimately lead to greater bioaccumulation of EDCs in biota.

Numerous studies have quantified such accumulation of EDCs. Phillips and Rainbow (1994), Brown (1979) and Kime (1994) have all extensively reviewed the concentrations end effects of EDCs in many fish and macro-invertebrate species. Furthermore, it should be noted that all these authors stressed that if bioaccumulation studies are attempted, a number of factors should be considered when resulting data is interpreted. These factors may include either external or internal influences, such as lipid content, age, sex, size, sexual maturity, sediment or water variables.

2.6 Effects of EDCs in fish and invertebrates

Biological effects of pollutants may be manifested at various levels of biological organisation, depending on whether the concentration is high enough and/or the duration of exposure is long enough (Smolders et al., 2003). When individuals are exposed to pollutants, a cascade of biological events may result. This cascade ultimately leads to the irreversible long-term effects occurring at higher hierarchical levels of biological organisation. However, such changes can be prevented by recognizing the causal relationship between various hierarchical level endpoints and utilizing the reversible endpoints as early warning tools to predict irreversible environmental damages (Hyne and Maher, 2003). Indeed, there is considerable interest in such relationships specifically between molecular effects in individuals and subsequent effects in populations and communities (Arcand-Hoy and Benson, 1998; Vasseur and Cossu-Leguille, 2006).

2.6.1 Individual effects of EDCs in fish and invertebrates

The nature of the effects of EDCs in biota can be very variable. Apart from the obvious lethal effect, EDCs has shown to have sub-lethal effects on many different biological systems in both fish and invertebrates (Holden, 1973; Bayley et al., 2002; Kime, 1998; Vasseur and Cossu-Leguille, 2006). In fish, it has been reported that most of the effects occurring from EDCs on nervous systems, behaviour, reproduction, and immune functions are predominantly induced by the endocrine disrupting mechanisms. According to Rodrigues et al. (2007), these disrupted endocrine functioning mechanisms usually result from EDCs altering hormone secretion, interfering with hormone-receptor interaction or modifying the metabolism of circulating hormones.

In invertebrates, however, very little information regarding the endocrine disrupting effects of EDCs is available. This is primarily due to the generally limited understanding of normal endocrine processes in invertebrates (Ketata et al., 2008). Nevertheless several (likely) endocrine disruptive effects have been demonstrated on invertebrates. The best documented cases were reviewed by Matozzo et al. (2008), Depledge et al. (2008) and Schramm et al. (2008) and included feminisation/masculinisation, sterility, abnormal sex ratio's, protein metabolism and emergence times. Since fish were the only group that individual effects were analysed for, there will be no further discussion on invertebrates and an extensive review on the reproductive effects on fish.

2.6.1.1 Reproduction in fish

Since the endocrine system is largely related to reproduction, the majority of the endocrine disrupting effects in fish are usually on the reproductive functioning. A large number of these effects have been reviewed by many, including large organisations such as USEPA and NOAA as well as authors such as Kime (1998), Huang et al. (2003) and Vouk and Sheen (1983). Some of the major effects of EDCs (with particular attention to DDT) and their measurable endpoints will be discussed in the following sections.

Vitellogenin

One of the most extensively reviewed mechanisms of action of EDCs in fish is the mimicking of the female hormone, estrogen (Cheek et al., 2001). Under normal conditions, estrogen stimulates the production of vitellogenin (VTG) in the fish liver, which is transported to the

ovary where it enters the mature female's oocyte to function as an essential protein in egg development (Marin and Matozzo, 2004). However, in the presence of EDCs such as DDT, such processes in the mature female may be inhibited. According to Overdorster and Cheek (2001), continuous estrogenic stimuli may trigger negative feedback on endogenous estradiol (estrogen) production which may lead to inhibited vitellogenin synthesis and ovarian growth. In contrast the male and juvenile fish exposed to estrogenic mimicking compounds may produce excess VTG. Hence, the abnormal presence of excessive VTG in male and juvenile plasma can be used as a biological indicator of exposure to estrogenic compounds (Lomax et al., 1998).

Donohoe and Curtis (1996) showed in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to DDT and DDE, that plasma VTG could be successfully used as a very sensitive marker of estrogen exposure. Similar conclusions were obtained using the Japanese medaka (*Oryzias latipes*), sunshine bass (Morone spp.) channel catfish (*Ictalurus punctatus*), Indian catfish (*Heteropneustes fossils*), common carp (*Cyprinus carpio*), and large-scale sucker (*Catostomus macrocheilus*) (Chatterjee et al., 2001; Hinck et al., 2005; Lavado et al., 2004; Nimrod and Benson, 1996; Thomson et al., 2000). Although VTG has been widely used as an indicator of exposure, the degree they adversely affect organismal characteristics and higher organizational levels remains unclear. However, Huang et al. (2003) suggested that VTG should be used in combination with other reproductive parameters so that their effects on organismal characteristics may become apparent.

Many assays for measuring VTG concentrations directly in fish plasma have been developed. For example, the enzyme-linked immunosorbent assay (ELISA), radio-immunoassays (RIA), western blot analyses, and VTG mRNA determination by DNA hybridization strategies (Marin and Matozzo, 2004). Undoubtedly the most favoured of these, is the ELISA as it is a relatively rapid and simple tool for monitoring EDC contamination. However, a disadvantage of this method is that it is limited to only a few species and not one of which is commercially available for any South African indigenous fish species (Verslycke et al., 2002). A rapid, cost effective alternative to the ELISA is the alkali-labile phosphate assay. This assay has been shown to successfully estimate the amount of VTG present in plasma by colorimetrically measuring the amount of phosphate groups bound to VTG. Similarly, concentrations of calcium, magnesium and total plasma proteins can be used as an indirect measure of VTG since these are increased significantly in presence of VTG.

Gonadosomatic index (GSI)

Gonad maturation and spawning readiness are frequently measured using fish condition indices such as gonadosomatic index (GSI) (Huang et al., 2003). This index, calculated as the percentage of gonad weight to total body weight, and is based on the broad assumption that proportionally larger gonads indicate greater development. Reduction in relative gonad mass can occur in response to increased EDC exposure therefore GSI can be used to estimate the degree of EDC effects on reproduction (Schweer, 2002). Indeed, numerous studies have successfully used this index to monitor the reproductive effects of EDC exposed fish. Fiest et al. (2005) showed, using white sturgeon (*Acipenser transmontanus*), significant negative correlations between DDT and its metabolites and GSI. Similar observations were found in the *Paralichthys dentatus* (summer flounder) (Mills et al., 2001), *O. latipes* (Papoulias et al., 2003), *Percopsis omiscomaycus* (trout-perch) (Gibbons et al., 1998), *O. mykiss* (Sheahan et al., 2002), and *Micropterus salmoides* (large mouth bass) (Sepulveda et al., 2003) among others, exposed to various EDCs.

Although measurements of the GSI are a successful index to monitor reproductive effects, care should be taken when interpreting results. According to the author of the *Verband der Chemischen Industrie* (VCI) position paper on “endocrine active substances” (2005), GSI may be influenced by many different factors which may occur alone or in combination. Some of which may include age, temperature, health, fish density, time of year or inter-individual variation in gonad weight during spawning season. In order to compensate for this large variability, the authors suggested that all examined samples should be of a sufficient size to rule out misinterpretation in the field. However, it was recommended that the more statistically sound multivariate analysis of covariance of the directly measured gonad and body weights be performed to assess the differences between treatment groups.

Condition factor

The condition factor (CF) is a general indicator of fish health, by correlating the fish body mass to its length. In fact this index is often used in aquaculture, to monitor feeding intensity, age and growth rates in fish (Anene, 2005). Within an ecotoxicological context however the CF is based on the fact that there is usually depletion in the energy resources available, as these resources are utilised to cope with the increased stresses posed by a toxicant. An astounding number of ecotoxicological based studies, including those measuring DDT, have incorporated this index and is particularly popular due to its simplistic and cost effective nature. To measure this CF, there are many different approaches available and are reviewed

by Stevenson et al. (2006). In the current study a more meaningful approach was used where a standardised mass was predicted on the basis of a statistical formula for a specified test species and then compared against the actual mass for each of the respective individuals.

Intersex

In comparison with GSI, histology is a more comprehensive and informative method for analyzing alterations in gonadal morphology brought about by EDCs (Pieterse, 2004). EDCs can induce a number of alterations in both ovarian and testicular morphology, which has been extensively reviewed by Kime (1998). One of the most ecologically damaging alterations observed in fish is the gonadal intersexuality induced by excess estrogen in males. This feminization was shown to have deleterious effects on fertilization success, which may ultimately have severe population-level consequences, although the extent of these conditions on fish populations remains to be thoroughly evaluated (Jobling and Tyler, 2003). Most studies have rather focused on identification and cause of intersex gonads in fish. For example, Cheek et al. (2001) observed intersex in most of the life stages of medaka exposed to DDT whilst Lye et al. (1997) found intersexuality in the flounder (*Pleuronectes flesus*). A field study by Hinck et al. (2005) in the pesticide ridden Columbia River showed intersex in the smallmouth bass (*Micropterus Dolomieu*). In South Africa, *C. gariepinus* present in the Rietvlei Nature Reserve, also showed signs of intersex due to exposure of *p*-NP (*p*-nonylphenol) (Barnhoorn et al., 2004).

Measure of reproductive performance

According to Kime (1998), early life stages of fish are very sensitive to pollution. This is however not surprising as there are so many modes of action in the fish reproductive system for toxicants to affect, especially if all life stages are exposed. This can include anything from changes that influence the functioning of sperm (McAllister and Kime, 2003), eggs (Lahnsteiner et al., 2006), fertilisation (Hose et al., 1989), hatch rate (Huang et al., 2003) and juvenile survival (Vouk and Sheehan, 1983). Hence, toxicity test on the survival of the juveniles are essential to identify the possible risk that toxicants pose on fish populations within their natural environment (Ankley et al., 2004).

Spermatology

For successful reproduction, viable gametes are required. Through spermatology the viability of sperm can be assessed. Reproductive dysfunction as a result of exposure to xenobiotic pollutants may be caused by either direct action on the gametes, or indirectly by modulation of the endocrine system so that gamete development takes place during an imbalance of the hormonal environment (Kime and Nash, 1999). Gamete viability has usually been assessed simply by determining fertilization rates, hatch rate or larval survival with eggs or sperm from exposed fish as the end point of standard toxicity tests. More recently however, there have been novel approaches used to assess the effects of potential endocrine disrupters on gamete viability (Kime and Nash, 1999). The quality and quantity of sperm produced by testis can be assessed through morphological and viability studies as well as computer assisted sperm analysis (CASA).

Gonadal histopathology

Macroscopic signs of toxicity are almost always preceded by changes at tissue, cellular or molecular levels (Segner and Braunbeck, 1990). According to Hinton and Laurén (1990) histopathological alterations are the overall result of unfavourable physiological and biochemical alterations in an organism. They may be used to foresee the effects of alterations on processes or activities such as predator avoidance, growth, reproduction, and population stability that occur at higher levels of biological organization (McKim, 1985; Meyers and Hendricks, 1985). Histopathological characteristics of specific organs express condition and represent time integrated endogenous and exogenous impacts on the organism stemming from alterations at lower levels of biological organization (Stebbing, 1985; Hinton and Laurén, 1990; Swee et al., 1996). For field assessment, histopathology provides a rapid method of detecting adverse acute and chronic effects of exposure in various tissues (Hinton et al., 1992). Histopathology does not solely depend upon qualitative light microscope observations at tissue level, as newer technologies allow cellular and molecular analyses (Hinton and Laurén, 1990; Wester et al., 2002). Histopathology alone has diagnostic strength. When combined with other disciplines such as analytical chemistry or xenobiotic physiology, histopathology may also reconstruct possible etiologies for the lesions found (Hinton and Laurén, 1990).

Gonadal staging

The synchronized process of spermatogenesis allows germ cells to advance (or change) within the seminiferous epithelium. In mammalian testes, the more mature cells are found away from the basement membrane and in specific associations with the younger that will divide and mature in time. Fish differ from mammals as spermatogenesis takes place in cysts within lobules. The cysts contain cells that are in different stages of development. The Stages Program used for mammalian tissue will not be ideal. The developmental stages of fish gonads will therefore be determined by identifying histological features as defined by the Biomonitoring of Environmental Status and Trends Program (BEST) (Schmidt and Dethloff, 2000).

2.6.2 Population and community effects of EDCs in fish and invertebrates

Populations and communities of fish and invertebrates may be influenced by EDCs through a variety of ways and varying degrees (Kerr and Vass, 1973). Direct influences are related to the lethal effect of EDCs of individuals within a population and community. According to Connell et al. (1999), when populations are exposed to high lethal concentrations they may respond by reducing or rising in abundance relative to their tolerance. A species which is more sensitive may be eliminated (show reduced abundance) before a more tolerant species is, which may increase in abundance in the absence of competitors. These alterations then in turn influence the entire community structure within an ecosystem. Many such effects have been shown in macro-invertebrates, fish populations and communities exposed to EDCs such as DDT (Kerr and Vass, 1973; Filteau, 1957), other organochlorines (Eaton and Lydy, 2000; Berenzen et al., 2005) and other pollutants such as metals and organophosphate pesticides (Lu, 2005; Ward et al., 1993; Phillips and Rainbow, 1994; Lawrence and Hemingway, 2003), just to name a few. Further reviews can be found in Johnson et al. (1993), Rolland (1997), and Peakall (1992).

In contrast to lethal concentrations, when organisms are exposed to low sub-lethal concentrations they may not necessarily eliminate a species directly, but may indirectly alter population structures and survival of species. That is, when an individual is exposed to sub-lethal concentrations of an EDC, endocrine disrupting mechanisms may occur that influences the reproductive output, the recruitment and/or growth that can lead to a reduction in the abundance of species within population. However according to the review by Mills and Chichester (2005) this approach should be done with care during field studies because even

though there is strong evidence that EDCs can affect the reproductive health of fish and macro-invertebrates mainly through endocrine disrupting effects (as shown previously), it is not to say that these impacts will influence the population level. For example, a study by Price and Depledge (1998) showed in polychaete worms exposed to an EDC (*p*-NP) showed that although there were endocrine induced changes in egg production and egg viability, there was no subsequent effects present on growth or sex ratios. Furthermore, very few studies linking the endocrine disruptive effects of individuals with consequences at population and community level have been done. According to Ford et al. (2007) the reason for this is firstly, not enough available data to create viable models for the population level effects of endocrine disruption. Secondly, reproductive disorders are mainly investigated in species that are difficult to model the populations. Then lastly the authors noted that a lack of population ecologists within this field and funding are further influencing factors. According to many, the best documented case within the aquatic ecosystem of endocrine disruption causing alterations in populations was in a study by Bryan and Gibbs (1991). The authors showed a causal decline in populations of gastropods exposed to tributyltin and associated with the imposex phenomenon. For a further review of the existing literature available on the potential of endocrine disruption to induce population level effects on both fish and macro-invertebrates refer to Taylor et al. (1999) and Taylor and Harrison (1999).

It has been found that some EDCs affect the motility and morphology of fish sperm in various ways. The hormonal environment or function of the Sertoli cells may be disrupted, the seminal fluid or sperm maturation may be changed, or there may be cytological damage to the sperm itself and altered efficiency of mitochondrial energy production (Kime, 2001). Environmental conditions during spawning activate the sperm, but not all factors involved are well understood. Attempts to change the environmental conditions, including water quality through addition of pollutants, may lead to a clearer understanding of these factors (Linhart et al., 1999).

There is an urgent need to know the environmental levels of DDT and what the precise effects will be on the affected systems and organisms. Fish were the selected study organism with regard to their reproductive capacity. Fish are constantly in contact with the water and are exposed at every level, from their body surfaces to their gills and through oral ingestion. They have a relatively long life span in comparison to other aquatic organisms. Thus, fish are expedient full-time monitors of aquatic ecosystems, as they integrate their responses through time and react to all synergistic and antagonistic effects of combined toxicants and stressors imposed on their environment (Hinton and Laurén, 1990; Van der Oost et al., 2003).

CHAPTER 3

ANALYSES OF WATER AND SEDIMENT

Bornman MS, Barnhoorn IEJ and Swemmer A

3.1 General water quality variables and other macro variables

The physico-chemical parameters were measured at all sites (1 to 7) during the sampling periods and included pH, temperature, oxygen (% and mg/L), conductivity and total dissolved salts (TDS). The macro variables were also measured at the three main sites during surveys three and four. These macro variables included the following parameters: chemical oxygen demand (COD), calcium (Ca) (mg/L), total hardness as $CaCO_3$ (calcium carbonate), total alkalinity as $CaCO_3$, ammonia as N, nitrate as N, nitrite as N, Kjeldahl nitrogen, chloride as Cl, sulphate as SO_4, total phosphate as P, fluoride as F and ortho-phosphate, all as mg/L.

3.2 Organic chemical analyses

For the purpose of this study, water and sediment samples from different localities in the Luvuvhu catchment were analysed for organochlorine pesticides (OCs), alkylphenols, phthalates (Surveys 3 and 4), and hormones in water samples only (Surveys 3 and 4). The list of OCs included: alpha-HCH, gamma-HCH (lindane), heptachlor, aldrin, dieldrin, beta-HCH, delta-HCH, 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. The alkylphenols included nonylphenol (*p*-NP) and octylphenol (OcP) while the phthalates included dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP) benzyl butyl phthalate (BBP), and di-(2-ethylhexyl) phthalate (DEHP) and the hormone analyses consisted of ethinylestradiol, estradiol, estrone and estriol.

The metal analyses are discussed separately in 3.6.

3.3 Materials and methods

3.3.1 Sampling and sampling sites

Water and sediment samples were collected at seven sites in the study area during Surveys 1 to 4. It should be noted that the area received very little rain during the so-called high flow season of 2007 (Survey 2).

Additional water samples were collected every second month at AD, ND and XW for DDT and metabolite analyses. Additional water samples were collected as a once-off sampling period from a thatched roof, a corrugated iron roof located in the DDT-sprayed villages and from run-off during a rainstorm at a lodge close to the AD. The sites where water and sediment were collected are listed below (GPS = Global Positioning System):

1	Lotanyanda tributary	GPS: S 22°58.366'; E 30°34.598'
2	Albasini Dam (AD)	GPS: S 23°05.974'; E 30°05.998', 758 m elevation.
3	Hasana	GPS: S 23°05.050'; E 30°28.204'
4	Nandoni Dam (ND)	GPS: S 22°88.945'; E 30°35.745', 490 m elevation.
5	Tshikonelo	GPS: 22°50.383'; E 30°44.294'
6	Xikundu Weir (XW)	GPS: S 22°48.506'; E 30°47.924', 450 m elevation.
7	Mhinga	GPS: 22°45.414'; E 30°53.412'

3.3.2 Water collection and extraction

Water samples were collected in 1 L glass bottles pre-washed with chromatography grade ethanol. Water samples were collected as deep as possible below the surface. Samples were stored at 4°C until analyses. No pH adjustment was done as all the pH readings in the laboratories were between 6 and 8.

The extraction method used is based on the method described by Cacho et al. (1995) where 1 L of the water sample was filtered into an acid washed reagent bottle. C_{18} solid phase cartridges (500 mg)/^{3}mL C_{18} cartridges (Waters-Microsep) were conditioned with water, methanol and water. The samples were then allowed to pass through the cartridge. Thereafter the cartridge was dried and eluted with 12 mL of hexane-diethylether (85:15, v/v). The samples were evaporated under nitrogen to dryness and reconstituted in 200 µL methanol, before 1 µL was injected. The OCs were analyzed and quantified by gas chromatography-mass spectrometry (GC-MS). A Hewlett Packard (HP7890) Gas

Chromatography system equipped with a HP 7683 auto injector and HP5975 mass selective detector (MSD), (Agilent Technologies, Palo Alto, CA, USA) was used for chromatographic separation and recording of mass spectra.

3.3.3 Sediment collection and extraction

Sediment samples were collected on the same day at approximately 1 m below the water surface into pre-washed clean wide mouth glass flasks. The sampling bottles were sealed and stored at 4°C in the laboratory, prior to sample preparation for chemical analyses.

The extraction method is based on a method published by Naudé et al. (1998). All sediment samples were dried at 110°C in an oven. 5 g samples were weighed, 100 mL Hexane-petroleum ether (50:50, v/v) added and extracted via reflux for a period of 12 hours. The samples were allowed to cool and evaporate to dryness under vacuum at 35°C. SPE clean-up was performed on C_{18} cartridges as mentioned under the section for water samples.

3.3.4 Analyses of water and sediment

3.3.4.1 Organochlorine pesticides

The OC residues were analyzed by a GC-MS. The column temperature was increased from 90 to 200°C at a rate of 40°C/min. The temperature of injector and detector was 250 and 200°C, respectively. High pure helium was used as carrier gas at a flow rate of 0.84 mL/min. Samples were injected under splitless injection mode (Villaverde et al., 2008; Bordet et al., 2002). The confirmation of the OCs was done using selected ion monitoring (SIM). The mass spectra were collected in the electron impact mode at 70 eV and the mass-to-charge ratios (*m/z*) of the ions were used for quantification in SIM mode.

3.3.4.2 Alkylphenols

The alkylphenols were analyzed by a GC-MS (Agilent 7890 A) equipped with a 5975C mass spectrometer and an Equity 1701 fused silica capillary column (15 m length × 0.1 mm i.d. × 0.1 µm film thickness, Supelco) according to a method by Croce et al. (2003). Data processing was done by the Agilent MSD ChemStation E.01.00.232 software. The mass spectrometer was operated in selected ion monitoring mode (SIM) with pulse splitless

injection mode (680kPa). The phenols were analysed as the pentafluoropropionic anhydride (PFPA) derivatives (Croce et al., 2003).

3.3.4.3 Phthalates

The phthalates were analyzed by a GC-MS (Agilent 7890 A) equipped with a 5975C mass spectrometer and an Equity 1701 fused silica capillary column (15 m length × 0.1 mm i.d. × 0.1 µm film thickness, Supelco). Data processing was done with Agilent MSD ChemStation E.01.00.232 software. The mass spectrometer was operated in electron impact (EI) mode with SIM detection. Pulse splitless injection mode (680 kPa) was used to enhance the detection of the phthalates (Petrovicè et al., 2001; Petrovicè et al., 2002).

3.3.4.4 Hormone analysed in water

The hormones were analysed with a Shimadzu LC 20 consisting of a binary pump, vacuum degasser, autosampler, column heater and mobile phase switching valve. An Applied Biosystems / MDX Sciex 4000 Q-Trap Tandem Mass Spectrometer was used for the detection system. Data processing was done by Analyst 1.4.2 Software. A Synergi Fusion C_{18} HPLC-column: 2.0 x 150 mm, 4 µm particle size, was used to separate the individual compounds. A gradient elution of water and acetonitrile was used to elute the hormone from the column. The mass spectrometer was operated in the multiple reaction monitoring mode (MRM). Two MRM transitions were used for identification purposes and one MRM per hormone for quantification (Diaz-Cruz et al., 2003; Isobe et al., 2003).

3.4 Results

3.4.1 Water

The general water quality parameter measurements and the macro variable results are shown in Table 3.1 and 3.2 respectively.

Most of the general water quality variables (Table 3.1) were in the range recommended by DWAF (1996 a,b,c,d,e,f,g,h). The pH during Survey 1 was higher at AD (9.72) and ND (10.2). During Survey 3 the conductivity was much higher at AD (825 µS/m) and at ND (424 µS/m)

while the TDS at both sites were also higher at AD (413 mg/L) and ND (214 mg/L)(Table 3.1).

All the macro variables (Table 3.2) measured over the 4 surveys were within the guidelines recommended for drinking water by the Federal-Provincial-Territorial Committee on Drinking Water (2008), WHO (2006) and the Department of Water Affairs and Forestry (DWAF, 1996a) of South Africa as well as SANS 241 (2006).

Table 3.3 summarises the OC residues that were detected during the study and shows that residues were only detected in the additional sampling taken in between the surveys. In February 2007, 0.27 µg/L *o,p'*-DDT was present in the run-off water from an old thatched roof house in Tshikudini, a village where DDT spraying occurs. 0.10 µg/L *p,p*-DDT was present in surface run-off water close at the Lodge close to AD.

In Survey 1, the ND had 0.12 µg/L *p,p*-DDT, and in Survey 2, ND water contained 0.3 µg/L *p,p'*-DDE and XW 0.4 µg/L *p,p*-DDE respectively. During Survey 3 AD contained *p,p*-DDT, *p,p*-DDD and *p,p*-DDE and the ΣDDT was 1.75 µg/L. All the sites with the exception of ND contained detectable *p,p*-DDE residues in Survey 4 and at XW the ΣDDT was 1.8 µg/L. 2.8 µg/L PCB153 was present at Hasana in Survey 4 and dieldrin residues were present at levels of 1.6 µg/L at AD, 2.4 µg/L at ND, 3.5 µg/L at XW and 1.9 µg/L at Mhinga. No nonyl- or octylphenol residues, ethinyl estradiol, estradiol, estrone or estriol or any of the phthalates were found in the water.

3.4.2 Sediment

No DDT residues were detected in any of the samples taken during Surveys 1 and 2. One sample had DDT in Survey 3, and *p,p*'-DDT and *p,p'*-DDE residues were found at ND, Tshikonelo and XW (Table 3.4).

During Survey 3, *p*-NP was present ranging from 29-851 µg/kg at sites downstream from AD. *p*-NP was present at all sites ranging between 52.7-309 µg/kg (Table 3.4).

No OcP residues or any of the phthalates (DMP, DEP, DBP, BBP, DEHP and DOP) were detected.

Table 3.1: The general water quality parameters measured at the three main sites during the four surveys.

Locality	Albasini Dam				Nandoni Dam				Xikundu Weir			
Parameter	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4
Temperature (°C)	20.9	24	24.7	28	25.8	27	22.4	29.5	24.8	28.2	18	28.9
pH	**9.72**	7.62	8.48	8.61	**10.2**	7.41	8.93	8.28	8.54	7.65	7.3	6.96
Conductivity (µS/m)	260	299	**825**	305	141.4	164.4	**424**	150.5	178.1	142	182.3	139.2
Total dissolved solids (TDS) (ppm)	126	149	**413**	153	71.1	82.2	**214**	75.2	89.1	72.1	n/a	69.5
Oxygen (mg/L)	5.53	5.4	8.74	10	5.31	6.09	9.81	10.38	5.58	6.1	12	9.27
Oxygen (%)	103.3	64.4	114.9	127.3	103.3	79.3	113.7	136.1	112.3	85	135.2	123.3

Table 3.2: The macro variables measured at the three main sites during four surveys.

Locality	Albasini Dam				Nandoni Dam				Xikundu Weir			
Parameter	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4
Chemical oxygen demand (COD)	bd	bd	n/a	n/a	bd	8.6	n/a	n/a	bd	bd	n/a	n/a
Calcium	1	18	n/a	n/a	bd	8	n/a	n/a	3	6	n/a	n/a
Total Hardness as $CaCO_3$ (mg/L)	n/a	n/a	152	152	n/a	n/a	124	76	n/a	n/a	100	64
Total Alkalinity as $CaCO_3$ (mg/L)	n/a	n/a	124	128	n/a	n/a	68	64	n/a	n/a	72	60
Ammonia as N (mg/L)	0.03	bd	<0.2	<0.2	0.03	bd	0.2	<0.2	0.1	0.02	<0.2	<0.2
Nitrate as N (mg/L)	2.5	bd	<0.2	4.6	bd	3.1	<0.2	1.9	0.3	0.7	0.2	0.7
Nitrite as N (mg/L)	0.01	0.02	<0.1	<0.1	0.01	bd	<0.1	<0.1	0.02	bd	<0.1	<0.1
Kjeldahl Nitrogen (mg/L)	n/a	n/a	<0.2	6.7	n/a	n/a	1.1	2.8	n/a	n/a	2.8	20
Chloride as Cl (mg/L)	n/a	n/a	19	22	n/a	n/a	15	13	n/a	n/a	11	10
Sulphate as SO_4 (mg/L)	bd	bd	<5	<5	7	bd	<5	<5	2	bd	<5	<5
Total Phosphate as P (mg/L)	n/a	n/a	<0.2	<0.2	n/a	n/a	<0.2	<0.2	n/a	n/a	0.2	<0.2
Fluoride as F (mg/l)	n/a	n/a	0.2	0.2	n/a	n/a	<0.2	<0.2	n/a	n/a	<0.2	<0.2
Ortho-phosphate (mg/L)	0.01	0.01	n/a	n/a	0.02	0.03	n/a	n/a	0.02	0.01	n/a	n/a

Table 3.3: The detected OC levels in the water samples collected from all the sites (µg/L).

Sample Feb'07	*o,p'*-DDE	*p,p'*-DDE	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDT	*p,p'*-DDT	ΣDDT	PCB 153	Dieldrin
Lodge at AD run-off	*	*	*	*	*	**0.10**		*	*
Thatch roof Tshikudini	*	*	*	*	**0.27**	*		*	*
Corrugated iron roof Tshikudini	*	*	*	*	*	0.12		*	*
Survey 1									
Albasini Dam	*	*	*	*	*	*		*	*
Nandoni Dam	*	*	*	*	*	**0.12**		*	*
Xikundu Weir	*	*	*	*	*	*		*	*
Survey 2									
Albasini Dam	*	*	*	*	*	*		*	*
Nandoni Dam	*	**0.3**	*	*	*	*		*	*
Xikundu Weir	*	**0.4**	*	*	*	*		*	*
Survey 3									
Albasini Dam	*	0.95	*	0.50	*	0.30	1.75	*	*
Nandoni Dam	*	0.11	*	*	*	*		*	*
Xikundu Weir	*	*	*	*	*	*		*	*
Survey 4									
Lotanyanda	**	**0.8**	**	**	**	**		**	**
Albasini Dam	**	**0.6**	**	**	**	**		**	**1.6**
Hasana	**	**1.0**	**	**	**	**		**2.8**	**
Nandoni Dam	**	**	**	**	**	**		**	**2.4**
Tshikonelo	**	**0.5**	**	**	**	**		**	**
Xikundu Weir	**1.2**	**0.6**	**	**	**	**	1.8	**	**3.5**
Mhinga	**	**0.6**	**	**	**	**		**	**1.9**

* = below detection limit of 0.1 µg/L (ARC lab)

** = below detection limit of 0.5 µg/L (FDA labs)

Table 3.4: The detected OC and phenol levels in the sediment samples collected from all the sites (µg/kg, dry mass).

Sediment Survey 3	*o,p'*-DDT	*p,p'*-DDT	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE	PCB 153	*p*-NP	OcP
Lotanyanda	*	0.1	*	0.1	*	*	*	29	**
Albasini Dam	*	*	*	*	*	*	*	**	**
Hasana	*	*	*	*	0.2	*	*	851	**
Nandoni Dam	*	*	*	*	*	*	0.59	29	**
Tshikonelo	*	*	*	*	*	*	*	225	**
Xikundu Weir	*	*	*	*	*	*	0.53	93	**
Mhinga	*	*	*	*	*	*	*	n/a	n/a
Survey 4									
Lotanyanda	***	***	***	***	***	***	***	52.7	**
Albasini Dam	***	***	***	***	***	***	***	309.5	**
Hasana	***	***	***	***	***	***	***	161.5	**
Nandoni Dam	***	1.4	***	***	***	3.7	***	207.5	**
Tshikonelo	***	1.8	***	***	***	3.8	***	69.3	**
Xikundu Weir	***	4.1	***	***	1.2	12.8	***	165	**
Mhinga	***	***	***	***	***	***	***	121.7	**

* = detection limit 01 µg/kg

** = detection limit 25 µg/kg

*** = detection limit = 0.5 µg/kg

n/a = not available

3.5 Discussion

The presence of *p,p'*-DDT and -DDE in water from ND was unexpected considering the high dilution factor of the newly constructed dam. Furthermore, the occurrence could have been missed as water is not necessarily the ideal matrix for *p,p'*-DDT and -DDE determinations. The higher levels downstream from ND at Tshikonelo and XW were anticipated, as these sites are located within the area where indoor DDT-spraying occurs. The ND is a newly constructed dam with a big holding capacity of 164 million m^3 (DWAF, 2005) and the sediment was not quite settled yet, therefore finding detectable *p,p*'-DDT and *p,p*'-DDE levels are quite significant. The water and sediment in Survey 4 probably reflects the only true 'high flow' sampling as there was little rain during the first 18 months of the study. The higher levels at Tshikonelo and the highest level at XW were expected as these sites were further downstream in the currently DDT-sprayed area. The ΣDDT levels at XW were above the WHO limit of 1 µg/L in drinking water. The dieldrin levels at both ND and XW were above the 2.0 µg/L EPA limit (EPA guidelines, 1986) for drinking water and could pose a problem to people collecting for drinking purposes. This will be further discussed in Chapter 14: Health Risk Assessment.

Aldrin and dieldrin are insecticides with similar chemical structures and aldrin quickly breaks down to dieldrin in the body and environment. These chemicals were used on corn and cotton, but the EPA banned the use of aldrin and dieldrin 1987. In South Africa, dieldrin was banned in 1983, and aldrin in 1993 (http://www.gov.za/). Both chemicals bioaccumulate in fat and decreased fertility was reported in some studies, as well as 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) (ATSDR, 2002).

Based on various *in vitro* and *in vivo* models, PCBs have been reported to have estrogenic and anti-estrogenic effects (Plíšková et al., 2005). Low-molecular weight PCBs appear to be estrogenic, while high-molecular weight PCB congeners are considered to be anti-estrogenic. The anti-estrogenic effects of three prevalent congeners (PCBS 138, 153 and 180) have been found both in a reporter gene and cell proliferation MCF-7 assays and ER-CALUX assay (Bonefeld-Jorgensen et al., 2001; Plíšková et al., 2005). In this study, the levels of PBC 153 (2.8 µg/L in water; 0.59 µg/g in sediment) were below the environmentally relevant concentration of 8 µg/ml (Gregoraszscuk et al., 2008). Despite the low concentration

of PCB 153 in this study, it is important to note that Radice et al. (2008) found that a mixture of PCBs including PCB 153 induces an anti-proliferative effect, ascribable to an apoptotic action.

p-NP was the major chemical in sediment at all the sites. In Surveys 3 and 4 levels in sediment were between 29 and 851 (155.3 ± 87.26) µg/kg. *p*-NP belongs to a group of alkylphenol polyethoxylates. These surfactants are used in detergents, paints, herbicides, pesticides and cosmetics, are relatively persistent, bioaccumulate in the lipids of living organisms and consequently have a more persistent effect than natural estrogens (Tapiero et al., 2002). *p*-NP a known estrogen, has adverse effects on the testis and epididymis of rodents in reproductive toxicology studies (Lee et al., 1999; De Jager et al., 1999a,b). The negative impact is further enhanced when animals are exposed to a relevant mixture of chemicals containing deltamethrin, DDT, phytoestrogens and *p*-NP (Kilian et al., 2005). The levels of *p*-NP were higher than the levels in a previous study at Rietvlei Dam (113 µg/kg) and Marais Dam (4.0 µg/kg) (Bornman et al., 2007). These dams are in an industrial area, whereas most of the sites in this study are in a rural area. Although *p*-NP is generally considered an industrial chemical, significant levels were reported in effluents from municipal sewage treatment plants (Sekela et al., 1999).

Diet seems to be the major source of human APEs intake and recently Ademollo et al. (2008) reported the presence of *p*-NP in breast milk samples from mothers in Italy. The concentrations in breast milk were close to the Tolerable Daily Intake (TDI) of 5 µg/kg body weight (bw) proposed by the Danish Institute of Safety and Toxicology. Dorea (2009) pointed out that breast milk, cow's milk and formula feeding may be contaminated with *p*-NP and thus expose nursing infants. Not only fish and water may be dietary sources, but *p*-NP may also come from animals raised on fishmeal or recycled animal products. In an area where breast milk will be inadvertently contaminated with DDT, it now seems that further studies including also industrial chemicals are warranted in the rural Limpopo area.

3.6 Metals

3.6.1 Materials and methods

Water and sediment were collected in 250 mL plastic containers as deep as possible below the surface at all the localities. The containers for water collection were pre-treated with an acid-based fixative to ensure preservation of the water sample. The collected samples were kept at 4°C until analyses. Metal levels were measured using Semi Quantitative ICP-MS by

Waterlab (PTY) Ltd. Although cadmium (Cd), arsenic (As), lead (Pb) and mercury (Hg) have been identified as the four endocrine disrupting metals (EDMs), the samples were also analysed for 66 other elements.

3.6.2 Results

The values for the elements in water from the different localities that were above the detection limit of 0.01 mg/L are shown in Table 3.5. All the levels found in water samples were below guideline values set by the Department of Water Affairs and Forestry (1996h).

The sediment collected at all the sites during the four sampling surveys had high levels of aluminium (Al) and manganese (Mn), while vanadium (V), silica (Si) and zinc (Zn) were also above the detection limit of 0.01 mg/kg (Table 3.6). The target water quality range (TWQR) and criteria for acid-soluble aluminium in aquatic ecosystems are < 5 mg/kg (pH < 6.5) and < 10 mg/kg (pH > 6.5), although in sediment the values (Table 3.6) were much higher than the recommended levels (DWAF, 1996a). The TWQR and criteria for dissolved Mn in aquatic ecosystems are < 180 µg/L in water, although in sediment the levels detected were much higher (Table 3.6). Although V was detected in all the sediment samples, most had levels below the TWQR of 0.1 mg/L recommended for human consumption (DWAF, 1996h). The TWQR and criteria for dissolved zinc in aquatic ecosystems are < 0.002 mg/L in water and in sediment samples levels were above 0.002 mg/L but below 1 mg/kg (Table 3.6).

Table 3.5: The concentrations of metals present in water samples above the detection limit of 0.001 mg/L.

Survey 1	Ba	Ca	K	Mg	Na	Si	Sr
Site 1	0.00	<0.01	0.42	3.04	3.09	2.69	0.01
Albasini Dam	0.02	1.35	1.07	7.30	4.62	2.34	0.05
Hasana	0.03	<0.01	0.17	3.56	3.81	2.69	0.05
Nandoni Dam	0.05	0.58	1.24	4.80	5.30	2.45	0.06
Tshikonela	0.02	<0.01	0.18	5.88	5.32	2.54	0.03
Xikundu Weir	0.02	0.84	0.39	3.61	4.17	2.93	0.04
Mhinga	0.02	0.83	0.58	7.15	8.25	2.44	0.04

Survey 2	Ba	Ca	K	Mg	Na	Si	Sr
Lotanyanda	0.01	<0.01	0.42	3.48	4.10	2.87	0.02
Albasini Dam	0.03	7.85	1.36	13.04	9.19	2.13	0.09
Hasana	0.02	<0.01	0.51	2.17	2.30	3.39	0.04
Nandoni Dam	0.06	7.25	1.51	4.02	4.19	2.20	0.09
Tshikonela	0.02	1.40	0.47	5.70	6.24	1.61	0.04
Xikundu Weir	0.02	1.32	0.68	5.74	6.51	2.12	0.04
Mhinga	0.05	12.3	0.40	4.04	3.97	9.21	0.08

Survey 3	Al	B	Ba	Ca	Cu	Fe	K	Mg	Mn	Na	Si	Sr	Zn
Lotanyanda	0.036	0.005	0.079	4.35	0.001	0.276	0.518	2.10	0.064	4.80	3.95	0.052	0.001
Albasini Dam	0.078	0.011	0.219	20.5	0.002	0.049	1.16	10.0	0.021	9.59	1.24	0.166	0.002
Hasana	0.044	0.004	0.180	6.97	0.001	0.284	0.702	3.95	0.051	6.30	3.25	0.111	0.002
Nandoni Dam	0.010	0.006	0.295	8.67	0.001	0.021	1.48	3.79	0.033	5.82	1.74	0.152	0.004
Tshikonela	0.041	0.006	0.195	9.11	0.001	0.197	0.861	4.14	0.035	6.22	2.44	0.109	0.003
Xikundu Weir	0.053	0.006	0.193	9.37	0.001	0.214	0.906	4.21	0.130	6.31	2.43	0.109	0.002
Mhinga	0.063	0.007	0.186	9.99	0.002	0.190	1.01	4.41	0.031	6.37	2.73	0.106	0.002

Survey 4	Al	B	Ba	Ca	Fe	K	Mg	Mn	Na	P	Si	Sr
Lotanyanda	0.055	<0.01	0.015	3.76	0.757	0.696	2.26	0.097	2.64	0.072	2.22	0.015
Albasini Dam	0.016	0.023	0.075	22.2	0.032	2.21	16.2	0.037	14.0	<0.01	1.25	0.142
Hasana	<0.01	<0.01	0.045	8.09	0.376	1.24	6.89	0.082	8.13	0.127	2.61	0.074
Nandoni Dam	<0.01	<0.01	0.077	10.4	<0.01	2.32	6.92	0.023	7.68	0.082	1.39	0.106
Tshikonela	0.045	<0.01	0.050	9.31	0.435	1.57	6.69	0.074	7.51	0.123	1.80	0.063
Xikundu Weir	0.034	<0.01	0.052	10.2	0.441	1.63	7.14	0.077	7.64	0.123	2.02	0.069
Mhinga	0.022	<0.01	0.044	7.97	0.250	1.04	4.51	0.061	5.24	0.068	1.46	0.055

Table 3.6: The metal concentrations present in sediment samples above the detection limit of 0.001 mg/kg, dry mass.

Survey 1	Al	Co	Cr	Cu	Fe	Mn	Si	V	Zn
Site 1	11.23	0.72	0.02	0.02	117.35	50.02	18.34	0.31	0.41
Albasini Dam	17.61	1.00	0.09	0.11	12.68	8.73	16.40	0.16	0.11
Hasana	5.07	0.14	0.04	0.06	14.01	5.04	5.12	0.05	0.14
Nandoni Dam	6.72	0.08	0.02	0.03	22.23	4.08	6.35	0.09	0.23
Tshikonela	12.45	0.48	0.04	0.13	59.84	24.35	20.55	0.27	0.17
Xikundu Weir	7.89	1.11	0.02	0.20	19.93	37.65	21.56	0.52	0.23
Mhinga	8.06	0.45	0.04	0.16	50.59	7.78	11.95	0.21	0.19
Survey 2	**Al**	**Co**	**Cr**	**Cu**	**Fe**	**Mn**	**Si**	**V**	**Zn**
Lotanyanda	11.03	0.39	0.02	0.13	23.02	18.66	6.87	0.12	0.14
Albasini Dam	12.56	0.45	0.04	0.06	41.41	17.76	22.17	0.22	0.26
Hasana	9.71	0.52	0.03	0.16	37.12	25.74	10.07	0.19	0.16
Nandoni Dam	4.35	0.15	0.02	0.05	8.91	7.33	3.68	0.04	0.15
Tshikonela	11.25	0.45	0.02	0.16	20.61	19.58	6.19	0.14	0.16
Xikundu Weir	5.43	0.21	0.03	0.10	18.96	9.60	5.64	0.10	0.21
Mhinga	4.35	0.15	0.02	0.05	8.91	7.33	3.68	0.04	0.15
Survey 3	**Al**	**Ba**	**Ca**	**Fe**	**Mn**	**Si**	**V**	**Zn**	
Lotanyanda	4.80	1.38	38.1	4.43	22.5	0.397	0.021	0.828	
Albasini Dam	9.42	3.25	67.2	3.18	5.58	4.50	0.119	0.682	
Hasana	3.29	0.953	29.1	3.15	2.69	0.128	0.018	0.261	
Nandoni Dam	7.70	5.40	72.2	0.186	7.88	5.12	0.286	0.290	
Tshikonela	5.64	1.69	31.2	5.16	8.44	1.64	0.086	0.527	
Xikundu Weir	5.22	5.70	111	0.87	23.5	5.90	0.375	0.351	
Mhinga	5.65	4.37	71.5	6.41	19.0	2.49	0.158	1.08	
Survey 4	**Al**	**Ba**	**Ca**	**Fe**	**Mn**	**Si**	**V**	**Zn**	
Lotanyanda	21.1	3.07	41.5	15.8	11.0	3.32	0.014	0.177	
Albasini Dam	4.99	4.25	161	22.9	9.86	10.3	0.064	0.119	
Hasana	6.97	3.30	58.0	14.8	12.7	3.29	0.024	0.209	
Nandoni Dam	5.45	8.27	223	46.2	7.51	15.8	0.021	0.145	
Tshikonela	13.8	5.55	102	17.0	26.8	7.01	0.017	0.201	
Xikundu Weir	2.94	4.59	213	42.9	18.4	8.89	0.056	0.067	
Mhinga	12.0	3.96	107	11.1	16.8	5.35	0.040	0.852	

3.6.3 Discussion

None of the so-called endocrine disrupting metals cadmium, arsenic, lead and mercury was present in the water and sediment samples collected. In water, Al, copper (Cu), iron (Fe), Mn, Si, strontium (Sr) and Zn were detected but at low concentrations and may therefore not be harmful to humans and aquatic biota.

In sediment, Al, Mn, and Si were present in concentrations higher than the allowed concentrations in water advised by DWAF (1996). A number of studies have been conducted on the effects of metals such as Al and Mn on freshwater fish and other freshwater biota. Most metals occur naturally in the environment and are thus omnipresent. It is known that metals in sediment may involve a threat for human/aquatic health when they seep from the sediment into the surrounding water. The release of these metals from sediments is influenced or controlled by parameters such as pH, redox potential and salinity (Cappuyns et al., 2004). A low availability of metals as a result of flooding can also stabilise chemical conditions and cause a decrease in ecological risk (Van Der Geest and Paumen, 2008).

3.7 Conclusions

In water samples from Survey 1 *p,p*-DDT was found at ND (0.12 µg/L) and no other OC residues were found. During the next Survey the following levels at ND and XW: 0.3 and 0.4 µg/L *p,p*-DDE respectively and no other OCs were found. During Survey 3, AD and ND had detectable levels of *p,p*-DDE, and ND levels of *p,p'*-DDD and *p,p'*-DDT at AD. In Survey 4 all the sites with the exception of ND contained *p,p*-DDE residues. At AD during Survey 3 and at XW in Survey 4 the ΣDDT were 1.75 and 1.8 µg/L respectively, which is higher than the WHO limit of 1 µg/L in drinking water. The presence of *p,p'*-DDT and –DDE in water from ND was unexpected considering the high dilution factor of the newly constructed dam. Furthermore, the occurrence could have been missed as water is not necessarily the ideal matrix for *p,p'*-DDT and –DDE determinations. The higher levels downstream from ND were anticipated, as these sites are located within the area where indoor DDT-spraying occurs.

In Survey 4 PCB153 was present at 2.8 µg/L at Hasana, and dieldrin at AD (1.6 µg/L), ND (1.1 µg/L), XW (3.5 µg/L) as well as at Mhinga (1.9 µg/L). The EPA limit (EPA guidelines, 1986) for drinking water of dieldrin is 2.0 µg/L and the water at both ND and XW could pose a problem to people collecting for drinking purposes. No nonyl- or octylphenol residues,

ethinyl-estradiol, estradiol, estrone or estriol or any of the phthalates were found in any water samples.

In the sediment samples collected during Surveys 1 to 3 no DDT residues were detected. In Survey 4 ND contained 1.4 µg/kg, Tshikonelo 1.8 µg/kg and XW 4.3 µg/kg *p,p*'-DDT and at the same sites 3.7, 3.8, 12.8 µg/kg *p,p*'-DDE respectively were found. No octylphenol residues, any of the phthalates (DMP, DEP, DBP, BBP, DEHP and DOP) or any of the hormones were detected. During Survey 3, *p*-NP was present ranging from 29 - 851 µg/kg at sites downstream from AD and during Survey 4 at all sites ranging between 52.7 – 309 µg/kg. *p*-NP, a known estrogenic, was the most prevalent chemical and in the highest concentrations in sediment of all the sites.

CHAPTER 4

EDC ACTIVITY IN WATER SAMPLES USING A BATTERY OF BIO-ASSAYS

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4.1 Introduction and objectives

In-vitro bio-assays have been widely used to determine if chemicals have endocrine disrupting activity. Many of these assays however have limited usefulness and are not freely available as a scientific tool (Wilson et al., 2004). Only by using a suite of bio-assays in this way will it be possible to minimize the chances of wrongly labelling chemicals as endocrine disruptors (Beresford et al., 2000).

4.1.1 The recombinant yeast estrogen screen (YES)

A recombinant yeast strain was developed in the Genetics Department at Glaxo for use in a test to identify compounds that can interact with the human estrogen receptor (ER)-α. 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 YES was performed according to the method described by Routledge and Sumpter (1996) with minor adjustments (Aneck-Hahn 2003; Aneck-Hahn et al., 2005; 2008; Bornman et al., 2007).

4.1.2 The T47D-KBluc reporter gene assay

The US EPA developed an estrogen-dependent stable cell line. The T47D human breast cancer cells, which contain both endogenous ERα and ERß, were transfected with an estrogen-responsive element (ERE) luciferase reporter gene construct. This provides an *in vitro* system that can be used to evaluate the ability of chemicals to modulate the activity of estrogen-dependent gene transcription. The 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 anti-estrogenic 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 (Wilson et al., 2004).

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. When testing chemicals using the T47D-KBluc cells, an estrogen is defined as a chemical that induces dose dependent luciferase activity, which could be specifically inhibited by the anti-estrogen ICI. Agonists stimulate luciferase expression and are compared to the vehicle control (media plus ethanol) or to the relative response of their respective E_2 control. Anti-estrogens block the E_2-induced luciferase expression, which is compared to the E_2 control (Wilson et al., 2004).

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

4.1.3 The MDA-kb2 reporter gene 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. They subsequently 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 receptor (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, eliminates the need for transfection, can be conducted in 96 well plates, and consistent results are produced. These make it an ideal assay for screening chemicals. Information on its use as an environmental screen is limited and this project was used to assess its usefulness as a screen on environmental water samples.

4.2 Experimental procedures

4.2.1 Sample collection and storage

Water samples were collected in 1 L glass Schott Suprax bottles (Cat. No. 21 802 54 56) pre-washed with ethanol (Cat. No. 27, 0741, Sigma-Aldrich). The samples were kept at 4°C until extracted.

4.2.2 Extraction procedure

For extraction and enrichment of potential estrogen-like compounds a solid phase extraction (SPE) was performed according to the protocol described in Bornman et al. (2007), without adjusting the pH to 3. The extracted samples were stored in sterile amber glass bottles (Cat. No.154515, Chromatography research supplies, 4 mL) at -20°C prior to analysis (Aneck-Hahn, 2003; Aneck-Hahn et al., 2008; Bornman et al., 2007).

4.3 Sample processing

4.3.1 YES assay

The YES assay was performed according to the assay procedure (Routledge and Sumpter, 1996) and analysis method described in Aneck-Hahn et al. (2008) and Bornman et al. (2007).

4.3.1.1 Maintenance of cell culture

The stock cultures and growth medium were prepared using medium components described in Routledge and Sumpter (1996). Short term stock cultures were used to prepare the assay medium for the experimental procedure (Aneck-Hahn, 2003; Routledge and Sumpter, 1996). The growth medium was inoculated with 125 µL of the 10 x concentrated yeast stock and incubated at 28°C in a rotating water bath at 150-155 µpm until turbid (Aneck-Hahn, 2003; Aneck-Hahn et al., 2005; 2008; Bornman et al., 2007).

4.3.1.2 Experimental procedure

Serial dilutions were made of the water sample extracts and controls, in 96 well microtiter plates (Cat. No. 95029780, Labsystems). 100 µL of the solvent, ethanol (Cat. No. 27, 0741,

Sigma-Aldrich) was placed in wells 2-12 on the plate. 200 µL of the sample extract was placed into the first well and this was serially diluted (100 µL) across the plate, using a multichannel pipette. 10 µL 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 (200 µL) of the assay medium containing the yeast and chromogenic substrate (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 1 x 10^{-8}M to 4.8 x 10^{-12}M (2.274 µg/l to 1.3 ng/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 5 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 540 nm for colour change and 620 nm 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 (540 nm) – test abs (620 nm) – median blank abs (620 nm)]

The 17 β-estradiol standard curve was fitted (sigmoidal 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 (EEq) of the water samples were interpolated from the estradiol standard curve and corrected with the appropriate dilution factor for each sample on day 4.

4.3.2 The T47D-KBluc reporter gene assay

The extracts were processed according to the chemical protocol described in Wilson et al. (2004).

4.3.2.1 Maintenance of cell cultures

T47D-KBluc cells were maintained in RPMI growth media (Cat. No. R8755, Sigma) supplemented with 2.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/L $NaHCO_3$, 10% fetal bovine serum (FBS) (characterized, Cat. No. SH30071.03, Hyclone, Separations, SA), 100 µg/mL penicillin, 100 U/mL streptomycin and 0.25 µg/ml amphotericin B (Cat. No. 15240-062, Gibco, Scientific Group, SA). One week prior to the assay, cells were placed in growth media modified by replacement of 10% FBS with 10% dextran-charcoal treated FBS (Cat. No. SH30068.03, Hyclone, Separations, SA) excluding antibiotic supplements (Wilson et al., 2004).

4.3.2.2 Experimental procedure

Cells were seeded at 5 x 10^4 cells per well in 96 – well luminometer plates and allowed to attach overnight. Dosing dilutions were prepared in growth media containing 5% dextran-charcoal treated FBS and vehicle (ethanol) did not exceed 0.2%. Each plate contained agonist positive control (E_2), negative control (vehicle only), antagonist control (E_2 plus ICI) and background control (vehicle plus ICI). Each sample was tested alone as well as in the presence of 0.1 nM E_2 or ICI. Cells were incubated 24h with 100 µL/well dosing solution at 37°C, 5% CO_2.

After the incubation period, cells were washed with phosphate buffered saline at room temperature and lysed with 25 µL lysis buffer (Cat. No. E3971, Promega, Whitehead Scientific, SA). Luciferase activity was determined using a microtiter plate luminometer and quantified as relative light units. Each well received 25 µL lysis buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/mL BSA, pH 7.8), followed by 25 µL 1 mM D-luciferin 5s later. Relative light units were converted to a fold induction above the vehicle control value.

The 17 β-estradiol standard curve was fitted (sigmoidal function, variable slope) using Graphpad Prism (version 2.01), which calculated the minimum, maximum, slope, EC_{50} value and 95% confidence limits. The estradiol equivalents (EEq) of extracts with greater than a twofold induction above the vehicle control were interpolated from the estradiol standard curve and corrected with the appropriate dilution factor for each sample.

4.3.3 The MDA-kb2 reporter gene assay

The extracts were processed according to the chemical protocol described in Wilson et al. (2002).

4.3.3.1 Maintenance of cell cultures

MDA-kb2 cells were maintained in Lebovitz's L – 15 growth media (Cat. No. 41300-021, Gibco, Scientific Group, SA) supplemented with 10% FBS (characterized, Cat. No. SH30071.03, Hyclone, Separations, SA), 100 µg/mL penicillin, 100 U/mL streptomycin and 0.25 µg/mL amphotericin B (Cat. No. 15240-062, Gibco, Scientific Group, SA).

4.3.3.2 Experimental procedure

Cells were seeded at 5 x 10^4 cells per well in 96 – well luminometer plates and allowed to attach overnight. Dosing solutions were prepared in growth media and vehicle (ethanol) did not exceed 0.2%. Each plate contained agonist positive control (dihydrotestosterone, DHT), negative control (vehicle only), antagonist control (DHT plus flutamide) and background control (vehicle plus flutamide). Each sample was tested alone as well as in the presence of 0.1 nM DHT or flutamide. Cells were incubated 24 h with 100 µL/well dosing solution at 37°C, without supplemental CO_2.

After the incubation period, cells were washed with phosphate buffered saline at room temperature and lysed with 25 µL lysis buffer (Cat. No. E3971, Promega, Whitehead Scientific, SA). Luciferase activity was determined using a microtiter plate luminometer and quantified as relative light units. Each well received 25 µL lysis buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/mL BSA, pH 7.8), followed by 25 µL 1 mM D-luciferin 5s later. Relative light units were converted to a fold induction above the vehicle control value.

The DHT standard curve was fitted (sigmoidal function, variable slope) using Graphpad Prism (version 2.01), which calculated the minimum, maximum, slope, EC_{50} value and 95% confidence limits. The DHT equivalents (DHTEq) of extracts with greater than a twofold induction above the vehicle control were interpolated from the DHT standard curve and corrected with the appropriate dilution factor for each sample.

4.4 Results

4.4.1 YES assay

None of the samples had 3 or more points above the detection limit and consequently EEqs could not be determined for any of the samples taken during all four surveys. Yeast growth was checked at an absorbance of 620 nm and compared to the reference wells; all samples showed a decrease in cell density, therefore cytoxicity was present. The YES screen is a suitable screening tool for the determination of the overall estrogenic activity in complex samples taken from aquatic environments (Beck et al., 2006). Beck et al. (2006) found that the inhibition of yeast growth possibly caused by acute toxic constituents was the reason for the masking of an estrogenic response in more highly concentrated extracts, this was also found to be the case in a study by Bornman et al. (2007). Similarly in this study, the toxicity found could be masking the estrogenic activity in the samples and therefore give a false negative result, as the estrogenic response may lie in the toxic range.

4.4.2 The T47D-KBluc reporter gene assay

The results of the water samples using the T47D-KBluc assay are tabulated in Table 4.1.

Table 4.1: Estrogenic and anti-estrogenic activity in water samples from the Vhembe District, Limpopo Province, South Africa.

Sample survey	Sample site	Anti-estrogenic activity	Estrogenic activity	EEq (ng/l) ± SD
Survey 2	Albasini dam	< dl	< dl	
	Nandoni dam	< dl	< dl	
	Xikundu Weir	< dl	< dl	
Survey 3	Albasini dam	< dl	Positive	0.57 ± 0.06
	Nandoni dam	< dl	Positive	0.11 ± 0.03
	Xikundu Weir	< dl	Positive	*0.04 ± 0.03
Survey 4	Albasini dam	< dl	< dl	
	Nandoni dam	< dl	< dl	
	Xikundu Weir	< dl	< dl	

< dl: Below detection limit of the assay plate

***** A two-fold induction relative to the vehicle control could not be reached; therefore the EEq is only an estimated value.

No estrogenic or anti-estrogenic activity could be detected in water samples from the AD, ND and XW taken in Survey 2 and Survey 4. However, samples taken in Survey 3 showed estrogenic activity at all three sites, ranging from 0.04 ng/L to 0.57 ng/L EEq. No cytotoxicity was observed in any of these samples.

4.4.3 The MDA-kb2 reporter gene assay

No anti-androgenic or androgenic activity was observed in any of the samples, as none of the samples were able to obtain a two-fold induction relative to the vehicle control. No evidence of cytotoxicity was observed.

4.5 Discussion

Although all samples had a toxic response in the YES, no cytotoxicity was observed in the T47D-KBluc and MDA-kb2 assays. In three of the cytotoxic samples the T47D-KBLuc assay was able to detect estrogenic activity (Survey 3). The toxic response in the YES could be underestimating estrogenic activity or giving a false negative result as occurred in the Rietvlei Nature Reserve study (Bornman et al., 2007). As the T47D-KBluc assay contains both ERα and ERß (Wilson et al., 2004) it is a more sensitive assay than the YES, which only detects

binding to ERα. The cause of the cytotoxicity is not clear, but it may indicate the presence of (a) non-estrogenic compound(s). The results strengthen the argument for the use of a battery of screening bioassays which can complement each other and give a more comprehensive assessment on the estrogenic activity in environmental samples.

Only samples collected in Survey 3 tested positive for estrogenicity, with AD dam having the highest EEq (0.57 ng/L) followed by ND (0.11 ng/L) and then XW. Although these values did not exceed the Predicted-No-Effect-Concentration (1 ng/L) for 17β-estradiol in water, it must be noted that this could be an under estimation as the pH of the water was not adjusted (pH 3) prior to extraction and the chemical analysis should also be taken into consideration. It should be kept in mind that a water sample consists of a complex mixture of chemicals with possible (anti)-androgenic and (anti)-estrogenic activity, but also chemicals not measured, or with other EDC effects could affect the outcome of the assay.

The absence of androgenic activity measured in the MDA assay might also be attributed to the complexity of the samples. At present the EPA is having difficulties in obtaining reliable responses in the MDA assay with environmental samples. Therefore, this assay might not be the ideal choice when screening environmental samples (personal communication Dr V Wilson, 20-23 October 2008).

4.6 Conclusions

The bioassays showed that all water samples collected from the dams had a toxic response in the YES assay. The toxic response in the YES may be an underestimation of true concentrations or false negatives as found in previous studies (Bornman et al., 2007). The cause of the cytotoxicity is not known but may indicate the presence of non-estrogenic compound(s). Estrogenic activity was detected using the T47D-KBLuc assay during Survey 3 with AD having the highest EEq (0.57 ng/L) followed by ND (0.11 ng/L) and then XW. Although these values did not exceed the Predicted-No-Effect-Concentration (1 ng/L) for 17β-estradiol in water, technical problems such as no pH adjustment, could underestimate the real situation. The estrogenic activity in the T47D-KBLuc assay while the YES showed cytotoxicity highlight the need for the use of a battery of screening bioassays which can complement each other and provide a more comprehensive assessment on the estrogenic activity in environmental samples. There was no androgenic activity detected using the MDA assay. This may be due to the complexity of the samples, or the assay itself. The US EPA

experiences similar difficulties and this assay may not be the ideal choice for screening environmental samples.

4.7 Recommendations

Further research and continuous monitoring using a relevant battery of assays is essential to establish the extent, sources and ecological consequences of estrogenic and androgenic contamination in the aquatic system. The sampling protocol of this study is not sufficient to accurately assess the endocrine disruptive status of the water in the study area as these the EDC status can change at any time depending on a number of environmental influences.

The EPA is currently in the process of developing a new assay for detecting androgenic activity in environmental samples. The assay used cells modified to contain the AR and multiple reporter genes to amplify androgenic responses, in order for the assay to be more sensitive. If the above-mentioned assay proves to be efficient in detecting androgenic activity in environmental samples, it is recommended to obtain the cell line and to include the assay in future projects.

CHAPTER 5

ANALYSES OF FOOD, SOIL, AND POTABLE WATER

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5.1 Introduction

Rural villages in the northern and eastern parts of the Vhembe District of Limpopo Province are located in an intermediate- to high-risk malaria area (Department of Health (DoH), 2005). DDT is annually used for the purpose of malaria vector control (The Lubombo Spatial Development Initiative (LSDI), 2003). Because of the extended half-life of DDT and its metabolites, DDD and DDE (ATSDR, 2002), residues of these chemicals can persist for years within the DDT-sprayed domestic environment. Through their daily activities in and around the house, residents could be exposed to DDT through a number of possible pathways including indoor air, dust, soil, water and food.

The main pathway for human exposure to DDT is through ingestion (Environmental Health Criteria 9 (EHC 9), 1979; Tao et al., 2009). Vegetables of subsistence farmers in the Vhembe district is very likely to be contaminated with DDT through both root uptake from contaminated soil and from drift of the DDT spray during indoor residual spraying (IRS). While vegetables make up a large portion of the diet of the VhaVenda people, chickens are one of their major sources of protein. Throughout the day, these animals have free range on the property, including access to the DDT-exposed floors inside the sprayed huts. DDT has been shown to accumulate in a number of chicken tissues, including fat (Vazquez-Moreno, 1999), muscle (Aulakh et al*.,* 2005) and liver (Tao et al., 2009).

This project was done to determine the DDT contamination level in potable water, vegetables and chicken meat (muscle), fat and liver.

5.2 Materials and methods

Twelve homesteads were selected within the exposed village Dididi (DV1-DV12) and nine within the control village Tshakhuma (TV1-TV9), comparable in terms of the traditional Venda architecture as well as socio-economic status. A single traditional Venda homestead consists of a number of round thatch-roof huts, 3-5 m in diameter, built in a close circular

arrangement. The walls are constructed by a mixture of mud and cement, and the floors by mud and/or cow manure or cement. The area between the huts is soil or a covering with a mixture of mud and dung. This is where children play; food is prepared on open fires, and chickens, goats and domestic pets frequent during the day. In almost all homesteads, the rest of the property is used for growing subsistence crops, mainly maize and leafy vegetables. The crops are planted in close proximity (± 1 m) to the huts. Water for drinking, cooking, and bathing, is collected on a regular basis from either a centrally located community tap (borehole water), from the water truck or from nearby natural springs. Water is usually stored on the property in large plastic containers before being used. In DV, the nearby Luvuvhu River is also used by local residents for the washing of clothes as well as for swimming and bathing.

5.2.1 Food

5.2.1.1 Vegetables

Three of the most common leafy vegetables found at the selected homestead were collected and mostly included pumpkin, cowpea and Lugu/Spinach. Samples were kept in paper bags and stored at -20°C until analysed. The analyses were done by the SABS according to a method developed by Anastassiades et al. (2003). This included a mini-multi residue method using acetonitrile extraction/partitioning and disperses solid phase extraction clean-up and the QuECHERS method. The final analyses were completed using Gas Chromatograph*(y)* – Electron Capture Detection (GC-ECD) and Gas Chromatography with Flame Photometric Detection (GC-FPD) and pesticides presence was confirmed by Gas Chromatograph*(y)* – Mass Spectrometry (GC-MS).

5.2.1.2 Chickens

One chicken was collected per homestead (DV, n = 12; TV, n = 9) or, if not available, from a neighbouring or nearby homestead. The chickens were sacrificed and muscle, fat, and liver samples were collected. Each sample was individually stored in aluminium foil at -20°C until chemical analyses. The chicken tissues were analysed for DDT and metabolites by FDA laboratories (Pretoria, South Africa) according to the method adapted from Bordet et al. (2002). Extraction was done using solid-phase C_{18} cartridges (500 mg)/3 mL cartridges (Waters-Microsep), conditioned with petroleum ether followed by acetone and methanol. The extracts were loaded onto the cartridge and allowed to flow through. The cartridge was

rinsed with acetonitrile and allowed to elute from the cartridges. The samples were evaporated under nitrogen at 35°C and reconstituted into 2 mL of hexane. A florisil cartridge was placed on the manifold; after conditioning it with 10 mL of hexane, the samples were loaded. The samples were eluted with 10 mL of petroleum ether/diethyl ether (98:2, v/v) and 12 mL of petroleum ether / diethyl ether (85:15, v/v). The two fractions were combined and evaporated under nitrogen to dryness. The samples were reconstituted into 250 µL hexane for GC-MS analysis.

5.2.2 Soil

From each homestead, five individual soil samples were collected in an area surrounding the huts. The collected soil were placed in a paper bag, sealed, and kept at room temperature until DDT analyses. Before extraction, the soil samples from each homestead were pooled and mixed. The samples were then dried at 110°C. From each sample, 5 g were mixed with 100mL hexane / petroleum ether (50:50, v/v) and extraction completed after 12 hours using a reflux method (Naudé et al., 1998). The extracted samples were analysed using GC-MS.

5.2.3 Potable water

Potable water was collected from a community tap in Dididi as the water supply to the village occurs central. The water sample was collected in a pre-washed ethanol glass container. The 2.5 L water samples were kept at ± 15°C until analyses by FDA laboratories (Pretoria, South-Africa). The method used is based on the method described by Cacho et al. (1995) where 1 L of water samples were filtered into an acid washed reagent bottle. C_{18} solid-phase cartridges (500 mg)/3 mL (Waters-Microsep) were conditioned with water, methanol, and water. The samples were then allowed to pass through the cartridge. Thereafter the cartridge were dried and eluted with 12 mL of hexane-diethylether (85:15, v/v). The samples were evaporated under nitrogen to dryness and reconstituted in methanol. Final analyses were done using GC-MS.

5.3 Results

The vegetables collected from the reference village had no detectable residues of DDT and metabolites. 92% (10 of 12) vegetable samples from the exposed area contained *p,p'*-DDD ranging from 10-80 µg/kg, but at two homesteads vegetables also had residues of 60 and 80

µg/kg *p,p'*-DDT, and 10 µg/kg *p,p'*-DDE respectively. No residues were detected in vegetables from the reference area (Table 5.1).

The chickens collected from Dididi had higher levels of DDT residues in their liver, muscle and fat tissues than those collected from Tshakhuma (Tables 5.1). Chicken livers from the exposed area contained *p,p'*-DDT in 9 of 12 (75%) of samples ranging from 50.6 to 604.8 µg/kg; *p,p'*-DDD in 83% ranging from 128.2 to 541 µg/kg, and 100% had *p,p'*-DDE at levels of 50.6 to 604.8 µg/kg. Moreover, 50% of the liver samples also contained *o,p'*-DDT ranging from 52.3 to 77.9 µg/kg. No liver residues of DDT and metabolites were found. In muscle tissue from chickens in Dididi *p,p'*-DDT and *p,p'*-DDE levels ranged from 52.5-326.3 µg/kg and 68.6-838.6 µg/kg respectively. Some chickens in the reference group also contained *p,p'*-DDT and *p,p'*-DDE albeit at much lower levels.

All the DDT isomers were detected in chicken fat samples from Dididi with *p,p'*-DDT and *p,p'*-DDE at extremely high levels of 45094.4 ± 2579.5 µg/kg and 192024.2 ± 35892.3 µg/kg respectively. The concentration of 409.4 ± 165.7 µg/kg of *o,p'*-DDT is also reason for concern as this isomer is estrogenic. Since chicken fat would form part of cooked chicken, this could be an important route of DDT exposure. At the reference site both *p,p'*-DDT and *p,p'*-DDE residues were found.

In soil samples from Dididi all the DDT isomers were detected and the *p,p'*-DDT level was 0.91 ± 1 µg/kg and *p,p'*-DDE 15.78 ± 16.1 µg/kg (Table 5.2). At the reference site all isomers were also detected, but in fewer samples at lower levels except for *p,p'*-DDE at one homestead (72.2 µg/kg).

Table 5.1: DDT and metabolite concentrations in the livers, muscle, and fat of chickens collected from Dididi (exposed) and Tshakhuma villages (unexposed)(mean ± SD; µg/kg; fresh mass).

	Exposed village						Unexposed village (control)					
Tissue	***o,p'*- DDT**	***p,p'*- DDT**	***o,p'*- DDD**	***p,p'*- DDD**	***o,p'*- DDE**	***p,p'*- DDE**	***o,p'*- DDT**	***p,p'*- DDT**	***o,p'*- DDD**	***p,p'*- DDD**	***o,p'*- DDE**	***p,p'*- DDE**
Liver	59 ± 10.2	218 ± 187.5		271 ± 147.2		1191 ± 1261.8						
Muscle		131 ± 103.7				286 ± 226						540 ± 784
Fat	409 ± 165	45094 ± 25795	101 ± 69	3019 ± 1229	97 ± 53	192024 ± 354892		432 ± 752				189 ± 167

Table 5.2: The mean DDT concentrations in soil collected from Dididi (exposed) and Tshakhuma (unexposed) villages (µg/kg, dry mass).

	Exposed village						Unexposed village (control)					
Soil	***o,p'*- DDT**	***p,p'*- DDT**	***o,p'*- DDD**	***p,p'*- DDD**	***o,p'*- DDE**	***p,p'*- DDE**	***o,p'*- DDT**	***p,p'*- DDT**	***o,p'*- DDD**	***p,p'*- DDD**	***o,p'*- DDE**	***p,p'*- DDE**
Mean	0.69	0.91	0.97	1.80	3.49	15.78	1.0	1.4				14.5
SD	0.1	0.1	0.4	1.1	1.6	16.1	0.7	1.2				28.4

5.4 Discussion

The vegetables from the exposed area mainly contained *p,p'*-DDD (10-80 µg/kg), the metabolite of *p,p'*-DDT, but at two homesteads vegetables also contained either *p,p'*-DDT *p,p'*-DDE. It should be noted that not all vegetables are cooked; some are eaten raw. The soil samples contained all the DDT isomers, but the *p,p'*-DDE 15.78 ± 16.1 µg/kg was high. Children play in the sand around the house and are probably exposed to residues. Also at the reference site all isomers were also detected, but in fewer samples at lower levels except for *p,p'*-DDE at one homestead (72.2 µg/kg). This finding further supports the possibility of illegal use of DDT outside the official DDT-sprayed area.

Chickens roam the open spaces and the gardens at the homesteads and at night sleep indoors. They may therefore be exposed to both the residues in the outdoor soil and the indoor house dust. Chicken meat had the lowest levels of both *p,p'*-DDT and *p,p'*-DDE, followed by concentrations in the liver and fat had extremely high values. Some chickens in the reference group also contained *p,p'*-DDT and *p,p'*-DDE albeit at much lower levels. Since these would all form part of cooked chicken, this could be an important route for human DDT exposure. At the reference site both *p,p'*-DDT and *p,p'*-DDE residues were found and since chicken would not have ranged 23 km to the DDT sprayed area, it seems fair to assume that some DDT was being illegally used and caused contamination and was therefore detected.

The *o,p'*-DDT levels (409.4 ± 165.7 µg/kg) in fat means an added estrogenic load to the insecticidal estrogenic *p,p'*-DDT. *o,p'*-DDT is a by product of DDT during production and if its presence in the final product used for IRS could be eliminated or at least lowered, a lot could be done to render the existing formulation safer while DDT is still being used.

The presence of *o,p'*-DDD in chicken liver and fat is also of concern. *o,p'*-DDD (Mitotane®) has been used to treat adrenocortical carcinoma and Cushing's disease in humans for almost four decades (Bergenstal et al., 1960; Wooten and King, 1993). *o,p'*-DDD selectively induces necrosis of the *zona fasciculata* and *zona reticularis* of the adrenal cortex and inhibition of cortisol synthesis.

Skinner-Adams et al. (2008) pointed out that the global epidemiology of HIV/AIDS and malaria overlap, as a considerable number of HIV-infected individuals live in regions with different levels of malaria transmission. The consequences of co-infection with HIV and malaria parasites are not fully understood, but it seems that the infections act synergistically and together produce devastating outcomes. DDT, DDE and DDD all induce differential degrees of humoral and cellular immune suppression, but the clear responses are by DDD and DDE. Suppression of immune responses by DDD and DDE is an important determinant of the toxicity of DDT (DDE > DDD > DDT) (Banerjee et al., 1996). To the best of our knowledge, the possible relationship between *o,p'*-DDD levels and the human immunodeficiency syndrome has not been studied, but it seems warranted in view of our findings.

The possible implications of the analytical chemistry and levels found will be fully discussed in Chapter 14.

5.5 Conclusions

Potable water from the community taps had no detectable DDT residues. The majority (92%) of the vegetables from the exposed area contained *p,p'*-DDD (ranging from 10-80 µg/kg), with 16.7 % (n=2) homesteads also having residues of 60 and 80 µg/kg *p,p'*-DDT, and 10 µg/kg *p,p'*-DDE respectively. No residues were detected in vegetables from the reference area.

Chicken livers from the exposed area contained *p,p'*-DDT in 75% of samples ranging from 50.6 to 604.8 µg/kg; *p,p'*-DDD in 83% ranging from 128.2 to 541 µg/kg, and 100% had *p,p'*-DDE at levels of 50.6 to 604.8 µg/kg. Moreover, 50% of the liver samples also contained *o,p'*-DDT ranging from 52.3 to 77.9 µg/kg. In muscle tissue from chickens in Dididi *p,p'*-DDT and *p,p'*-DDE levels ranged from 52.5 – 326.3 µg/kg and 68.6 – 838.6 µg/kg respectively. Single chicken liver and meat samples in the reference group also contained *p,p'*-DDT and *p,p'*-DDE albeit at much lower levels. All the DDT isomers were detected in chicken fat samples from Dididi with *p,p'*-DDT and *p,p'*-DDE at extremely high levels of 45094.4 ± 2579.5 µg/kg and 192024.2 ± 35892.3 µg/kg respectively. The concentration of 409.4 ± 165.7 µg/kg of *o,p'*-DDT is also reason for concern as this isomer is estrogenic. Since chicken fat would form part of cooked chicken, this could be an important route of DDT exposure. At the reference site 440 ± 760 µg/kg *p,p'*-DDT and 190 ± 170 µg/kg *p,p'*-DDE were found.

In soil samples from Dididi all the DDT isomers were detected and the *p,p'*-DDT level was 0.9 ± 1 µg/kg and *p,p'*-DDE 15.78 ± 16.1 µg/kg. At the reference site all isomers were also detected, but in fewer samples (n = 6), 66.7%) at lower levels except for *p,p'*-DDE at one homestead (72.2 µg/kg). Since chicken could not have ranged 35 km to the DDT sprayed area, it seems fair to assume that some DDT was being illegally used in the reference area and caused the subsequent contamination and detection. The high soil values at one homestead further supports the possibility of illegal use of DDT outside the official DDT-sprayed area.

CHAPTER 6

CHEMICAL ANALYSES OF FISH FAT AND BRAIN

Barnhoorn IEJ, Bornman MS, Van Dyk JC, Swemmer A and Patrick S

6.1 Introduction

In a study performed by Bornman et al. (2007) at the Rietvlei Dam, it was clear that although even frequently sampled water and/or sediment samples were analysed, some chemicals were only detected in fish fat. Fat contains bioconcentrated and bio-accumulated lipophilic chemicals and is, therefore, a suitable matrix to reflect the historical chemical exposure of an individual. Some of the fish collected in the present study had no or insufficient fat tissue to sample and it was decided to evaluate the possible use of brain tissue as alternative substrate. DDT residue values in the brain would at the same time provide an indication of possible organ involvement as some studies (Clotfelter et al., 2004; Clotfelter and Rodriques, 2006) reported changed neuron behaviour in fish exposed to chemicals.

6.2 Materials and methods

Available abdominal fat of each fish was removed as well as the brain (only during Survey 3) and individually wrapped in foil, placed in plastic bags, and kept at -20°C until analysed. The fish fat and brain samples were chemically analysed for DDT residues by FDA laboratories (Pretoria, South Africa) according to a method adapted from Bordet et al. (2002). Extractions were done using solid-phase C_{18} cartridges (500 mg)/3 mL (Waters-Microsep) conditioned with petroleum ether followed by acetone and methanol. The collected eluate was rinsed with acetonitrile and allowed to elute from the cartridges. The samples were evaporated under nitrogen at 35°C and reconstituted into 2 mL of hexane. A florisil cartridge was placed on the manifold and after conditioning with 10 mL of hexane, the samples were loaded. The samples were eluted with 10 mL of petroleum ether-diethyl ether (98:2, v/v) and 12 mL of petroleum ether-diethyl ether (85:15, v/v). The two fractions were combined and evaporated under nitrogen to dryness. The samples were reconstituted into 250 µL hexane for GC/MS analysis.

Statistical analyses

Correlation of DDT isomers in fish brain to fish fat was calculated using Pearson's Correlation Coefficient (r) to identify potential strong positive (r > 0.75), strong negative (r < -0.75) and/or similar linear relationships between the isomers.

6.3 Results

The DDT residues in the fat of *C. gariepinus* and *O. mossambicus* at the AD, ND and XW are shown in Tables 6.1-6.3. The detection limit was 0.010 µg/kg for fish fat samples.

Table 6.1 summarizes the levels in fish fat from AD. The major metabolite was *p,p'*-DDE (mean 2722 ± 1901 µg/kg), but both *o,p'*-DDT, *p,p'*-DDD and *p,p'*-DDT were present in considerable concentrations.

Table 6.1: The mean DDT residues present in fat from *C. gariepinus* and *O. mossambicus* collected from AD (µg/kg, fresh mass).

FISH #	*o,p'*-DDT	*p,p'*-DDT	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE	FISH #	*o,p'*-DDT	*p,p'*-DDT	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE
Survey 1		***C. gariepinus***					**Survey 1**	***O. mossambicus***					
AD 1	*	*	*	31	*	311							
AD 2	*	*	*	*	*	54		**NO FISH/FAT**					
AD 3	*	*	*	81	*	272							
Mean													
SD													
Survey 3							**Survey 3**						
Mean	96	238		270		2722							
SD	16	100		95		1901		**NO FISH/FAT**					
Survey 4							**Survey 4**						
Mean	71	134		2529		399							
SD	11	73		1086		246		**NO FISH/FAT**					

* = single samples no DDT or metabolites detected

At ND, limited numbers of *C. gariepinus* were caught during Survey 1 and 4, but 9 during Survey 3. The mean *p,p'*-DDE level in *C. gariepinus* was three times higher in Survey 3 (11515 µg/kg) compared to Survey 4 (3497 µg/kg). In *O. mossambicus* from Survey 4, *p,p'*-DDE was 13 times higher than in Survey 1 (Table 6.2).

Table 6.2: The mean DDT residues present in fat from *C. gariepinus* and *O. mossambicus* collected from ND (µg/kg, fresh mass).

FISH #	*o,p'*-DDT	*p,p'*-DDT	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE	FISH #	*o,p'*-DDT	*p,p'*-DDT	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE
Survey 1		***C. gariepinus***							***O. mossambicus***				
Mean				341		873	**Mean**				72		86
SD				67		338	**SD**				29		47
Survey 3													
Mean	170	1057	157	2233		11515	**Mean**						
SD	59	488	94	873		6253	**SD**						
Survey 4													
Mean	87	662	331	16573	474	3497	**Mean**	81	421	80	2575	87	1082
SD	28	932	446	17103	692	3352	**SD**	12	509	10	1063	12	343

At XW, only Survey 4 had *C. gariepinus,* but *O. mossambicus* numbers were sufficient during Surveys 1 and 4. The mean levels in Survey 4 (6135 µg/kg) were almost three times higher than in Survey 1 (2045 µg/kg) (Table 6.3).

Table 6.3: The mean DDT residues present in fat from both species collected from the XW (µg/kg, fresh mass).

FISH #	*o,p'*-DDT	*p,p'*-DDT	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE	FISH #	*o,p'*-DDT	*p,p'*-DDT	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE
Survey 1		***C. gariepinus***							***O. mossambicus***				
Mean							**Mean**	79	2661	23	2351		2045
SD							**SD**	51	1305	12	921		644
Survey 3													
Mean	2249	18786	535	8050	90	37527	**Mean**						
SD	178	1359	109	956	32	3686	**SD**						
Survey 4													
Mean	418	4541	285	34467	564	8198	**Mean**	312	6571	171	12076	270	6135
SD	264	4279	145	26542	317	3865	**SD**	132	3098	45	4121	115	1970

Figures 6.1 and 6.2 show the trend of total DDT values in the two species over the Surveys. At ND, the levels were lower in *C. gariepinus*, intermediate at ND and highest at XW (Figure 6.1). The same trend was observed for *O. mossambicus*, but catfish in general had the highest values (Figure 6.2).

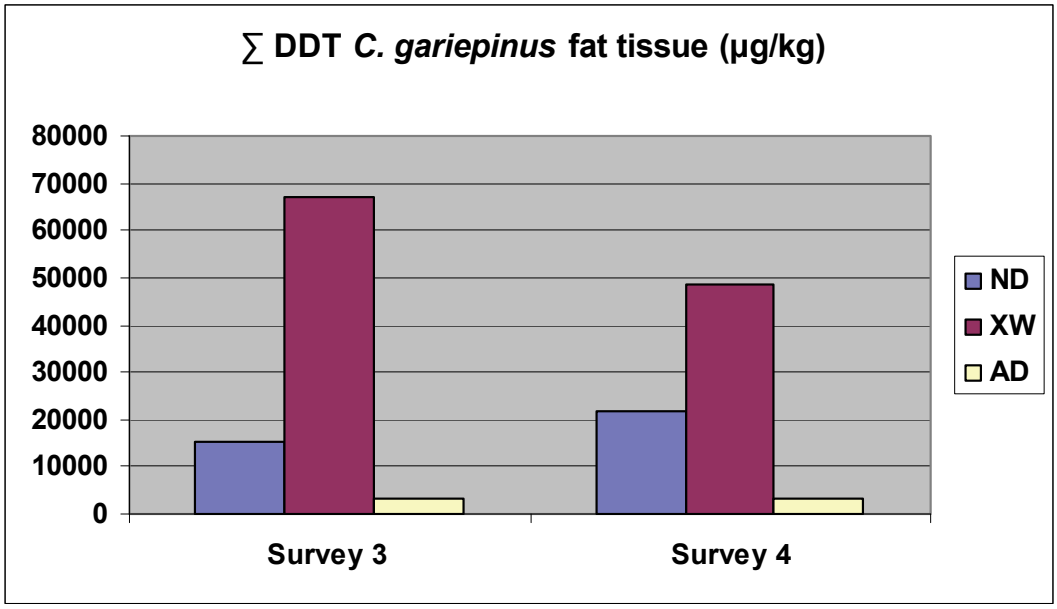

Figure 6.1: The total DDT residues (fresh mass) found in the fat of *C. gariepinus* during Surveys 3 and 4.

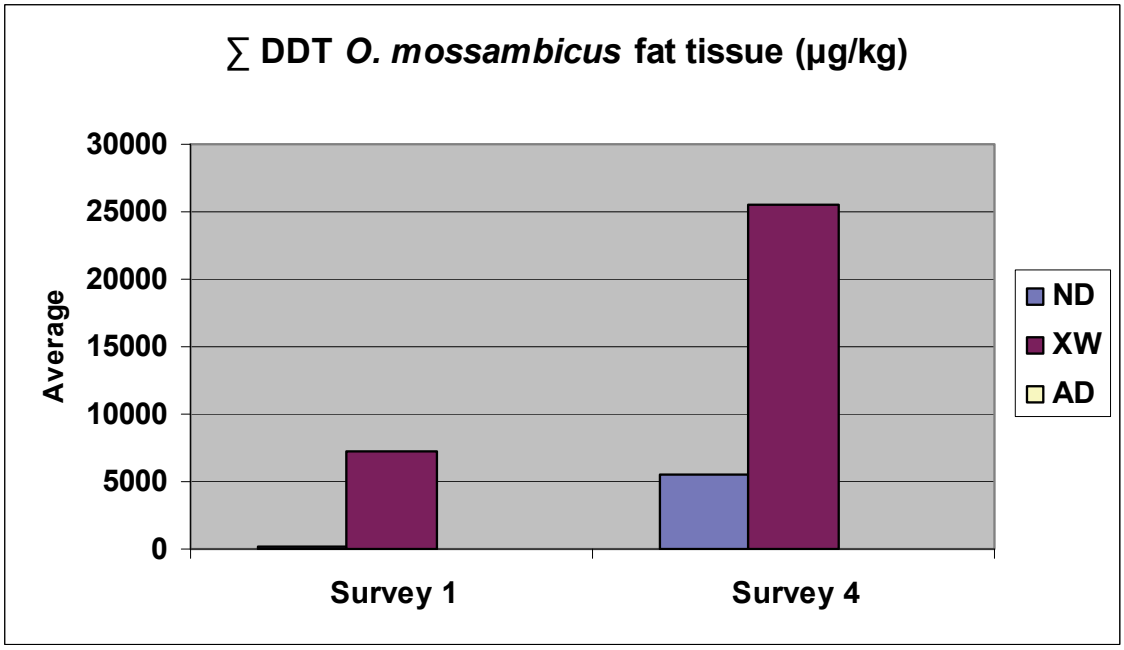

Figure 6.2: The total DDT residues (fresh mass) found in the fat of *O. mossambicus* during Surveys 1 and 4.

No *C. gariepinus* brains were available from any of the major sites and no *O. mossambicus* tissue from AD either. The levels (mean ± SD) of the different DDT residues from D and XW are summarized in Table 6.4. At ND *o-p'*-DDT and *p,p'*-DDE were detected, but at XW *o-p'*-DDT and the *p,p'*-isomers- DDT, -DDD and -DDE. There were strong positive correlations of *o,p'-DDT* (r = 0.968) and *p,p'-DDT* (r = 0.895) between fat and brain tissue.

Table 6.4: The levels of DDT and metabolites detected in the brain of *O. mossambicus* from the three sites during Survey 3 (µg/kg, fresh mass).

FISH #	*o,p'*-DDT	*p,p'*-DDT	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE
Survey 3			AD			
Mean						
SD						

FISH #	*o,p'*-DDT	*p,p'*-DDT	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE
			ND			
Mean	65					108
SD						81

FISH #	*o,p'*-DDT	*p,p'*-DDT	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE
			XW			
Mean	64	84		92		284
SD	1	24		37		202

6.4 Discussion

This study confirmed that DDT and metabolites also accumulate in fish brain tissue, but the isomer dynamic might be different and brain tissue could not substitute fat tissue to address the historical exposure of an individual fish. However, the high concentrations may have an effect on brain function. In a previous study on chicken embryo brain cells, Bornman et al. (2007) showed that the DDT isomers, especially DDE, cause ultrastructural changes to neuronal tissue that may contribute to neurotoxicity in chickens. Therefore, future studies should include light and electron microscopy of the brain.

6.5 Conclusions

The high levels detected in fish fat and brain tissue emphasizes the bio-concentration of DDT in various tissues of exposed fish. In this regard bioaccumulated levels in fat tissue provide an indication as to what the fish might have been exposed to over a life time. It also raises the possibility that fish behaviour may be adversely affected as fat tissue levels are in equilibrium with blood levels.

CHAPTER 7

THE EFFECTS OF DDT ON FISH REPRODUCTION AND MACRO-INVERTEBRATE COMMUNITIES

Brink KA, Van Vuren JHJ and Bornman MS

7.1 Introduction

1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) is a highly lipophilic and persistent toxicant. This very nature allows organisms to bioaccumulate sub-lethal concentrations, through exposure to the ambient environment (bioconcentration) and/or through dietary uptake (biomagnification) (Borga et al., 2001). These sub-lethal concentrations may manifest a wide range of responses measured at various levels of biological organisation, from biochemical and cellular levels to organismal and community levels. While most studies on DDT have focused on its effects at a biochemical level of biological organisation, DDT or its metabolites can have indirect effects on community structures (Vasseur and Cossu-Leguille, 2006).

In this chapter, the aim was to assess the effects that DDT on community structures in the Luvuvhu River, through spatial and temporal comparisons. Both fish and macro-invertebrate communities were assessed as they occupy different ecological niches within an ecosystem and thus have varying responses to the same environmental stressor. Therefore fish communities are valuable indicators as they are high-order consumers and are thus able to reflect the responses of the entire trophic structure (Berkman et al., 1989; Sanchez et al., 2008). While macro-invertebrates are more sensitive to water quality disturbances and although the level of biological organisation is high they are still able to act as an early warning to deterioration of the local water quality (Dallas, 2002).

7.2 Experimental

7.2.1 Field procedures

7.2.1.1 Fish and invertebrate collection

Sampling for the purpose of assessing the status of fish communities occurred at five riverine sites in the Luvuvhu River, namely Lotanyanda, Hasana, Tshikonelo, XW and Mhinga (Figure 7.1). These sites were sampled in October 2006 (survey 1), March 2007 (survey 2), October 2007 (survey 3) and February 2008 (survey 4). The Lotanyanda and Xikundu Weir (XW) however were not sampled during survey 1. The Albasini (AD) and Nandoni (ND) Dams were not sampled the surveys due to two main reasons: firstly, as is well known, reservoirs have substantially different physical, chemical and biological properties from that of flowing rivers therefore making comparisons between these types of environments difficult and secondly, even if one was to sample these reservoirs, accurate population and community assemblages are not practical as sampling is very intensive; requiring specialised sampling techniques, funds, time, and labour.

At each of the riverine sites, fish were collected using electronarcosis and a seine net. Electronarcosis was primarily used to sample fish from riffles, runs, rapids, and vegetated habitats in a known amount of time. Whilst the seine net was used to sample fish primarily from sandy pools (the amount of drags were also noted at each site). Following collection, fish were identified to species level using the key from Skelton (2001). All unwanted fish species collected were all safely returned to the water.

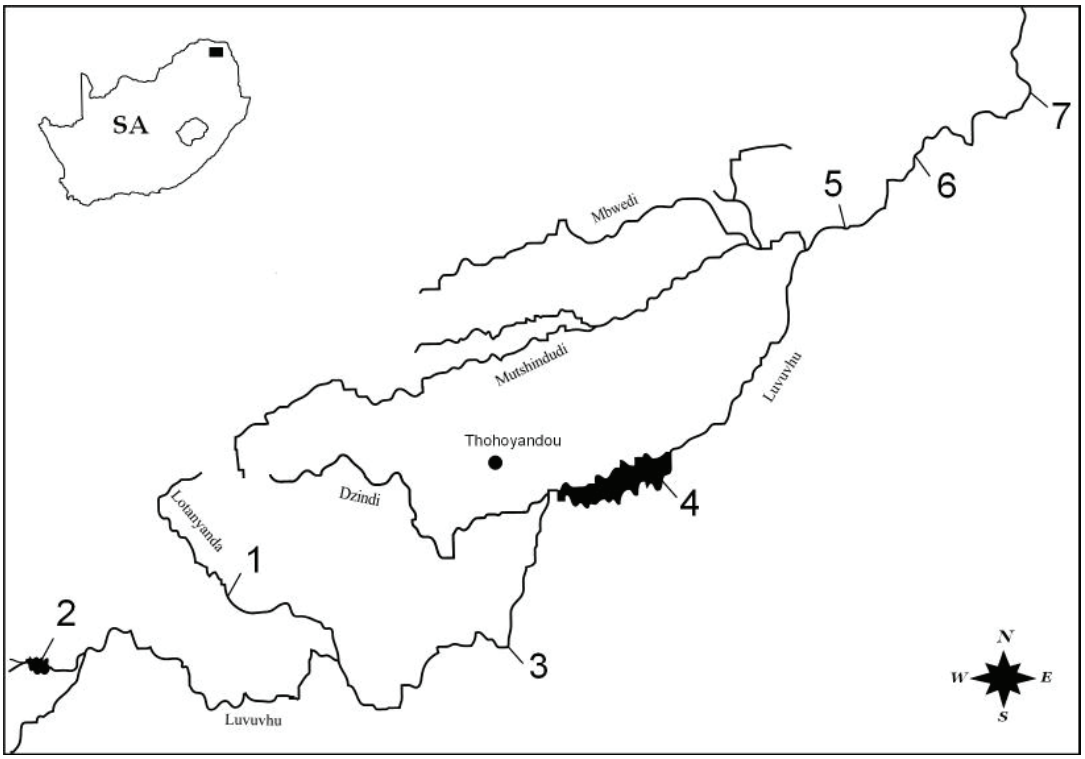

Figure 7.1: The sampling sites within the Luvuvhu River including (1) Lotanyanda, (2) Albasini Dam, (3) Hasana, (4) Nandoni Dam, (5) Tshikonelo, (6) Xikundu Weir, and (7) Mhinga.

The macro-invertebrates were collected as per the standard South African Scoring System (SASS) 5 sampling protocol developed by Chutter (1998). A net with a 1 mm mesh size (30 cm x 30 cm) was used to collect specimens that were dislodged from their respective habitats of stones, mud or vegetation. This sampling was standardised to disturbing cobbles and bedrock for 6 minutes, gravel sand and mud for 1 minute and marginal vegetation for 2 square meters. The organisms were then preserved in 10% neutral buffered formalin (100 mL 40% formaldehyde, 4 g NaH_2PO_4 and 6.5 g Na_2HPO_4 made up to 1 L) and then transferred and stored in 70% ethanol in the laboratory. Each specimen was then identified to family level and classified according to sensitivity groups and expressed as average score per taxon (ASPT). The family level taxonomic resolution was selected as it is the most cost and time effective and is able to distinguish between polluted sites (Krassulya, 2001; Marshal et al, 2006; Baley et al., 2001; Waite et al., 2004).

7.2.1.1 Habitat

As the physical habitat can influence fish and invertebrate community structures the general physical characteristics and impacts thereupon at each site was evaluated. The physical characteristics of the instream channel and riparian habitat of each site was described by using a number of criterions from DWAF's site characterisation field-manual (Dallas, 2005). The criterion included channel morphology, stream dimensions, canopy cover, riparian vegetation, aquatic vegetation, and substratum composition. The impacts on the physical habitat were assessed using the index of habitat integrity (IHI) derived by Kleynhans (1996). For each disturbance, an impact rating and respective confidence score was assigned. These two values were then used to calculate the impact scores for each criterion i.e. (impact rating/25) x (the weight of that impact). The resulting scores for each criterion were all summed, expressed as a percentage and subtracted from 100 to yield the IHI score.

In order to determine if the available habitat is sufficient to satisfy all indigenous fish species at each site the habitat cover rating (HCR) index was used (Kleynhans, 1997). The protocol required that the abundance of each velocity-depth class (fish habitat) present was to be estimated according to the guidelines 0 = absent; 1 = rare; 2 = sparse; 3 = common; 4 = abundant; 5 = very abundant (Dallas, 2005). Thereafter, the abundance values were added together and divided by the total number of velocity—depth classes. This value was then multiplied by the sum of the abundance values for the various cover features (including overhanging vegetation, aquatic macrophytes, substrate and bank undercut rootwads) present, which ultimately lead to the final HCR score. As for the invertebrates, there was no habitat index available for assessment of habitat changes, although the biotopes available were noted (Dallas, 2005).

7.2.2 Data treatment

7.2.2.1 Occurrence of fish and invertebrates

In order to informally identify changes in the distribution of fish species in the current study comparisons were made with reference frequency of occurrence (FO) data. This is a reference list of species that should occur within a segment of a river. Each species was rated, by specialists, according to its expected presence within a specific reach, where 1 = < 10%, 2 = 10-

25%, 3 = 25-50%, 4 = 50-75% and 5 = > 75% (Kleynhans, 2007). Although the same importance to reference conditions can be said for invertebrates, historic data for the Luvuvhu River invertebrate population was limited. However according to Thirion (2007) it is acceptable to utilise a reference site that is within the same level II ecoregion and geomorphological zone. For the purposes of the present study, the Lotanyanda was utilised as a reference site to the Luvuvhu River.

7.2.2.2 Relative abundance

Collection of biota for absolute abundance of populations is very intensive and according to Barbour et al. (1999) is not a required endpoint to determine the effects of pollution in lotic ecosystems. Therefore only the relative abundance was used as an indicator of contaminant stress on fish and invertebrate communities. Furthermore manipulation of fish data into catch per unit effort (CPUE) values was required to overcome the differences in the sampling efforts between the sites (Schneider, 2000; Kleynhans, 1999). To determine the CPUE, the total catch for the relevant sampling technique was divided by their total sampling effort, which would be time (minutes) for electronarcosis and number of drags for seine net. This CPUE was then finally standardised to 30 minutes and 2 drags for each respective technique, and rounded off to the nearest 1.

7.2.2.3 Non-parametric diversity indices

Margalef's species richness, Pilou's evenness index, Shannon diversity index and log series diversity index of fish can be used to describe the response of a community to the quality of environment. The Margalef's species richness was selected to measure of the number of taxa (species/families) present for a given number of individuals, incorporating total number of individuals and total number of taxa. Pilou's evenness index was selected as it represents how individuals are distributed over the taxa, and Shannon diversity index because it incorporates both the species richness and evenness components. In addition, the log series diversity index (α) was also included as an alternative to the controversial Shannon diversity index, and was calculated as according to Magurran (1988). All of the other indices were then calculated on the statistical program PRIMER v5.

7.2.3 Statistics

In order to identify a cause-effect relationship a two-tailed Pearson's correlation analyses was computed using SPSS release 12.0.1. The community endpoints, described earlier in this chapter, were pooled regardless of survey number and sites, since no significant differences between the flow regimes were found for fish species, while macro-invertebrates families were pooled according to flow regimes. The pooled data for fish and macro-invertebrate communities included the number of species (S), the abundance of species (N), Margalef's species richness, Pilou's evenness index, Shannon diversity index, and for the fish only the log series-alpha diversity index. A number of possible environmental influences were then correlated with these data and include the following. Correlations were done with standard physico-chemical water quality measurements, including temperature (°C), dissolved oxygen (mg/L), pH, conductivity, TDS, and nutrients measured in mg/l (ammonia, calcium, nitrate, nitrite, ortho-phosphate, sulphate). Then all the metals in water and sediment, with concentration units of mg/L and mg/g respectively, were correlated. Similarly, DDT and its metabolites measured in µg/L in the water and µg/kg in the sediments were analysed. Physical anthropogenic habitat disturbances (measured using IHI index) were also correlated along with HCR (Kleynhans, 2007). Invertebrates had no habitat availability index and so correlations could not be successfully done (Dallas, 2002).

Multivariate statistics were used to compare community patterns because subtle changes in the community composition across sites may be masked when the characteristics of a site is combined, as is in the case of univariate analyses, such as ANOVA. In order to assess any spatial and temporal changes in the community structures, the Bray Curtis cluster determination, non-metric multi-dimensional scaling (NMDS) and analysis of similarities were conducted using PRIMERv5 (Clarke and Warwick, 1994). For these analyses, the abundance data for each species were initially square root transformed to yield a similarity matrix that was used to plot both a bray-Curtis dendrogram and an NMDS plot. The bray-curtis dendrogram was then used to visually identify the extent of spatial differences, while the MDS ordinations were used to visually identify seasonal differences. Once the (dis)similarities have been identified for each site and season, the similarity percentages (SIMPER) were then calculated in order to identify which species/family were principally responsible for the differences or similarities and the ANOSIM was calculated to identify any significant differences between the sites or seasons. For further explanations on the interpretation of these statistics, refer to Clarke and Warwick, 1994.

7.3 Results

7.3.1 Habitat

In order to get a clearer understanding of the habitat and impacts at each site, a site-specific description of the physical components was necessary. Since there was very little variation between each season, all the physical habitat descriptions were based on summarised observations from all four surveys (Table 7.1)

Table 7.1: Generalised physical characteristics of each site within the Luvuvhu River.

	Lotanyanda	Hasana	Tshikonelo	XW	Mhinga
Riverine zone	head-middle zone	middle zone	middle zone	middle zone	middle zone
Slope	steep-gradual	gradual	gradual	gradual	gradual
Velocity	slower	slower	slower	slower	slower
Active width of stream	2-5	20-50	20-50	20-50	20-50
Dominant depth	<0.5m	>0.5m	>0.5m	>0.5m	>0.5m
Dominant substratum type	cobbles/ pebbles/GSM	bedrock	GSM/cobbles/ pebbles	cobbles/pebbles/ gravel	boulders/cobble/ pebble
Temperature	21-23	26-30	23-33	24-28	25-28
Turbidity	low (clear)	intermediate	intermediate	Intermediate-high	Intermediate-high
Light reaching stream (canopy)	partially open	open	open	Open	open
Riparian vegetation type	grasses/shrubs/ trees	grasses/reeds	reeds	Reeds	reeds

The IHI was utilised in order to quantify the individual impacts that may influence the physical instream and riparian habitats as well as their accumulative impacts on the habitat integrity at each site (Figure 7.2). The site along the Lotanyanda River had a score of 80% which can be classified as largely natural with a few modifications mainly as a result of water abstraction. The habitat integrity at the sites within the Luvuvhu River were also influenced by water abstraction from two major dams (Albasini and Nandoni dams) as well as a number of weirs with Hasana, and Tshikonelo having habitat scores of 65%, and 67%,respectively. XW had a largely modified habitat of 51% due to its proximity to the weir, but it did recover downstream at Mhinga where the habitat improved to a 70%. The riparian integrity was good at Lotanyanda, while the other sites along the Luvuvhu River suffered from severe erosion and indigenous vegetation removal that resulted in scores of 78%, 75%, 52%, and 72%.

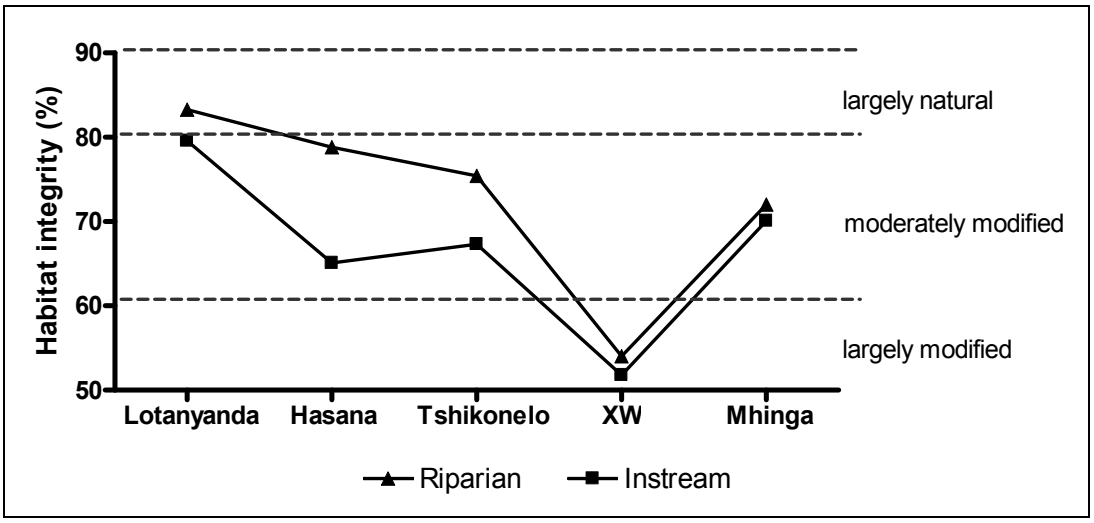

Figure 7.2: The instream and riparian zone habitat integrity of the Luvuvhu River at each of the lotic sites

The fish habitat quantified using HCR index was represented in Figure 7.3. The scores were all generally high ranging from 9 at Hasana in Survey 2 to 16.5 at Mhinga in Survey 3. In all four surveys, the amount of habitat available for fish showed similar spatial variations. The sites with the lowest available habitat in the Luvuvhu River catchment were the Lotanyanda River site and Hasana. This was primarily due to the lack of cover available for fish at these sites. Lotanyanda was particularly susceptible to this as it had head-water characteristics, whilst Hasana was susceptible as bedrock was the predominant substratum type. In contrast, Tshikonelo, XW and Mhinga had the greatest availability for fish habitat, with great variety of depth-flow classes and ample cover.

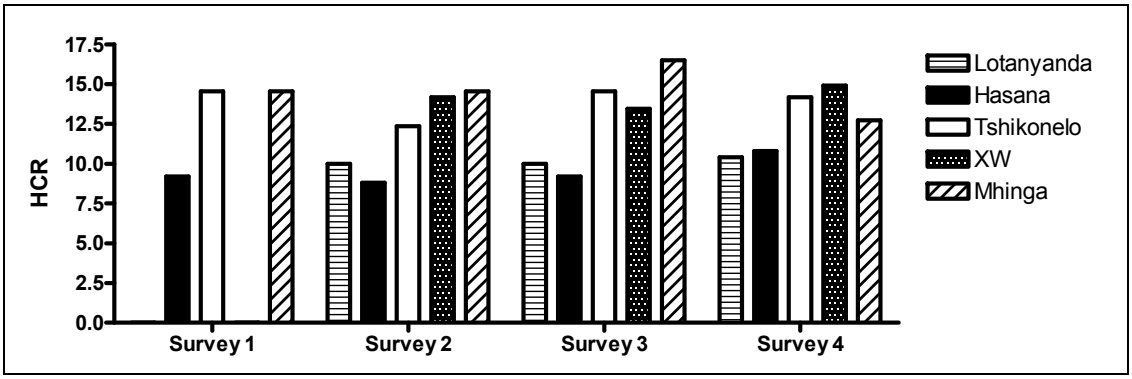

Figure 7.3: The fish habitat cover rating (HCR) index scores in the Luvuvhu River.

7.3.2 Fish communities

7.3.2.1 Occurrence of fish

The fish species composition differed with a substantial decline in species distribution from the reference (Frequency of occurrence data [FO]) data for all sites (Table 7.2). That is in the Lotanyanda River, 13 indigenous species were observed throughout the seasons in the present study. Comparison with the FO data showed that there was 57% fish species still found in the system of the total fish species that should have been present. Of the species that were not found, four were previously wide spread (> 50% chance that species should have occurred within the reach), namely *A. benalensis, A. uronoscopus, B. eutaenia* and *B. neefi*, and 8 were previously scarce (< 50% chance that species should have occurred within the reach). At Hasana there were a total of 15 species that were collected during this survey, which was 62% of the total species that should have been collected. The unsampled species included 13 species, none of which were previously wide spread in this reach of the river. At Tshikonelo 60% of the total species that should be present was not collected. However, only 8% of this, including *B. viviparous, L. molybdinus*, and *P. philander*, was of real concern as these species should have been widely spread along this reach of the river. The remaining 52% of species not found were those that had been previously scarce according to FO data. Downstream from XW 15 different species were collected out of the 36 species that should have been collected if under reference conditions. However, out of the remaining 60% of unsampled species only 2 were wide spread (*B. viviparus* and *P. philander*) and 18 were scarce species. Then at the last site measured, Mhinga, 13 different fish species were observed, but this was only 36% of that which should have been caught. Of the unsampled fish species most were previously scarce except for *M. brevianalis*.

7.3.2.2 Informal assessment of number of species and relative abundances

The total number of fish, measured in CPUE, from each site and survey is shown in Table 7.2. A total of 353 specimens representing 41 different species, were caught between 2006 and 2008. As was expected from literature, there was a large spatial heterogeneity in the Luvuvhu River with differences, between the sites, in both fish species type and abundance. For each of the 5 sampling sites only 6 species including *Labeobarbus marequensis*, *Barbus trimaculatus*, *Chiloglanis pretoriae, Labeo cylindricus*, *Micralestes acutidens*, and *Tilapia sparrmanii* were

common throughout and most of which had the highest abundances. Despite this heterogeneity, comparisons between sites could still be made. That is, it was evident in all seasonal samples that there was a definite smaller number of fish species found at Mhinga (5-7) and to an even lesser extent, at Tshikonelo (3-9), compared to XW and Hasana fish communities with species numbers between 8 and 13, and 7 and 11, respectively. In fact XW had the most thriving fish community with the highest number of fish species and abundances in all surveys, with the exception of the survey 3 where Hasana showed greater fish species diversity. With regards to seasonality, there were a few differences in species and abundances observed between the seasons. Lotanyanda showed a seasonal fluctuation in the presence of four fish species, *B lineomaculatus*, *T. sparrmanii*, *B. radiatus* and *B. trimaculatus*. The former two were only present during the surveys 2 and 4, while the latter two were only found during the surveys 1 and 3. Seasonal changes in remaining sites were not really visible, but there was a tendency toward an increase in number of species from 2006 to 2008 at Tshikonelo and towards a higher diversity and abundance in surveys 2 and 4 at XW.

List of the species names abbreviations used in the tables to follow

ABEN	*Anguilla bengalensis*	BUNI	*Barbus eutaeniatus*	MACU	*Micralestes acutidens*
AMOS	*Anguilla mossambica*	BVIV	*Barbus viviparus*	MBRE	*Mesobola Brevianalis*
AURA	*Amphilus uranoscopus*	CGAR	*Clarias gariepinus*	MMAC	*Marcusenius macrolepidotus*
BANN	*Barbus annectens*	CPAR	*Chiloglanis paratus*	MSAL	*Micropterus salmoides*
BEUT	*Barbus eutaeniatus*	CPRE	*Chiloglanis pretoriae*	OMOS	*Oreochromis mossambicus*
BFRI	*Barbus bifrenatus*	CSWI	*Chiloglanis swierstrai*	OPER	*Opsaridium peringueyi*
BIMB	*Brycinus imberi*	GCAL	*Glossogobius callidus*	PCAT	*Petrocephalus wesselsi*
BLIN	*Barbus lineomaculatus*	GGIU	*Glossogobius giuris*	PPHI	*Pseudocrenilabrus philander*
BMAR	*Labeobarbus marequensis*	HVIT	*Hydrocynus vittatus*	SINT	*Schilbe intermedius*
BNEE	*Barbus neefi*	LCON	*Labeo congoro*	SZAM	*Synodontis zambezensis*
BPAU	*Barbus paludinosus*	LCYL	*Labeo cylindricus*	TREN	*Tilapia rendalli*
BRAD	*Barbus radiatus*	LMOL	*Labeo molybdinus*	TSPA	*Tilapia sparrmanii*
BTOP	*Barbus toppini*	LROS	*Labeo rosae*		
BTRI	*Barbus trimaculatus*	LRUD	*Labeo ruddi*		

Table 7.2: The frequency of occurrence (FO) of fish species within the segment and the number of individuals (CPUE) caught at each site.

	Lotanyanda				Hasana					Tshikonelo					XW				Mhinga				
	FO	S2	S3	S4	**FO**	S1	S2	S3	S4	**FO**	S1	S2	S3	S4	**FO**	S2	S3	S4	**FO**	S1	S2	S3	S4
ABEN	**4**	-	-	-	**1**	-	-	-	-	**1**	-	-	-	-	**1**	-	-	-	**1**	-	-	-	-
AMOS	**1**	-	-	-	**1**	-	-	-	-	**1**	-	1	-	-	**1**	-	-	-	**1**	-	-	-	-
AURA	**4**	10	1	-	**3**	-	-	-	-	**2**	-	-	-	-	**1**	-	-	-	**1**	-	-	-	-
BANN	**-**	-	-	-	**-**	1	-	-	-	**3**	-	-	-	-	**2**	-	-	-	**2**	-	-	-	-
BEUT	**4**	-	-	-	**2**	-	-	-	-	**3**	-	-	-	-	**1**	-	-	-	**1**	-	-	-	-
BFRI	**-**	-	-	-	**-**	2	-	-	-	**-**	-	-	-	-	**1**	-	2	1	**1**	1	-	-	-
BIMB	**-**	-	-	-	**-**	-	-	-	-	**1**	-	-	-	-	**2**	-	-	-	**2**	-	-	-	-
BLIN	**4**	2	-	2	**2**	-	-	-	-	**2**	-	-	-	-	**-**	-	-	-	**-**	-	-	-	-
BMAR	**4**	66	16	23	**5**	-	35	1	1	**5**	1	4	3	1	**5**	39	2	2	**5**	-	28	69	7
BNEE	**5**	-	-	-	**2**	-	-	-	-	**2**	-	-	-	-	**-**	-	-	-	**-**	-	-	-	-
BPAU	**3**	-	-	2	**1**	1	-	-	-	**3**	-	-	-	-	**-**	-	-	-	**-**	-	-	-	-
BRAD	**-**	-	2	-	**1**	-	-	-	-	**2**	-	-	-	-	**1**	-	-	-	**1**	-	-	-	-
BTOP	**-**	-	-	-	**2**	-	-	-	-	**2**	-	-	-	-	**2**	-	-	-	**2**	-	-	-	-
BTRI	**4**	-	2	-	**3**	-	6	6	6	**3**	-	-	-	1	**4**	-	-	2	**4**	3	-	6	-
BUNI	**4**	2	2	5	**3**	-	6	11	-	**3**	-	-	-	-	**3**	1	-	1	**3**	-	-	-	-
BVIV	**3**	-	-	-	**3**	-	-	-	-	**4**	-	-	-	-	**4**	-	-	-	**4**	-	-	22	-
CCAR	**-**	-	-	-	**2**	-	-	-	-	**-**	-	-	-	-	**-**	-	-	-	**-**	-	-	-	-
CGAR	**1**	-	-	-	**-**	1	-	-	-	**2**	-	-	-	2	**3**	-	-	2	**3**	1	-	-	-
CPAR	**-**	-	-	-	**-**	-	-	-	-	**2**	-	-	-	-	**3**	-	-	-	**3**	-	-	-	-
CPRE	**3**	4	-	-	**5**	19	2	3	1	**5**	3	2	5	3	**5**	31	51	13	**5**	50	31	29	13
CSWI	**-**	-	-	-	**-**	-	-	-	-	**2**	-	-	-	-	**2**	-	-	-	**2**	-	-	-	-
GCAL	**-**	-	-	-	**-**	-	-	-	-	**1**	-	-	-	1	**1**	9	-	11	**1**	-	-	-	-

Table 7.2 Continued

	Lotanyanda				Hasana					Tshikonelo					XW				Mhinga				
	FO	S2	S3	S4	**FO**	S1	S2	S3	S4	**FO**	S1	S2	S3	S4	**FO**	S2	S3	S4	**FO**	S1	S2	S3	S4
GGIU	**-**	-	-	-	**-**	-	-	-	-	**2**	-	-	-	-	**2**	-	1	-	**2**	-	-	-	-
HVIT	**-**	-	-	-	**-**	-	-	-	-	**1**	-	-	-	-	**2**	-	-	-	**2**	-	-	-	-
LCON	**-**	-	-	-	**-**	-	-	-	-	**-**	-	-	-	-	**2**	-	-	-	**2**	-	-	-	-
LCYL	**3**	16	1	-	**3**	12	-	1	1	**4**	1	-	-	-	**4**	31	-	4	**4**	7	3	7	1
LMOL	**3**	-	-	-	**3**	-	1	-	-	**4**	-	-	-	-	**4**	1	-	-	**4**	-	-	-	1
LROS	**-**	-	-	-	**-**	-	-	-	-	**2**	-	-	-	2	**3**	-	-	-	**3**	-	-	-	-
LRUD	**-**	-	-	-	**-**	-	-	-	-	**-**	-	-	-	-	**2**	-	-	-	**2**	-	-	-	-
MACU	**3**	90	-	17	**4**	-	-	2	-	**5**	-	-	77	1	**5**	40	4	3	**5**	-	-	194	-
MBRE	**2**	-	-	1	**4**	-	-	4	-	**5**	-	-	1	1	**5**	50	4	3	**5**	-	-	-	-
MMAC	**2**	-	-	-	**-**	-	-	-	-	**2**	-	-	-	-	**2**	3	-	-	**2**	-	-	-	-
MSAL	**-**	-	-	-	**-**	-	-	-	-	**-**	-	-	-	-	**-**	-	-	-	**-**	-	-	-	-
OMOS	**2**	-	-	-	**3**	5	11	9	1	**4**	-	2	-	-	**4**	3	1	1	**4**	4	27	2	-
OPER	**-**	-	-	-	**1**	-	-	-	-	**2**	-	-	-	-	**2**	-	-	-	**2**	-	-	-	-
PCAT	**2**	-	-	-	**-**	-	1	-	-	**2**	-	-	-	-	**2**	-	-	-	**2**	-	-	-	-
PPHI	**4**	-	2	1	**4**	-	3	11	24	**4**	-	-	-	-	**4**	-	-	-	**4**	1	-	-	-
SINT	**-**	-	-	-	**2**	-	1	-	-	**2**	-	-	-	-	**2**	-	-	1	**2**	-	-	-	-
SZAM	**-**	-	-	-	**-**	-	-	-	-	**1**	-	-	-	-	**2**	-	-	-	**2**	-	2	-	-
TREN	**1**	-	-	-	**3**	-	4	8	-	**3**	-	-	-	2	**3**	6	1	2	**3**	-	2	-	1
TSPA	**3**	2	-	2	**1**	1	3	4	7	**3**	-	-	2	-	**3**	6	-	-	**3**	-	1	-	-
S		**8**	**7**	**8**		**8**	**11**	**11**	**7**		**3**	**4**	**5**	**9**		**12**	**8**	**13**		**7**	**7**	**7**	**5**
N		**192**	**26**	**53**		**42**	**73**	**60**	**41**		**5**	**9**	**88**	**14**		**220**	**66**	**46**		**67**	**94**	**329**	**23**

Dash represents species that are not present. S is the number of species and N is the number of individuals. CPUE values for site 1 survey 1 and site 6 survey 1 not represented.

7.3.2.3 Species diversity indices

Non-parametric diversity indices represented in Figure 7.4 were used to describe different attributes of the fish communities obtained in the present study. No real seasonal trends were observed, but there were a number of interesting spatial fluctuations that were identified. The graphs showed that there was an increase in the fish species richness and diversity between Lotanyanda and Hasana during all surveys, except for the slight deterioration in species richness and diversity observed at Hasana in survey 4. Then from Hasana to Tshikonelo, the species diversity was greatly reduced in the first three surveys and in fact showed the lowest scores, but then had a dramatic recovery in the last survey especially seen by the log series-alpha index. At XW the species richness and diversity showed conflicting results with a decrease in diversity and an increase in richness from Tshikonelo in survey 2 and a much higher diversity than richness in survey 3. These differences were mainly due to the low and high evenness values, respectively, which had a direct influence on the Shannon diversity index values. That is in survey 2 and 4 there was a low species' evenness, indicating a largely unbalanced number of species to abundances which negatively affect the diversity values at this site. While the high evenness during surveys 3 indicates that there is minimal imbalance between the number of species and abundance and therefore positively influencing the diversity values. Nevertheless there was a generally good recovery from the low index scores at Tshikonelo at all the sites, before the species richness and diversity once again dropped at Mhinga in all surveys.

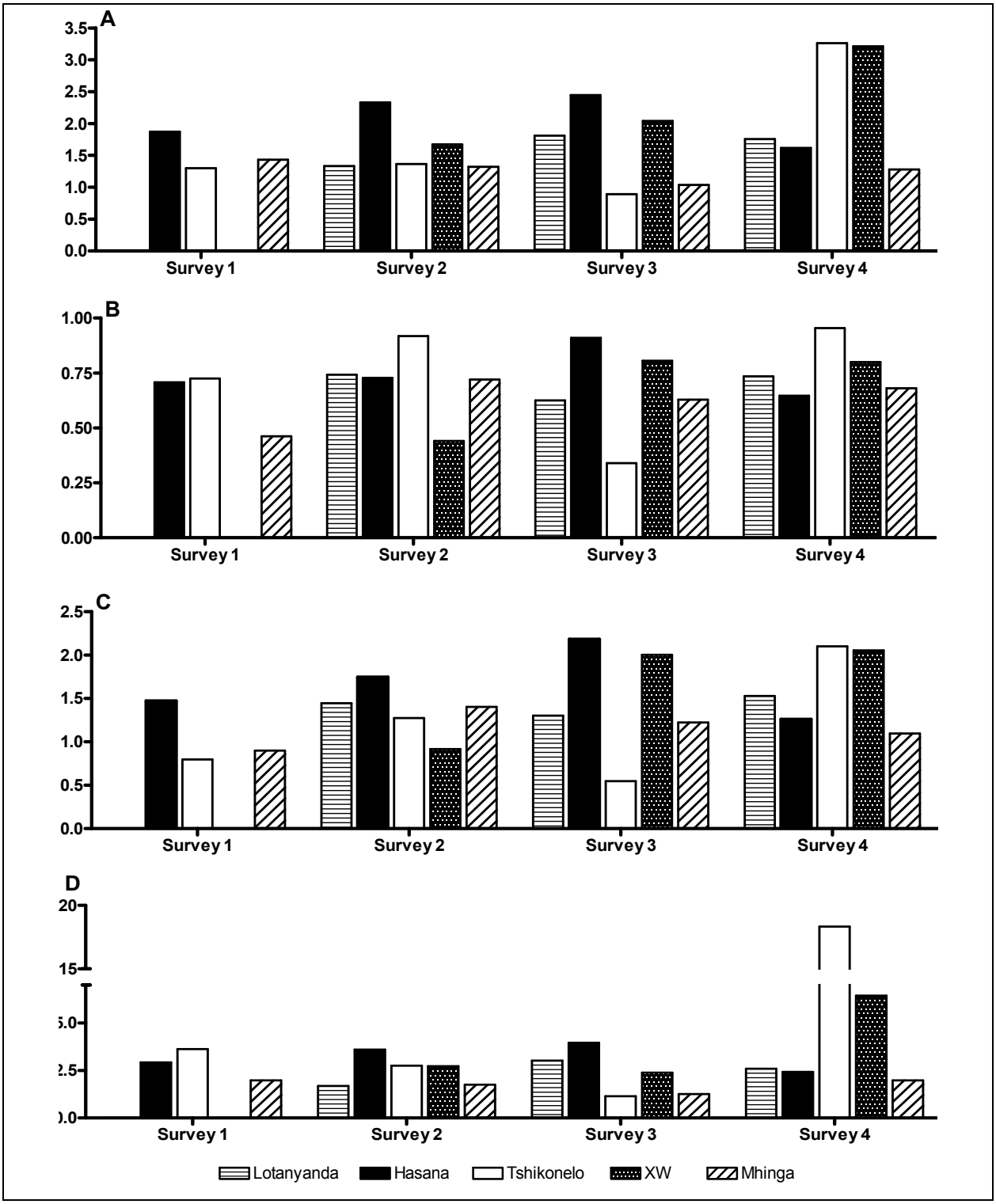

Figure 7.4: Non-parametric fish diversity indices including Margalef's species richness (A), Pilou's evenness index (B) Shannon diversity index (C) and log series diversity index (D).

7.3.2.4 Multivariate statistics

According to the MDS plot and ANOSIM in Figure 7.5, no significant temporal differences were observed for any of the sites. There were however, some significant spatial differences shown by ANOSIM between both Lotanyanda and Mhinga, and Hasana and Tshikonelo (Table 7.3). In fact as can be seen in Figure 7.6, the Bray-curtis dendrograms showed that all of the sites were largely different with no similar spatial clusters/groups and similarities of 60% or more, except for survey 1 (Figure 7.6a) where Hasana and Mhinga had an average similarity of 58, primarily due to similarly high *C. pretoriae* abundances (Table 7.4). Nevertheless, the multivariate analysis of communities in survey 2 revealed that Tshikonelo had a predominantly different fish community structure, with an average dissimilarity of 94 primarily due to the generally low species present at this site (Figure 7.6b). The remaining sites in this season were also distributed on the MDS ordination by the previously mention species, with Lotanyanda and XW having a higher abundances of *M. acutidens* and *M. brevianalis* than at Hasana and Mhinga. During survey 3 (Figure 7.6c), Lotanyanda, Hasana and XW were separated from the other two sites due to their low *M. acutidens* abundances, while XW was separated from Lotanyanda and Hasana due to its higher abundances of *C. pretoriae*. During survey 4 (Figure 7.6d), Hasana had the least common fish assemblage according to dendrograms, primarily due to high abundances of P. philander species. Lotanyanda was also different from the other sites and this was attributed to the *L. marequensis* and *M. acutidens* found in high abundances, whilst XW had high *Glossogobius callidus* and *C. pretoriae* at this site.

Table 7.3: Significant spatial differences of fish assemblage structures observed using one-way analysis of similarity (ANOSIM).

	Global R	P
Site 1 vs. Site 7	0.76	0.03
Site 3 vs. Site 5	0.64	0.03

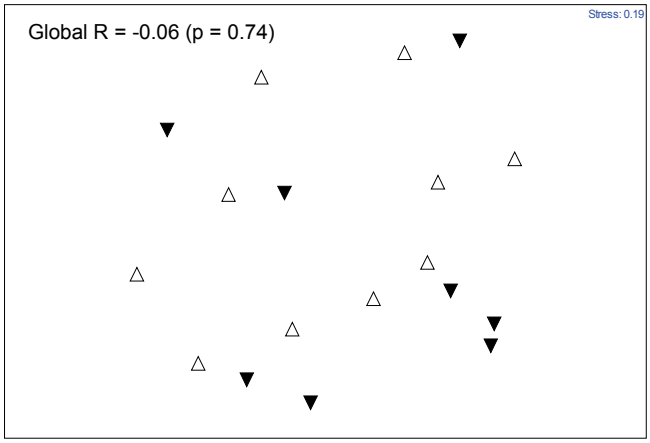

Figure 7.5: MDS ordination showing the seasonal difference between combined fish taxa of low (▼) and high (Δ) flow regimes with one-way analysis of similarity (ANOSIM).

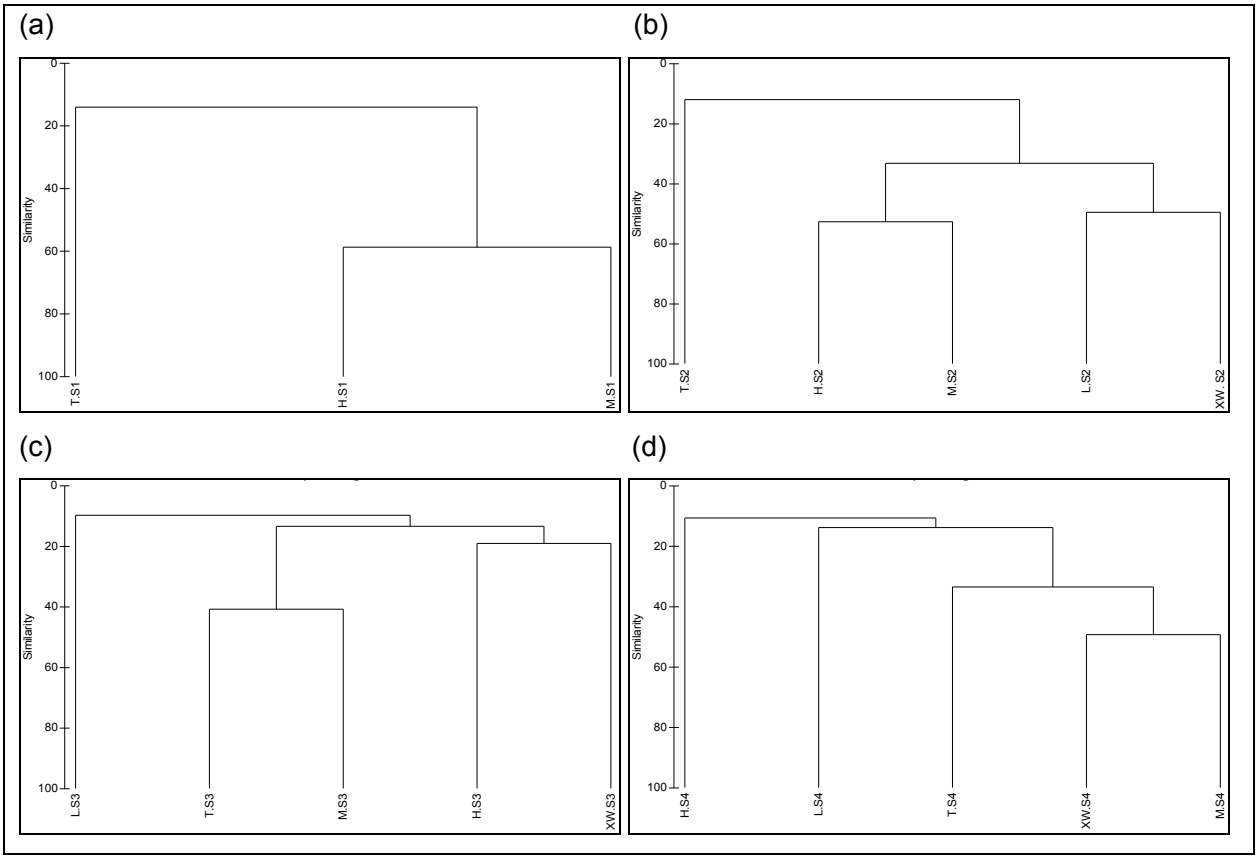

Figure 7.6: Bray-curtis dendrograms of fish assemblages showing spatial differences in (a) Survey 1, (b) Survey 2, (c) survey 3 and (d) survey 4 at Lotanyanda, Hasana, Tshikonelo, XW and Mhinga.

Table 7.4: Fish community attributes contributing toward spatial dissimilarities observed in Bray-Curtis dendrograms.

Surveys	Sites	Attributes
SURVEY 1	Lotanyanda	-
	Hasana	High abundances of CPRE
	Tshikonelo	Dissimilarities defined by other site species
	XW	-
	Mhinga	High abundances of CPRE
SURVEY 2	Lotanyanda	High abundances of MACU and BMAR
	Hasana	Dissimilarities defined by other site species
	Tshikonelo	General low abundances of species
	XW	General high abundances of species
	Mhinga	High abundances of CPRE and OMOS
SURVEY 3	Lotanyanda	Dissimilarities defined by other site species
	Hasana	High abundances of BUNI, OMOS and PPHI
	Tshikonelo	High abundances of MACU
	XW	High abundances of CPRE
	Mhinga	High abundances of MACU and BMAR
SURVEY 4	Lotanyanda	High abundances of BMAR and MACU
	Hasana	High abundances of PPHI
	Tshikonelo	General low abundances of species
	XW	High abundances of CGAL and CPRE
	Mhinga	High abundances of CPRE and BMAR

7.3.2.5 Factors influencing fish abundance and diversity

In Table 7.5, only the variables that showed a significant correlation with the indicators were noted. As can be seen there were very few correlations between the indices and the possible causes and no meaningfully significant ($p < 0.05$) correlations were observed for the abundance and evenness, while the number of species, Margalef's species richness, Shannon diversity index and log series diversity index showed some correlations. pH was negatively correlated with the number of species present, the Shannon and log series diversity indices, while TDS also had a negative correlation, but only with the log series diversity index. Zinc in the sediment also showed a significant negative correlation with fish species richness and log series – alpha, suggesting a possible influence on fish communities. In contrast, the DDE and DDD concentrations were significantly positively correlated with number of species and species

richness, however this relationship was more likely due to other influences. As can be expected there were positive correlations observed between species richness and phosphate as well as between fish abundance and nitrates.

Table 7.5: Pearson's correlation coefficients between variables and fish endpoints that were significantly correlated (P<0.05).*

	S	N	D	J	H	α
pH	**-0.59**	-0.16	-0.38	-0.10	-0.47	**-0.59**
TDS	-0.24	0.14	-0.17	-0.16	-0.34	**-0.78**
Phosphate	0.29	-0.35	**0.50**	0.31	0.43	-0.04
***o,p-'*DDE (s)**	**0.53**	-0.12	**0.57**	0.21	0.41	-0.09
***p,p-'*DDD (s)**	**0.50**	-0.17	**0.68**	0.27	*0.44	-0.03
***o,p-'*DDE (w)**	**0.48**	-0.11	**0.53**	0.16	0.34	-
potassium (s)	0.13	**0.76**	-0.36	-0.27	-0.10	**-0.74**
Zinc (s)	-0.61	0.47	**-0.68**	-0.40	-0.55	**-0.13**

*Including number of species (S), number of individuals (N), Margalef's species richness (D), Pilou's evenness (J), Shannon Wiener (H), log series diversity index (α). Bold-faced values indicate significant correlations ($p < 0.05$).

7.3.3 Invertebrate communities

7.3.3.1 Occurrence of invertebrate families

Although the variations were very slight, some spatial and seasonal differences were present as shown in Table 7.6. Seasonally, the low flow regimes had higher numbers of families and abundances, with averagely lower number of sensitive taxon than in the high flow regimes. Spatially, the Lotanyanda had the lowest number of species and abundances of all surveys with a good ASPT score, while Xikundu showed an opposite trend with the highest number of taxon and abundances with lower ASPT scores.

7.3.3.2 Species diversity

The diversity indices, represented in Figure 7.7, were used to describe the response of the macro-invertebrate community to the water quality of each site. There was a seasonally low species richness and diversity found in surveys 2 and 4 with two exceptions. Firstly, at Lotanyanda and Tshikonelo there was a slightly lower diversity during survey 3 however this was

due to an increase in unevenness. Secondly there was a lower than normal low flow invertebrate community at Hasana in 2007 (survey 3), with a lower family richness.

Upon spatial comparison, there were no sites that particularly stood out throughout the four surveys, with Tshikonelo, XW, Hasana and Mhinga having the lowest community diversities in the four surveys. During survey 1, Tshikonelo showed the lowest family richness and an exceptionally high abundance of baetidae (Table 7.6). During survey 2, XW had the lowest diversity as a result of low evenness particularly caused by generally low family abundances and a high abundance of hydropsychidae. Whilst in survey 3, Hasana had a slightly lower richness. Then in survey 4, there was a lower invertebrate diversity found at Mhinga. This was a primarily due to a higher than normal abundance of atyidae and chironomidae at this site, with a much lower overall abundance.

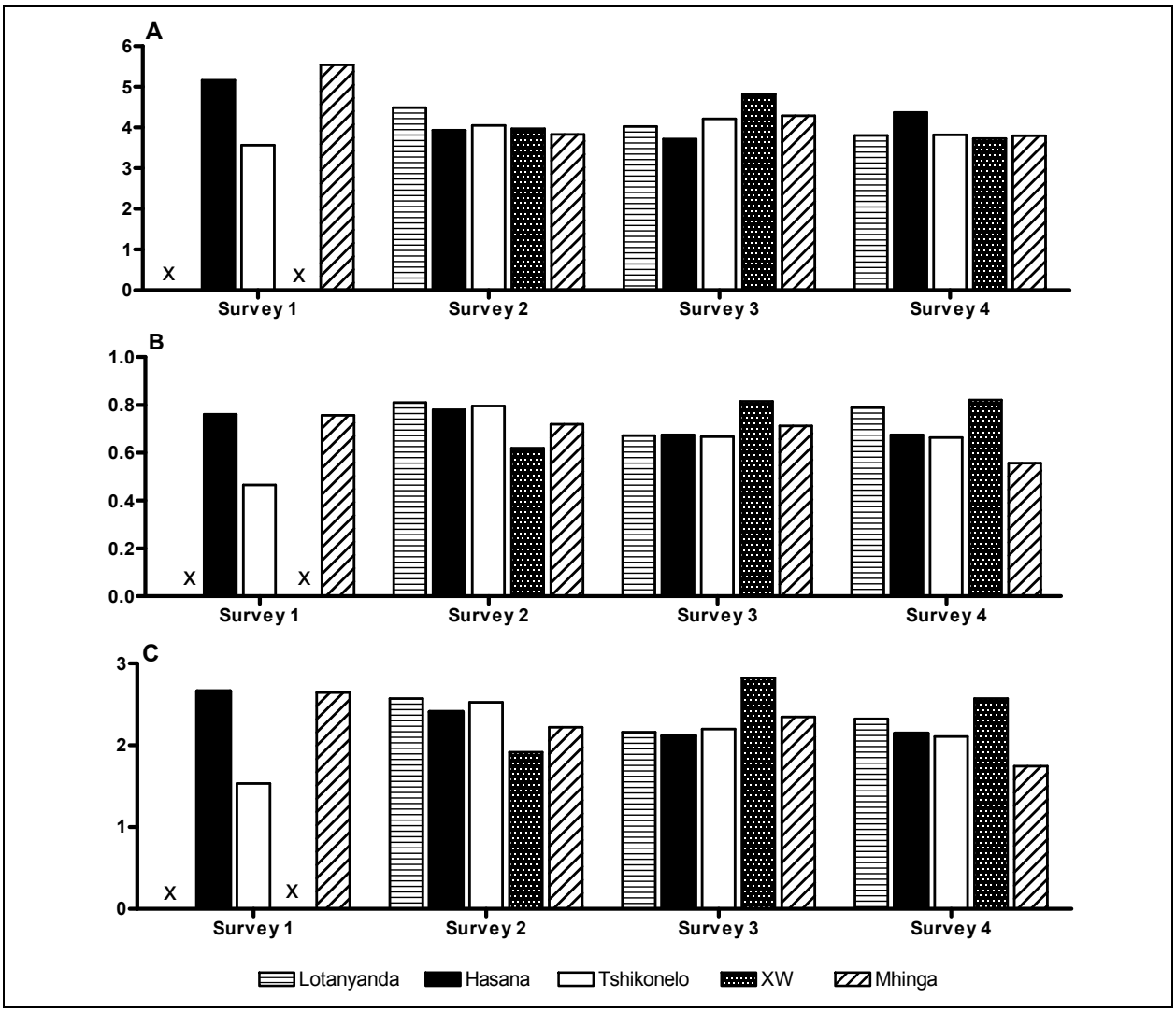

Figure 7.7: Non-parametric diversity indices for macro-invertebrates including Margalef's species richness (A), Pilou's evenness index (B) and Shannon diversity index (C).

Table 7.6: Number of individuals for each macro-invertebrate family caught along with sensitivity scores obtained from SASS 5 scoring sheet from Dallas (2005). The hyphen represents zero families sampled.

SPECIES & SENSITIVITIES		Lotanyanda			Hasana				Tshikonelo				XW			Mhinga			
		S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	S2	S3	S4	S1	S2	S3	S4
Oligochaeta	1	16	3	1	2	6	19	3	-	2	2	-	4	10	12	1	8	5	6
Potamonautidae	3	3	-	15	1	-	-	1	-	-	1	1	1	1	1	-	-	-	-
Atyidae	8	2	4	-	105	33	-	6	-	-	3	32	20	96	80	27	15	11	174
Palaemonidae	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Hydracarina	8	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Perlidae	12	-	-	-	7	1	-	2	-	-	-	2	-	-	4	17	2	7	4
Baetidae	4	3	52	17	16	19	101	71	897	74	164	20	2	24	30	2	8	35	17
Caenidae	6	3	10	2	16	21	96	1	26	2	17	-	12	26	6	7	3	6	9
Heptageniidae	13	24	2	12	9	3	-	1	7	-	3	16	-	-	21	3	1	-	9
Leptophlebiidae	9	34	44	-	12	56	5	18	5	24	14	7	1	9	29	1	91	20	5
oligoneuridae	15	-	-	-	-	-	-	-	-	3	-	37	3	-	7	-	-	-	6
Tricorythidae	9	21	-	-	-	1	-	2	-	30	3	183	-	1	39	-	7	3	3
Synlestidae	8	-	-	1	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Coenagrionidae	4	2	18	24	108	4	4	1	11	1	14	-	1	62	-	2	2	7	3
Playcnemidae	10	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aeshnidae	8	-	-	-	15	-	-	-	-	-	-	-	-	1	-	7	-	-	-
Gomphidae	6	1	17	1	30	1	-	2	9	2	19	7	-	3	5	9	-	5	3
Libellulidae	6	3	3	2	4	-	-	-	28	14	1	10	1	9	3	11	6	4	2
Pyralidae	12	-	-	-	-	1	-	-	-	3	-	2	3	16	-	-	-	3	-
Belostomatidae	3	-	2	-	23	-	-	-	5	-	-	-	-	-	-	1	-	2	2
Corixidae	3	-	2	-	1	-	-	-	5	-	-	-	-	1	-	9	-	-	-
Gerridae	5	1	9	-	4	-	-	-	4	5	-	-	1	-	-	2	-	-	-
Naucoridae	7	5	1	1	3	-	-	-	12	6	7	1	-	6	-	9	-	1	-
Nepidae	3	1	2	-	4	-	-	-	1	-	-	-	-	1	-	7	-	-	-
Notonectidae	3	9	-	-	4	1	1	-	-	-	-	-	2	-	-	25	1	1	-
Pleidae	4	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Veliidae	5	4	84	-	10	3	1	3	36	10	1	1	2	5	4	2	-	4	1
Ecnomidae	8	-	-	-	2	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Hydropsychidae	4	-	1	7	29	12	1	16	251	48	91	33	104	21	28	9	44	16	2

Table 7.6: Continued.

SPECIES & SENSITIVITIES		Lotanyanda			Hasana				Tshikonelo				XW			Mhinga			
		S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	S2	S3	S4	S1	S2	S3	S4
Philopotamidae	10	-	-	-	2	2	-	1	-	5	-	3	-	-	-	-	5	1	-
Polycentropodidae	12	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
Hydroptilidae	13	-	-	-	-	-	-	-	-	7	-	-	-	-	-	-	3	-	-
Leptoceridae	6	2	-	-	-	-	1	1	16	-	13	12	-	3	1	1	-	-	1
Dytiscidae	5	-	-	3	-	-	-	-	2	2	-	-	1	-	-	3	-	-	-
Elmidae	8	-	1	2	14	1	8	4	23	10	51	11	4	68	33	63	5	18	6
Gyrinidae	5	-	2	-	7	-	-	-	9	-	1	-	-	2	-	1	-	-	-
Helodidae	12	5	-	-	-	-	-	-	-	-	-	-	-	15	-	-	-	-	-
Hydraenidae	8	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Hydrophilidae	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1
Athericidae	10	1	1	1	-	-	-	-	1	-	1	1	-	-	1	-	-	-	-
Blephariceridae	15	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ceratopogonidae	5	19	-	-	1	3	3	-	9	1	3	-	2	21	2	1	-	-	-
Chironomidae	2	-	117	19	15	13	55	42	81	17	9	21	7	93	37	19	10	11	65
Culicidae	1	-	5	1	8	5	10	5	6	10	2	1	6	32	3	-	2	2	2
Dixidae	10	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simuliidae	5	-	2	-	-	-	13	3	13	-	1	7	-	3	3	2	1	-	1
Syrphidae	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tabanidae	5	2	-	-	2	1	1	-	18	9	6	1	4	12	11	1	10	12	3
Tipulidae	5	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ancylidae	6	-	2	-	-	-	1	2	1	-	1	-	-	12	-	-	-	1	-
Lymnaeidae	3	-	-	-	1	-	2	-	-	1	1	-	-	11	-	3	1	19	-
Physidae	3	1	-	-	-	-	1	-	-	-	-	-	-	2	-	-	-	2	-
Planorbinae	3	1	-	-	-	-	5	2	-	-	3	-	-	16	-	2	2	114	-
Thiaridae	3	-	-	-	1	14	4	2	-	-	-	-	2	4	-	-	-	2	-
corbiculidae	5	-	-	-	37	8	36	3	-	6	48	1	15	35	7	73	14	117	2
Sphaeriidae	3	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Unionidae	6	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S		**24**	**25**	**18**	**33**	**22**	**22**	**24**	**27**	**24**	**27**	**24**	**22**	**32**	**23**	**33**	**22**	**27**	**23**
N		**168**	**389**	**113**	**495**	**209**	**369**	**193**	**1478**	**292**	**480**	**411**	**198**	**621**	**367**	**323**	**241**	**429**	**327**
ASPT		**5.7**	**5.8**	**5.9**	**5.3**	**6**	**4.6**	**6.1**	**5.6**	**6.4**	**5.7**	**7.6**	**5.7**	**5.1**	**6.4**	**5.7**	**6**	**5.6**	**6.2**

7.3.4 Multivariate statistics

The multi-dimensional scaling (MDS) plot shown in Figure 7.8 illustrates the significant difference between the surveys 1 and 3 (low flow regimes) and surveys 2 and 4 (high flow regimes) obtained by ANOSIM calculations. These differences were generally a result of higher abundances in surveys 1 and 3 (Table 7.6), although surveys 2 and 4 did have higher abundances of atyidae, tricorythridae, leptophlebiidae, heptageniidae, oligochaeta and oligoneuridae that contributed toward the dissimilarity of the two flow regimes. Due to this high difference between flow regimes, the spatial variations were separated according to each sampling regime.

As shown in Figure 7.9, there were no consistent spatial trends between any of the flow regimes. In survey 1, comparisons between all the sites showed that Tshikonelo had the most dissimilar macro-invertebrate community assemblage (Table 7.8). This was primarily based on the dominance of the mayfly family baetidae, which then differed in survey 2, 3 and 4. In survey 2, the Lotanyanda invertebrate community was predominantly dissimilar based on higher abundances of heptagenidae and ceratopogonidae. Similarly large dissimilarities in Lotanyanda communities were found during survey 3, although during this survey, the chironomides and velidae where the characterising taxa contributing toward the average dissimilarity. It should also be noted that Hasana was also fairly dissimilar during this survey, which was a direct result of an above normal number of caenidae and baetidae present. The remaining sites including Tshikonelo, XW and Mhinga had community structures that could be grouped primarily due to high abundances of corbiculidae, elmidae and baetidae. Then during survey 4, the Lotanyanda communities were again the most dissimilar due to coenagrionidae populations, Hasana was characterised by baetidae and chironomidae and the remaining sites were again grouped according to their similar atyidae and tricorythridae populations.

Table 7.7: Significant spatial differences of the invertebrate assemblages using one-way analysis of similarity (ANOSIM).

	Global R	P
Site 1 vs. Site 3	0.41	0.03
Site 1 vs. Site 5	0.41	0.03

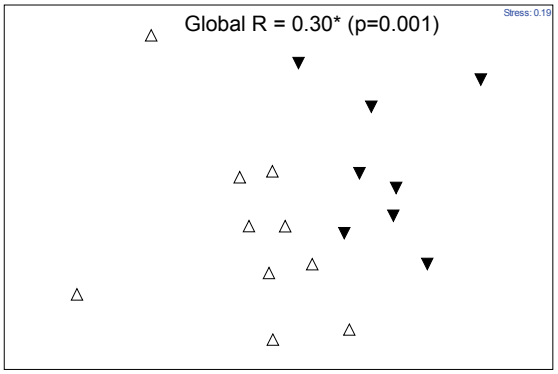

Figure 7.8: MDS ordination showing the significant (ANOSIM) seasonal differences between low flow and high flow regimes based on macro-invertebrate taxa of low (▼) and high (Δ) flow regimes.

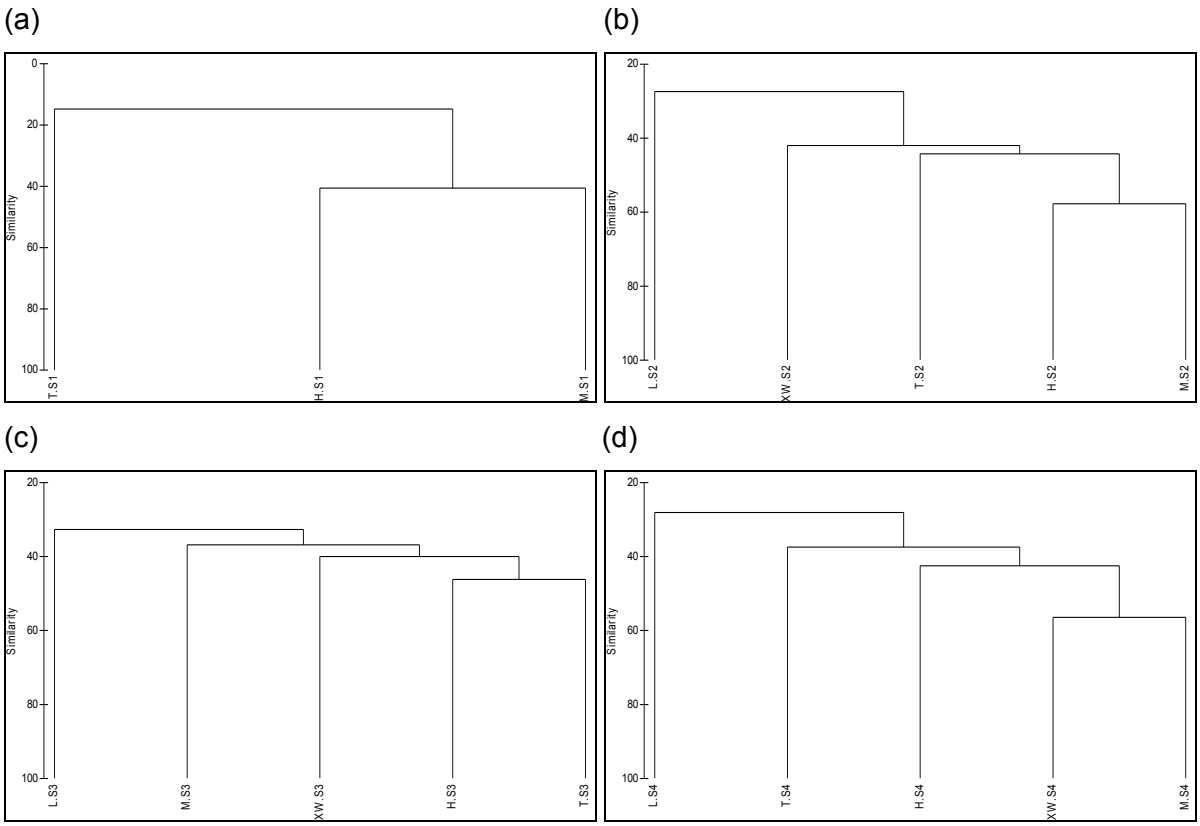

Figure 7.9: Bray-Curtis dendrograms showing spatial differences in (a) survey 1, (b) survey 2, (c) survey 3 and (d) survey 4 for macro-invertebrate assemblages at the Lotanyanda, Hasana, Tshikonelo, XW and Mhinga.

7.3.5 Factors influencing invertebrate abundances and diversity

As shown in Table 9 only those variables with significant correlations were noted. In the low flow surveys there was a significantly negative correlation between the ASPT scores and phosphate and *o,p'*-DDE in the sediment. The latter was primarily a result of the 0.2 µg/kg DDE in the sediment of Hasana during survey 3. Then the species richness, number of species and abundance were significantly correlated with the oxygen in the water. As for the high flow surveys ammonia was significantly correlated with the number of species, nitrate was negatively correlated with evenness and zinc in the sediment was correlated with the Shannon diversity index.

Table 7.8: Fish community attributes contributing toward spatial dissimilarities observed in Bray-Curtis dendrograms.

Surveys	Sites	Attributes
SURVEY 1	Lotanyanda	-
	Hasana	High abundances of Corbiculidae and Atyidae
	Tshikonelo	High abundances of Baetidae
	XW	-
	Mhinga	Average similarity of 41 with Hasana: same attributes
SURVEY 2	Lotanyanda	High abundances of Heptagenidae
	Hasana	Generally high abundances with high Hydrophysidae and Leptophlebidae
	Tshikonelo	High abundances of Baetidae
	XW	Average similarity of 48 with Hasana and Mhinga: same attributes
	Mhinga	Average similarity of 48 with Hasana and XW: same attributes
SURVEY 3	Lotanyanda	High abundances of Chironomidae and Veliidae
	Hasana	High abundances of Caenidae and Baetidae
	Tshikonelo	Generally higher abundances with high Corbiculidae, Elmidae and Hydropsychidae
	XW	Average similarity of 40 with Tshikonelo and Mhinga: same attributes
	Mhinga	Average similarity of 40 with Tshikonelo and XW: same attributes
SURVEY 4	Lotanyanda	High abundances of Coenagrionidae
	Hasana	High abundances of Baetidae and Chironomidae
	Tshikonelo	High abundances of Atyidae and Tricorythridae
	XW	Average similarity of 40 with Tshikonelo and Mhinga: same attributes
	Mhinga	Average similarity of 40 with Tshikonelo and XW: same attributes

Table 7.9: Pearson's correlation coefficients between variables and macro-invertebrate endpoints that were significantly correlated (P < 0.05) for the low flow regimes (S1 and S3) and high flow regimes (S2 and S4).

SURVEY		S	N	D	J	H	ASPT
SURVEY 1 & 3	**Oxygen**	**0.95**	**0.96**	**0.92**	0.73	0.82	0.19
	Phosphate	0.69	-0.10	0.47	0.41	0.49	**-0.98**
	***o,p-'*DDE (s)**	-0.63	-0.22	-0.41	-0.06	-0.19	**-0.83**
SURVEY 2 & 4	**Ammonia**	**-0.85**	-0.50	-0.23	0.29	0.13	-0.19
	Nitrite	0.26	0.42	-0.14	**-0.64**	-0.59	0.18
	Zinc (s)	0.14	0.13	-0.11	-0.84	**-0.89**	-0.19

7.4 Discussion

Aquatic community structures have been widely used to assess the effects of humans on water quality and habitat within an aquatic ecosystem (Bervoets et al., 2005). However few studies have directly assessed the effect of a specific stressor such as DDT on the aquatic ecosystem communities. Therefore in the current survey the effects on fish and macro-invertebrate communities were assessed in the Luvuvhu River catchment specifically focusing on the influences of DDT contamination.

7.4.1 Fish

In the Lotanyanda River there was only a slight reduction in the fish community diversity compared to the other sites, with a definite reduction in *B. eutaenia and B. neefi* distribution. The significantly reduced abundances of these species were perhaps due to an altered environmental condition, similar to the findings by Fouché et al. (2005). The authors also showed a lack of these two species in the upper reaches of the Luvuvhu River and attributed this to the fact that these species were more specialist and sensitive species. With regards to seasonal changes at this site, there was lower fish diversity as shown by Shannon diversity index and informal assessments in the survey 3 with a sudden absence of *C. pretoriae*, *T. sparrmanii*, *M*. *acutidens,* and *B. lineomaculatus*. *C. pretoriae* was not found again at this site (also not present in the survey 4) possibly due to deterioration in either the water flow requirements or water quality changes (Kleynhans, 2005). Indeed there was 0.1 µg/kg of bioavailable DDE and DDT in the sediment as well as higher than normal nitrate levels, which could have influenced these sensitive populations negatively. With regards to *M*. *acutidens,* and *B. lineomaculatus* these species did recover in the following high flow suggesting that these species showed natural seasonal fluctuations. Indeed, similar

fluctuations of these species were shown by Fouché et al. (2001), where the species' were only found in the summer months. In fact according to the informal assessments, there was a generally lower fish species' abundance in the survey 3. This could perhaps be due to a reduction in an already lower habitat availability at this site, with an average water surface width of 2 meters and depth of less than 0.5 meters (Bicknell, 2005; Kaemingk et al*.,* 2007; Fouche et al*.,* 2001) that yielded this site as strongly dissimilar to sites within the Luvuvhu River (as shown by the multivariate analyses).

The log series showed slightly different temporal trends. In this index the high flow regimes (survey 2 and 4) showed a reduced diversity compared to the low flow regimes (survey 1 and 3). This was surprising since the informal assessment showed that there were more species and number of individuals present in the high flow regimes than in the low flow regime. Similar results were shown by Russel (1997) that explained the reason for this inconsistency is because the indices utilise species richness that depicts the ratio between the number of individuals and number of species and as such a sample set with a lower number of species and abundance can produce the same or higher index value as a sample set with higher number of species and abundances. Although these attributes can and have been shown to be advantageous, it can lead to a misinterpretation of data. Thus in the present study the log series diversity index should be utilised with caution. Upon comparison between this index and the other indicators utilised in the current study it was evident that the good diversities depicted in survey 3 and survey 4 was indeed misleading, especially in survey 3 that was shown to have a reduction in important species such as *C. pretoriae*.

The fish assemblages of Hasana were generally unchanged with the majority of the species not sampled due to sampling inefficiencies. A large percentage of fish species that were absence at this site had high preferences to slow deep habitats (as defined by Kleynhans, 2007). This suggests that sampling of slow deep habitats were not as efficient at this site (as well as the remaining sites). The habitats were sampled with a small seine net which could have been too small for the average depth. According to Hayes (1983), seining is not very effective in deep water over rough bottoms as the fish then often escape underneath the lead line. Furthermore no gill nets were used and cast nets were largely ineffective. Once these sampling inefficiencies were corrected for, the fish assemblages at Hasana seemed to be in generally good condition compared to the other sites with *A. uronoscopus*, *B. viviparous* and *L. molybdinus* the only species that were not sampled that were predicted as being widespread within this reach. In the survey 2 and survey 3 the diversity indices showed that fish assemblages were moderately natural with only slight changes. However in the survey 4

the fish diversity was slightly reduced as shown by all the indicators. The possible cause for this reduced fish community integrity was perhaps changes in water quality since there was a lower abundance of sensitive species at this site including C. pretoriae and *L. marequensis*. Indeed upon comparisons with contaminants there was a presence of the combination of DDE and PCB 153 within the water that may have resulted in changes of the fish community. A similar study by Eaton and Lydy (2000) showed similar reduction in fish communities in the presence of a combination of OC contaminants, although as was shown in this study there was significant relationship between the indicators and the individual OC concentrations.

Tshikonelo had the most dissimilar fish community structure compared to all the other sites in the first three seasons. This was generally attributed to lower diversities and abundances, which was observed with the informal descriptions and the diversity indices including Shannon diversity index and the log series diversity index. Upon initial evaluation of these indicators it was evident that no consistently high contaminants were correlated with the low fish diversity at this site and that the possible cause for the large absence of fish could be due to sampling inefficiency or fish movement. Once the sampling inefficiencies were accounted for, the primary cause of change from reference was due to a reduced amount of habitat available for fish, with a large lack of species that have a high preference for vegetation including *B. annectens*, *B. eutaenia*, *B. paludinosus*, *B. viviparous*, *L. molybdinus* and *P. philander*. Indeed many habitat perturbations were evident including water extraction trucks, actual removal of river sand, and cattle grazing within the riparian zone which resulted in erosion, and general loss of habitat. According to Russel (1997), there has been a marked reduction of marginal vegetation in Luvuvhu River over the years and in fact also showed a reduction in both *B. viviparus* and *P. philander* in the Luvuvhu.

With regards to the recovery of the species diversity in the last season (survey 4) it was due to a increased number of juveniles from adult species that were generally not caught using the techniques in this study such as *C. gariepinus*, *L. rosae*, and *T. rendalli*. This increased FO (frequency of occurrence) of juvenile specimens indicates that there was an increase spawning success from the previous season suggesting a recovery in the fish community in this season. The possible reason could be due to increased habitat availability at this site as shown by higher HCR scores compared to previous spawning seasons (Figure 7.3).

The multivariate analysis seemed to identify more subtle changes between the fish communities than the other indicators. For instance the reduced abundance of *C. pretoriae* and *L. marequensis* were a major cause of dissimilarity compared to other sites in all of seasons. These species were shown as a very sensitive species to changes in flow and/or

water quality suggesting an increased perturbation at this site. As was shown in previous chapters the water quality at this site was not significantly reduced, suggesting that reduced flow (combined with reduced habitat) may have influenced the presence of these species at this site probably due to reduced discharges from ND and Mutshindudi River (Figure 7.1). Then in survey 3 the large dissimilarity was primarily a result of the appearance of a shoal of *M. acutidens* that was probably migrating upstream after the first summer rains (Skelton, 2001).

In comparison to all the sites XW had the most thriving fish community according to the diversity indices. It had high numbers of sensitive species such as *C. pretoriae, L. molybdinus* and *M. acutidens*, despite that this site had high pesticide contamination. As was seen in a previous chapter the majority of contamination occurred at XW in survey 4, however the fish communities did not reflect this in any of the indicators utilised. This contradicts some studies that have shown negative effects on fish populations when exposed to contamination (Vasseur and Cossu-Leguille, 2006; Eaton and Lydy, 2000). The probable reason for this was because the concentrations of the contaminants were either not high enough or the exposure period was not long enough to induce a population/community level effect (Connell, 1999). Apart from the pesticide contamination there were also large changes within the habitat at XW including degradation of stream integrity due to the weir as well as a substantial amount of bank erosion downstream from the weir. The possible reason for the good fish community assemblage at this site may be because of the presence of the fish ladder. The fish ladder probably provided a fair amount of protection and habitat for the fish communities as was demonstrated by Jensen et al. (1999).

Upon evaluation of the seasonal changes that occurred at XW it was evident that the survey 3 had the most changed fish community assemblage, which was apparent in all of the indices. The reason for this was due to the absence of a large amount of specimens that had a high probability of being sampled including *C. paratus*, *T. sparrmanii*, *B. unitaeniatus*, *L. cylindricus*, *L. molybdinus*, *P. philander*, *B. trimaculatus*. Most of these species had a strong sensitivity to reduced vegetation, which suggested that these specimens may have migrated upstream in search of better habitats as a consequence of the weir or in the case of *M. acutidens* and *L. cylindricus* for an annual breeding migration upstream (Skelton, 2001; Wyle and Booth, 1999). As for the log series index, it seemed to accurately identify change in species abundance in contrast to the previous sites. This may have been due to the higher number of species present at this site allowing for greater discriminatory abilities between the seasons, although this was in contrast to Magurran (1988) who emphasised that this index is not unduly influenced by sample size.

Mhinga had a similar fish community structure to Tshikonelo, with slightly higher fish diversities throughout the sampling periods. However there were many fish species that should have been sampled compared to reference data, although most were scarce. As described in previous sites the lack of sampling of these species was not entirely due to their extinction but rather that they were not sampled due to the sampling methodologies. Once these inefficiencies were accounted for there was still a low number of species with varying abundances throughout the sampling regimes. The lowest species and abundances (high evenness) were observed in survey 4 by the informal assessments. The informal assessments showed that the majority of species were either absent or largely reduced compared to the other seasons. They included many various types of species was differing habitat preferences. These results suggest that in the survey 4 there was a large impact on the fish community structures at Mhinga. Although no species with the same preferences were consistently absent, there was a combination of contaminants above water quality guidelines in this flow regime. As shown in a previous chapter there was a combination of DDE, dieldrin and lindane in the water which may have resulted in the change in fish community observed similar to that of Hasana.

These results were however not reflected in the species richness index and log series diversity index. All these indicators showed that the fish diversity in survey 3 was the most reduced. These reduced values in the diversity indices suggested that there was a decline in the desired state of a fish community. However upon inspection of the informal abundances and species numbers in the survey 3 there were higher abundances and a high number of species. The main reason for this is because the indices essentially depict the ratio between the number of individuals and the number of species and as such result in conflicting results as explained for the Lotanyanda fish community above. This suggests that the results depicted by the log series diversity and species richness indices are not an adequate representation of fish community structures and that the survey 3 fish communities were largely misrepresented. Nevertheless the main cause for the high abundances in the survey 3 at Mhinga was the collection of a *M. acutidens* shoal, perhaps also migrating upstream as shown in Tshikonelo.

7.4.2 Invertebrates

Stream macro-invertebrate populations and communities are highly recommended as their sedentary nature allows for effective spatial analysis of pollution and disturbance effects. In the present study, Hasana community structures during survey 3 was most deteriorated with

an average sensitivity score of 4.5, which is (according to Thirion et al., 1995) an indicator of a “fair” water quality (most of the sites had good scores ranging from 5 to 7.6). A possible explanation for the deterioration could be due to the DDE concentrations in the sediment, which were significantly correlated with the ASPT score, or due to the higher nitrites and phosphate concentrations. The former results were, however, surprising as the concentration of 0.1 µg/g DDE in sediment was much lower than what was found in other sites with seemingly unperturbed assemblages. For example the assemblage at Tshikonelo during survey 4 (2008) had DDD and DDE concentrations of 3.8 µg/g in sediment and 0.5 µg/l in water, respectively, with an average score per taxon of 7.6, which is an indication of “excellent” water quality according to Thirion et al. (1995). This suggests that the combination of pollutants including DDT and its isomer concentrations in both the water and sediment are not an influencing factor on invertebrate community structures. According to a review done by EXTOXNET (1996), aquatic invertebrates can experience mortality at ranges between 0.18 µg/L to 7 µg/L after 96 hours of continuous exposure, while mortalities are only observed at concentrations of 4.7 µg/L-15 µg/L after 48 hours of exposure. Thus the lack of DDE influence on communities could be due to the invertebrate families being exposed in lower durations and if there are effects they are only present at sub-cellular level, which was not assessed in the present study. The higher nitrites and phosphate concentrations however could have resulted in the lower number of sensitive species at Hasana as well as the higher abundance of caenidae and baetidae. These two families, contributing toward the high dissimilarity of Hasana, are predominantly collector gatherers, primarily feeding on particulate matter including diatoms, algae and phytoplankton (Barbour, 1999). The high abundances of the families could thus be due to increased availability of these diatoms, algae or phytoplankton that may have resulted from higher nutrient levels (Alanson, 1995; Davies and Day, 1998; Rudek et al. 1991). Similarly higher nutrient levels were found during survey 1 at Tshikonelo, which could have perhaps also explained the 167 baetidae individuals that caused a disturbance in the community structures at this site. However it should be noted that none of the nutrient levels were above the ideal scoring range of the water quality guidelines (DWAF, 1996a) and that there were no significant correlations observed. According to Barbour et al. (1999), a relative increase in baetidae abundance can also be an indicator of organic pollution such as sewage outfall, which could also be a possible explanation for both low flow sites community structural changes.

In the high flow regimes different spatial trends were apparent. Specifically, during survey 2 it was evident those XW macro-invertebrates also had higher abundances of hydropsychidae than normal; also shown by Barbour et al. (1999) as an indicator of increased organic

pollution. However, deviation in community assemblage was not reflected in the Bray-Curtis similarity matrices analysis, suggesting that at the community assemblage level, the impact of this family dominance was perhaps negligible. Then lastly, during survey 4, Figure 7.7 shows that Mhinga had the most uneven macro-invertebrate assemblage, with a contrastingly good ASPT score (Table 7.6). Thus, suggesting that factors other than water quality were influencing the assemblages at this site. Perhaps the higher number of atyidae and chironomidae resulted from increased organic matter, while the generally lower abundance found in the stones and GSM habitats may have resulted from reduced sampling success due to increased water velocities in the high flow.

Most documented macro-invertebrate assemblages undergo a significant natural seasonal succession within a lotic environment. This same seasonality can be clearly seen in the MDS plots in Figure 7.8 and further confirmed with informal descriptions and diversity indices. Evidently, the low flow regimes' invertebrate assemblages had a significantly higher distribution and abundance than the high flow regimes. Explanations for this natural distribution is however extensive and thus won't be dealt with here; an extensive review for South African stream macro-invertebrate variability's can be found in Dallas (2002).

This seasonal trend however did not coincide with ASPT score variations. The taxa sensitivities were in fact lesser in the low flow regimes than in the high flow regimes. This corresponds with many studies that have shown an increase in community structural perturbations in the low flow. What was evident from the studies was that the lower water flows usually resulted in either a reduction of suitable habitat for sensitive species (Henning, 2001) or an increase in nutrient and toxicant concentrations, which were not found at such high concentrations in the more diluted high flow regime (Garnier et al*.,* 1995; Gasith and Resh, 1999; Moreau et al., 1998)

7.5 Conclusions

In conclusion, the fish communities showed a number of influencing factors in the Luvuvhu River. Firstly, it was found that the major decline in the species distributions from reference distributions was not due to the absence of fish species within in the Luvuvhu River but mainly because the species were not sampled due to the lack of specialised sampling techniques such as angling and gill nets. Nevertheless once these sampling inefficiencies were accounted for, the fish communities did highlight some important results. None of the fish communities were influenced by DDT or its isomers, nor any other contaminant

measured in this study. This was probably due to the fact that the exposure duration and/or concentrations were too low in the Luvuvhu River to induce an effect at the community level of biological organisation. The fish communities were however affected by changes in the physical habitat, both natural and anthropogenically induced such as reduced vegetation and increased erosion in the riparian zone from cattle and human activity, as well as changes habitat availability due to the changes in flow as a result of the presence of impoundments (weir at Xikundu and the Nandoni Dam upstream from Tshikonelo). As for the spatial changes, Tshikonelo and Mhinga showed the most altered fish community structures, whilst Xikundu and to a lesser extent Hasana had less modified community structures. Then upon evaluation of temporal trends there were a number of fluctuations apparent, with no real seasonal changes contributing toward the changes. What was not evident from the results in this study was the effect of fishing by the local communities on the health of the fish communities within the Luvuvhu River catchment. At all of the sites within the Luvuvhu River extensive fishing using gill nets, seine nets, cast nets and traps was observed and therefore it is recommended that further investigations be done on this matter.

As for the macro-invertebrate community structures, they were strongly influenced by seasonal changes, but were still able to identify negative effects within the Luvuvhu River. As was shown by the fish communities, there were no significant effects on invertebrate communities due to DDT on the other contamination. The changes that were apparent at most of the sites, within the Luvuvhu River, were hypothesised as being due to organic (sewage) pollution and increased nutrient levels as a result of the locals washing and bathing within the Luvuvhu River, on a daily basis. The Lotanyanda invertebrate community was the most dissimilar compared to all the other sites in the Luvuvhu River, however the site that showed the most change from natural conditions was Hasana.

CHAPTER 8

THE USE OF *OREOCHROMIS MOSSAMBICUS* AS A SENTINEL SPECIES OF THE POSSIBLE EFFECTS ON HEALTH AND REPRODUCTION OF DDT EXPOSURE FROM A DDT SPRAYED AREA

Bremner KJ and Van Vuren JHJ

8.1 Introduction

Pesticides are becoming increasingly predominant environmental contaminants due to their extensive use all over the world, particularly in the developing countries (Parvez and Raisuddin, 2005). The organochlorine insecticides were amongst the first pollutants shown to cause adverse population effects. The potential adverse effects of these pollutants on wildlife are a cause for great concern. The severity of their effects was sometimes surprising given the low levels of the compounds in environmental compartments such as surface waters and soils. These toxic chemicals with characteristically long half lives in the environment were released on a large scale all over the world while there was still very little knowledge of their potential effects on the environment and are now commonly known worldwide as POPs (persistent organic pollutants). High lipophilicity combined with chemical stability and very slow biodegration are characteristic features (Vasseur and Cossu-Leguille, 2006).

Adverse effects upon populations may be avoided if the potential of chemicals to cause these effects are recognized before problems arise. This approach needs to take into account the potential of chemicals to have long term effects. Long term effects may be simply due to compounds showing marked biological persistence and therefore the tendency to undergo longer term biomagnification with movement along food chains, as in the case of POPs. In adopting a mechanistic approach to identifying chemicals that may have adverse ecological effects, attention should be given to the potential of these chemicals to induce long term effects (Vasseur and Cossu-Leguille, 2006).

In recent years, biochemical, physiological and histological measurements have been used increasingly in ecological assessments to assess the effects of toxic exposure on biota (De Coen, 1999). These biomarkers can be defined as xenobiotically induced variations in cellular or biochemical components or processes, structures, or functions that are measurable in a biological system or sample. They are endpoints of ecotoxicological tests which register a potential effect on a living organism (Connel et al., 1999), measurable

responses to the exposure of an organism to xenobiotics. They usually respond to the mechanism of toxicity rather than the presence of a specific toxicant and are increasingly being used as valid indicators of aquatic pollution (Riffat and Ahmad, 2006).

Biomarkers offer a number of advantages compared to the conventional ecotoxicity tests. According to De Coen (1999), biomarkers can generally be considered as measures of the first toxicological interactions between the chemical and the biological receptor site. This interaction induces a cascade of events starting at the subcellular level and eventually leads to adverse effects at higher levels of biological organization. The effects normally studied in conventional toxicity tests can be considered as the final result of accumulating damage at the suborganismal level (De Coen, 1999). Toxicity appears first in the individuals before populations are affected. Individual effects of greatest concern include neurotoxicity, behavioural changes, endocrine disruption, effects upon reproduction, immune dysfunction and genotoxicity (Vasseur and Cossu-Leguille, 2006). Biomarkers are thus extremely good indicators of toxic stress, with stress being defined by De Coen (1999) as “a state produced by an environmental or other factor which extends the adaptive responses of the organism beyond the normal range or which disturbs the normal functioning to such an extent that the chances of survival and/or reproduction are significantly reduced”.

Fish (*Oreochromis mossambicus*) were collected using gill nets and biomarker analyses include the following:

8.1.1 Cellular energy allocation (CEA)

Organisms exposed to suboptimal conditions face an a priori cost of combating stress in terms of metabolic resources. The energy available for maintenance, growth and reproduction, based on the biochemical analysis of the energy budget rather than on the direct measurement of those endpoints, thus provides a sensitive measure of stress in an organism. Under normal circumstances, fish will use available resources for maintenance, growth and reproduction. When facing an additional cost like restoring damage from pollutant exposure, fish must relocate some of the acquired energy to repair and/or maintain their physiological integrity. If fish use more energy than can be generated, the energy budget may even turn negative. Thus, because changes in energy budget reflect how energy availability and accumulation evolves within an organism in time, it is often a sensitive and ecologically relevant sublethal indicator of stress in organisms (Smolders et al., 2003a).

8.1.2 7-ethoxy resorufin O-deethylase (EROD)

The mechanism of cytochrome P450 (CYP) dependent mixed function oxidases with particular reference to 7-ethoxy resorufin O-deethylase (EROD) activity, as a response of the organism to the presence of pollutants in the aquatic environment has shown to be very popular. The advantages of using EROD as a biomarker are specificity, high sensitivity, feasibility and simplicity of its measurement. CYP450 activity measured in terms of EROD activity has been used as a biomarker by a number of investigators (Riffat and Ahmad, 2006). EROD activity has been shown to respond to a variety of organic pollutants like polychlorobiphenyls, polyaromatic hydrocarbons and different pesticides. Induction in EROD with respect to the control has been observed in a variety of systems (Riffat and Ahmad, 2006). According to Zapata-Perez et al. (2000), EROD activity correlates with *o,p'*-DDE, *p,p'*-DDE and *p,p'*-DDD concentrations. Most studies conducted in fish have reported significant induction in EROD following exposure to classical CYP1A1 inducers such as PAHs and PCBs (Riffat and Ahmad, 2006). EROD induction by the pesticides may be inhibited to a degree by the presence of metals (Riffat and Ahmad, 2006).

8.1.3 Catalase (CAT) – gills

All aerobic organisms need molecular oxygen for their oxidative metabolic processes but as a consequence must also deal with the formation of dangerous reactive oxygen species (ROS) including superoxide anion (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical (-OH). ROS attack and chemically alter cellular macromolecules including proteins, lipids and DNA causing metabolic damage that is often severe (Bagnyukova et al., 2005) and often resulting in cumulative organ damage (Lushack et al., 2005). All organisms therefore possess well-developed systems of antioxidant defense that include both low molecular weight antioxidants and antioxidant enzymes. The main antioxidant enzymes are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). Under normal physiological conditions there is the balance between ROS generation and their elimination by different antioxidant scavengers. If prooxidant processes are enhanced and/or the power of the antioxidant system decreased, oxidative stress arises. Although all protective components are needed to cope with oxidative stress, each particular enzyme accomplishes specific functions that can only be partly replaced by the actions of other antioxidant defenses (Bagnyukova et al., 2005).

Fish have been known to possess high antioxidant defenses particularly in the liver and the kidney, which are both metabolically active tissues. These tissues are sites of xenobiotic detoxification and are thus considered to be powerful ROS generators (Bagnyukova et al., 2005).

8.2 Materials and methods

8.2.1 Data collection and location of sample sites

Fieldwork was carried out along the Luvuvhu River in the Limpopo Province, South Africa, during both the "low and high flow surveys" of 2006 and 2007 (Surveys 1 to 3). Three sites were selected, namely the Albasini dam (AD) (to be used as a reference site), the Nandoni dam (ND) and then the Xikundu Weir (XW).

8.2.2 Equipment preparation

All glassware used for water and sediment collection was washed before use. Glassware was washed and placed in a soap bath containing a two percent Contrad TM (Merck Chemicals) solution for 24 hrs. Thereafter, it was rinsed with dH_2O and placed in a 1 M hydrochloric acid bath (Merck Chemicals) for another 24 hrs. It was then rinsed again with dH_2O, allowed to dry and re-rinsed with chromatography grade ethanol. Sterile eppendorf tubes were used for the collection of all biomarker analysis tissue.

8.2.3 Sampling protocol

One sampling survey was conducted during the high flow hydrological regime (10-11 March 2007, survey 2) and two sampling surveys were conducted during the low flow hydrological regime (1-11 October 2006 and 22 October-2 November 2007; surveys 1 and 3).

8.2.3.1 Fish collection

Mozambique tilapias (*O. mossambicus*) were collected using gill nets and were sexed according to external features. The male:female ratio was determined and 10 males were kept from each site. Each fish was weighed and measured and blood was drawn. The fish was then killed using cerebral severing and the ventrally open fish was then examined

macroscopically for abnormalities. The testes were removed, weighed and measured in order to calculate the gonadosomatic index (GSI) using gonadal weight/(total body weight – gonadal weight) and being expressed as a percentage. The liver was also removed, weighed and measured in order to calculate the hepatosomatic index (HSI) using liver weight/(total body weight – liver weight).

8.2.3.2 Biomarker sample collection

Sections of liver, gills and muscle were collected in sterile eppendorf tubes with stabilizing Hendrikson buffer and stored in liquid nitrogen until being placed in -80°C freezer.

8.2.4 Tissue analyses

8.2.4.1 Biomarker analyses

Selected tissue samples were used for biomarker analysis. Tissues were thawed and homogenised on ice, in the appropriate homogenising buffer. The biomarkers assayed were: CAT activity, EROD and CEA. All samples were analysed in triplicate. The methods and apparatus used in the selected biomarker analysis are summarised in table 13B1.

CAT activity (Catalase)

Catalase activity was determined by method of Cohen et al. (1969). Gill samples were homogenized with 0.01 M phosphate buffer (pH 7.0) on ice at a ratio of 1:2 and then centrifuged at 10 000 rpm for 10 min at 4°C. To an aliquot of the supernatant was then added cold 6 mM H_2O_2 to start an enzyme catalyzed decomposition reaction of the H_2O_2. After an incubation period of 3 min, the reaction was stopped with the addition of 6 N H_2SO_4. The remaining H_2O_2 was measured by allowing it to react with a standard excess of 2 mM $KMnO_4$ and then measuring the residual $KMnO_4$ spectrophotometrically at a wavelength of 490 nm within 30 to 60 seconds of the addition of the $KMnO_4$.

EROD (Ethoxyresorufin-O-Deethylase)

EROD was determined by method of Burke and Mayer (1978), with some modifications. Liver samples were homogenized in 0.25 M sucrose – 0.05 M Tris (pH 7.4) on ice at a ratio of 1:3 and then centrifuged at 13 500 rpm for 20 min at 4°C. To the supernatant of each

sample was added a general reaction mixture of 0.1 M potassium phosphate buffer (pH 7.8) and 0.05 M Ethoxyresorufin in 1.25% Tween 80. A 0.05 M aliquot of nicotinamide adenine dinucleotide phosphate (NADPH) was stirred into the mixture to start the reaction and the progressive increase in fluorescence, as Ethoxyresorufin was deethylated to resorufin. A baseline of fluorescence was recorded at an excitation wavelength of 544 nm and an emission wavelength of 590 nm with a fluorimeter and using resorufin as a standard. The excitation emissions were then recorded another six times at one minute intervals measuring the progressive increase in resorufin. Ethoxyresorufin and resorufin are both light sensitive compounds and the solutions of these compounds were thus prepared and stored in the dark and the metabolic reaction was performed in a room with dim infrared lighting.

CEA (Cellular Energy Allocation)

The CEA was measured according to De Coen and Janssen (1997), with some modifications. Sampled muscle tissue was homogenised in 3 parts of ice-cold homogenizing buffer before centrifuging at 3 000 rpm for 10 minutes at 4°C separating the homogenate into 4 different fractions for energy reserve (lipids, carbohydrate and protein) quantification and energy consumption (ETS) quantification.

E_r (Available energy reserves)

Lipids were separated, using one of the most common lipid extraction procedures derived by Bligh and Dye (1959). Thereafter, lipids were quantified by measuring absorbance at 405 nm, on an automated microplate reader and using cholesterol (tripalmitin) as a standard. The total carbohydrate concentration was determined using the glucose GOD-PAP colorimetric assay (Roche) at a 560 nm absorbance. Lastly, the protein fraction was quantified using Bradford's reagent (Bradford, 1976) and a bovine serum albumin standard at a wavelength of 630 nm. The lipid, carbohydrate and protein fractions were then transformed into energetic equivalents using their respective energy combustion values (39 500 mJ/mg lipid, 17 500 mJ/mg glycogen and 24 000 mJ/mg protein) (Gnaiger, 1983).

E_c (Energy consumption)

The energy consumed was estimated by measuring the ETS originally described by King and Packard (1975). A 25 µL aliquot of the resultant supernatant was added to 75 µL buffered substrate solution and 25 µL NAD(P)H. The reaction was initiated with 50 µL 8mM *p*-IodoNitroTetrazolium and the absorbance was read kinetically at 492 nm for 10 minutes. The cellular respiration rate was quantified using the theoretical stoichiometrical relationship that

for each 2 µmol formazan formed, 1µmol O_2 was consumed. The respiration rate was then transformed into energetic equivalents using an average oxyenthalpic equivalent of 484 KJ/mol O_2 (Gnaiger, 1983).

CEA

The difference between total energy reserves and energy consumed represents the net energy budget. The following equations illustrate how the E_r, E_c and CEA values were calculated (Moolman, 2004):

$E_r = E_{lipid} + E_{carbohydrate} + E_{protein}$

$E_c = E_{ETS}$

$CEA = E_r - E_c$

8.2.5 Statistical methods

All values are given as means ± SE (standard error) and were calculated using Microsoft Excel. Statistical analysis was performed, using SPSS 11.0 and the homogeneity of data was assessed using Levene's test. If the criterion of homogeneity was met, one-way analysis of variance (ANOVA) was used, to determine significant differences between the metal content and the biomarker responses of the test organisms and between the exposure periods. ANOVA was followed by Scheffé's multiple comparison tests as post-hoc criterion for homogeneity. If there was no homogeneity of variance, Dunnett's-T3 test was applied to assign significant differences ($p < 0.05$) between the variables. Spearman's rank correlation (two-tailed) was used to determine if there was a relationship between the organochlorine and metal concentrations and the biomarker responses. Significant differences are indicated as the letters a, b and c, which indicate a significant difference between the control and the different exposure periods.

8.3 Results and discussion

8.3.1 Tissue analysis

8.3.1.1 Biomarker analyses

Table 8.1 shows the biomarker concentrations derived from the selected tissue samples taken from the three selected sites at the time of sampling.

Table 8.1: Shows the concentration measures for the selected biomarkers used in the samples taken from the three selected sites at the time of each of the fieldtrips.

Site			Oct-06			Mar-07			Oct-07		
Sampling period			AD	ND	XW	AD	ND	XW	AD	ND	XW
EROD Activity		Average	—	11.61	27.23	6.48	—	12.76		—	
(nM/min.mg protein)		Std. Dev (±)	—	14.70	21.74	4.77	—	7.94		—	
Catalase Activity		Average	—	22.75	22.26	20.46	—	11.24	42.00	—	46.12
		Std. Dev (±)	—	14.26	6.13	3.64	—	2.98	6.96	—	6.22
CEA	Glucose	Average	—	35.76	32.26	37.58	—	43.23	50.70	—	41.64
		Std. Dev (±)	—	26.94	17.71	7.66	—	24.31	7.98	—	6.47
	Protein	Average	—	65.17	65.54	39.52	—	38.60	33.23	—	33.93
		Std. Dev (±)	—	0.90	2.57	1.34	—	1.76	1.00	—	0.94
	Lipids	Average	—	586.36	471.62	277.72	—	216.07	184.31	—	259.97
		Std. Dev (±)	—	171.87	212.08	122.20	—	87.25	59.91	—	78.09
	Ea	Average	—	68.73	56.94	35.48	—	29.79	26.82	—	33.55
		Std. Dev (±)	—	17.24	21.21	12.48	—	9.42	6.33	—	8.07
	Ec	Average	—	3.95	3.57	0.25	—	0.30	1.21	—	1.00
		Std. Dev (±)	—	0.57	0.83	0.41	—	0.49	0.28	—	0.25
	CEA	Average	—	683.33	565.86	354.58	—	297.60	267.03	—	334.56
		Std. Dev (±)	—	172.50	211.65	124.37	—	93.90	63.19	—	80.50

Albasini dam (AD) (reference site), the Nandoni dam (ND) and then the Xikundu Weir (XW).

EROD

EROD activity has been proven to be induced specifically by exposure to certain planar organic compounds such as PAHs, dioxins, and non-ortho-PCB (Schiedek et al., 2005). It was, therefore, unexpected to find that there were EROD inductions present in samples obtained from the reference site, AD. It was even more unexpected to find that the EROD inductions obtained at AD were higher than those present in the samples obtained at XW. This said, it should be noted that EROD activity has been shown to be influenced by a number of abiotic and biotic factors such as temperature, age as well as the reproductive stages of the organisms sampled (Schiedek et al*.,* 2005), all of which were shown, in this

particular study, to vary to a large degree. Much higher EROD inductions were noticed during surveys 1 and 3. It is also logical that DDT and DDE concentrations would be higher in a dam environment rather than in a riverine environment where water flow is continuous, thus being less conducive to the settling out of the DDT and DDE into the organic compartments of the environment and thus, meaning less exposure to DDT and its metabolites. ND is situated in the heartland of the DDT sprayed area. It is thus logical to assume that there should be a greater possibility of DDT-runoff and its metabolites into this dam. The fact that ND constitutes a dam environment means that there is more time for DDT and its metabolites to settle out and be taken up by the surrounding organic compartments. This however is not evident in the organochlorine concentrations obtained in this particular field study, with all sediment and water concentrations found to be less than 0.02 and 0.1 µg/L (Chapter 3).

Additionally, as was previously stated, DDT and its metabolites are extremely volatile and are able to traverse through many different watercourses in the environment, thus, perhaps the possibility of exposure to DDT and its metabolites, even at the reference site, should not be ruled out completely.

Catalase activity

Catalase activity concentrations obtained from XW during survey 2 showed significantly lower catalase inductions than were obtained at any of the other sites during any of the periods sampled. The catalase activity concentrations obtained from XW and AD during survey 3 differed significantly from any results obtained in previous sampling periods with much higher catalase inductions. Results obtained during survey 1 show similar results for both XW and ND. This is not surprising as, although ND is situated in the heartland of the DDT sprayed area; catalase is a very general biomarker and, as such, will indicate any number of impacts occurring in the area. In conjunction with direct DDT spraying, additional impacts to the ND is; construction, recreation as well as sideline fishing by the local population. Additionally XW is impacted by the presence of contaminants transported downstream from the DDT sprayed area as well as with livestock, agricultural, domestic and fishing activities. Results obtained during survey 1 show significantly higher catalase inductions at AD than those obtained at XW. This may be an indication of the general nature of the catalase biomarker and as such, indicates the presence of a variety of environmental stressors. Thus, even though AD was considered the control site, being situated upstream and outside of the DDT sprayed area, there are a number of other environmental impacts in

the area including the presence of orchards and other agricultural activities, recreational activities and livestock. It is likely that any number of environmental contaminants such as fertilizers and pesticides are being washed into the dam at any given time, thus explaining the higher inductions of catalase obtained at this site. When comparing the “high” and “low flow” results obtained at XW and AD, it is seen that catalase inductions were reduced at both sites during the high flow season (survey 2), thus suggesting lower contaminant concentrations in the surrounding environment during this time. October 2007 was particularly dry with water levels significantly reduced and elevated TDS and conductivity readings. However, it is unlikely that this alone would explain the elevated catalase induction results from samples obtained at this time.

CEA

The measurements of the available energy reserves and the energy consumption of *O. mossambicus* at each of the sites during each of the exposure periods can be seen in table 8.1. Significantly lower energy values for the glucose content were observed at XW and ND during survey 1, while both sites also showed both higher lipid and protein measurements, than were obtained in survey 2 and 3. The higher lipid content at these sites indicate that the effect of environmental disturbances and pollutants in these two areas were more recent than at AD, while the depleted lipid and protein reserves at AD indicate more of a constant historical impact than recent. However, with repeated sampling, XW, during survey 2, appears to have undergone a continued impact from the October 2006 period, with lower levels of lipids as well as proteins measured in these samples. The increased glucose levels indicate recent recovery in the fish.

The total energy reserves (Ea) gave an integrated overview of the temporal changes of the available energy reserves of *O. mossambicus*, obtained from each of the selected study sites (Table 8.1). XW and ND during survey 1 appeared to be the least affected of all the surveys with significantly higher overall energy reserves available than when compared to surveys 2 and 3. AD and XW during survey 2 shows significantly lower energy reserves compared to from survey 1. While energy reserves from AD continued to deteriorate, it seems that fish from XW recovered slightly by the time of survey 3. The cellular respiration rate (Ec) decreased very significantly from survey 1 to survey 2, with respiration rates then increasing at AD and XW, but with AD showing more of an increase in the respiration rate at this period than was found at XW. It can be said with all surety that changes in the glucose, lipid and protein reserves contributed to the changes in the Ea and CEA. According to Muyssen and

Janssen (2001) lipids are known as more efficient energy-storage products than either proteins or glucose and consequently, lipid reserves can be the first energy source to be consumed under conditions of toxic stress. This was observed in the results obtained during survey 2 and 3.

8.4 Conclusion

In many of the individuals found in a specific area, large differences were noted in the various activities of each of the biomarkers used in this study. In the case of each of the biomarkers used, field results when compared to the results for the relevant controlled aquarium exposures (discussed in chapter 13b), results showed ample experimental evidence that if an exposure is prolonged, the induced response may decline, or even return to background levels (adaptation). Adaptation processes are generally species-specific and/or contaminant-specific and specific biomarker levels are each uniquely affected. Thus, it can be assumed that the possibility does indeed exist that because of the prolonged exposure of *O. mossambicus* to the polluted selected environments, that any induced responses with regard to exposure to pollutants may have declined to nearly background levels and that the variations noted in the measurements obtained are as a result of natural environmental variations at each of the selected study sites. *O. mossambicus* were successfully utilized to identify contamination within the surrounding environment, but this species was not sensitive enough to assess small variations in organochlorine (and other contaminants) concentrations within the environment.

CHAPTER 9

A HISTO-MORPHOLOGICAL STUDY OF THE HEART OF *CLARIAS GARIEPINUS* AND *OREOCHROMIS MOSSAMBICUS.*

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9.1 Introduction

The Luvuvhu River catchment is located in a tropical, high-risk malaria area in the Limpopo Province, South Africa. DDT was introduced in this region for vector control purposes. There is an ongoing debate concerning the potential threat of environmental estrogenic pollutants, including DDT, regarding human and animal health. More specifically, little information is available on the cardiovascular actions of environmental estrogenic pollutants in animals and particularly fish.

Epidemiological studies suggest that exposure to POPs might induce cardiovascular disorders. PCBs have been linked to ventricular hypertrophy in rodents (Lind et al*.,* 2004). This condition often accompanies hypertension and is a risk factor for cardiovascular disease. Lindane absorption resulted in ECG modifications and produced histological alterations (ventricular hypertrophy and necrosis of the ventricular wall) of cardiac tissue as well as cardiovascular dystrophy (Sauviat and Pages, 2002). *4*-Octylphenol, *p*-Np, and *o,p'*-DDT have been shown to inhibit Ca^{2+} channels in vascular smooth muscle and relaxation of the coronary arteries (Ruehlmann et al*.,* 1998). DDT has also been linked to cardiovascular disease in humans (Waliszewski, 1995). The endocrine disruptor metals lead, arsenic and cadmium have been linked to hypertension and cardiac arrhythmia (Taylor, 1995).

According to Taylor (1995), possible mechanisms that could cause cardiovascular disease include (1) damage to endothelial cells in the vascular system (2) activation of leucocytes and platelets (3) initiation of plaque formation (4) stimulation of an inflammatory response and (5) direct damage to cardiac tissue. Although fish heart is not always regarded as a primary target organ for exposure to toxicants, several studies have shown histological lesion in cardiac tissue especially with regard to metal pollution (Exley, 1996; Borges et al*.,* 2003). The two most recognisable histopathological changes in the heart are muscular necrosis and inflammation of the cardiac muscle, endocardium and epicardium.

The presence of cardiac inflammation in feral fish has been described before in various species including roach (Haaparanta et al*.,* 1993), halibut (Johansen and Poppe, 2002) and salmon (Kongtorp et al*.,* 2004; Ferguson et al*.,* 2005; Kongtorp et al*.,* 2006). Inflammation of the cardiac muscle is characterised by the presence of excessive accumulation of leucocytes and lymphocytes in the spaces among the muscle fibres. Collagen fibres in these spaces also show a tendency to proliferate (Takashima and Hibiya, 1995). However, these cardiac histological alterations have not yet been described in South African freshwater fish species.

During the second survey (March 2007) of the prroject, it was decided to sample the heart of the Sharptooth catfish (*Clarias gariepinus*) and the Mozambique tilapia (*Oreochromis mossambicus)* as a potential target organ for histological assessment. The histological results of this pilot investigation revealed cardiac inflammatory responses in a number of specimens. It was subsequently decided to perform a histo-morphological study of the heart as a potential target organ of fish collected during survey 3 (October 2007) and survey 4 (February 2008).

The aim of this part of the project was to qualitatively and quantitatively assess the cardiac histo-morphology of the above mentioned freshwater indicator fish species from two water bodies in the Luvuvhu River catchment within the DDT sprayed area (Nandoni Dam [ND] and Xikundu Weir [XW]) and from a reference site in the same catchment outside the sprayed area (Albasini Dam [AD]).

9.2 Material and methods

9.2.1 Fish and tissue sampling

Adult specimens of both species (n = 185) (Table 9.1) were collected during three surveys using gill nets. The sex of each specimen, was determined according to secondary sexually characteristics i.e. the presence of a distinct urogenital papilla and/or according to their breeding colours.

Table 9.1: Number of fish collected per sampling period.

	Survey 2		**Survey 3**		**Survey 4**	
Species	***O. mossambicus***	***C .gariepinus***	***O .mossambicus***	***C. gariepinus***	***O. mossambicus***	***C. gariepinus***
AD	8	15	9	16	8	20
ND	1	3	3	10	12	7
XW	20	7	11	9	20	6

The total body mass and total lengths of each fish were recorded to calculate a condition factor (CF) for each specimen (total body mass x 10^5 / total length^3). Blood was drawn from the dorsal caudal artery at the posterior region of the lateral line to measure the haematocrit (Hct) and leukocrit (Lct). Following the blood collection, a standard necropsy was performed including a macroscopic examination of the external features of each specimen (opercula, eyes, skin and fins) to identify any aberrations, erosion or abnormalities as well as the presence of any possible external parasites.

The fish were sacrificed by severing the spinal cord anterior to the dorsal fin. The hearts were dissected out and the heart mass was recorded (heart mass was not recorded during the pilot investigation – survey 2) to calculate the cardiac somatic index (CSI) (heart mass / body mass x 100). A macroscopic examination of the heart was performed to identify any abnormalities i.e. nodules, cysts, growths or discolouration.

9.2.2 Tissue processing

The hearts were immediately fixed in 10% neutrally buffered formalin for 48 hours. Following fixation, the samples were washed in tap water and dehydrated in rising concentrations of ethanol (30%, 50% & 70%). The samples were then sent to the Onderstepoort Veterinary Institute, University of Pretoria, for further processing and preparation (H&E staining) for light microscopy analyses.

9.2.3 Qualitative and quantitative histological assessment

Longitudinal mid-sections (5 µm) of each heart were analysed using light microscopy (Leica *DMLS – ICCA*). Depending on the histological detail of the different structures within the heart, slides were examined using four objective levels (4 X, 20 X 40 X and 100 X -oil). Digital micrographs were produced using a digital camera with IM50 Image Manager Software (Pixel IT (Pty) Ltd).

A protocol for the quantitative assessment of histological alterations in fish, published by Bernet et al. (1999), was adapted to be used specifically for cardiac histology. This assessment tool as proposed by Bernet et al. (1999) has formed part of various published papers (e.g. Schmidt et al., 1999; Schmidt-Posthaus et al., 2001; Chun-Yao et al., 2004; Bernet et al., 2004; Nero et al., 2006).

Using this protocol, cardiac alterations were quantified as follows: An importance factor of 1, 2 or 3 (indicating the specific alteration's pathological importance) and a score value of 0, 2, 4 or 6 (indicating the extent of the alteration within the heart) were assigned to each alteration. Using these two values, a reaction index value is calculated for each alteration. Subsequently, an organ index (in this case, a cardiac inflammatory index (I_{CII})) is calculated as the sum of the various reaction indices. Higher index values indicate an increase in pathology.

9.2.4 Target chemical analyses

The determination of DDT levels in water, sediment (chapter 3) and tissue (chapter 6) samples are presented as part of the larger project methodology.

9.2.5 Statistical analyses

For surveys 3 and 4, Pearson's Correlation Coefficient (r) and the two-sample T-test was calculated to identify potential strong and/or similar linear relationships between the quantitative histological results and related aspects including, body mass, condition factor, cardiac somatic index, haematocrit, leukocrit, age, DDT levels in fat tissue and parasite infection. Nematodes identified in the visceral cavity were quantified according to the extent of the infection i.e. few (= 2), moderate (= 4) and severe (= 6).

9.3 Results

9.3.1 Necropsy

No macroscopic abnormalities of the heart were identified in any of the fish from the three sampling sites. A nematode infection was, however, identified in three *O. mossambicus* specimens from the XW. The presence of this parasitic infection was confirmed during the histological evaluation. The presence of nematodes between organs in the visceral cavity was also observed in the majority of *C. gariepinus* specimens.

The morphometric data recorded throughout this study are presented in Table 9.2. The mean cardiac somatic index (CSI) values for *C. gariepinus* varied between 0.12 and 0.13% and 0.09 and 0.12% for *O. mossambicus* respectively. The mean condition factor (CF) for *C. gariepinus* ranged between 0.65 and 0.98 and between 1.34 and 1.78 for *O. mossambicus*.

Table 9.2: Morphometric data of sampled fish specimens (mean values are presented).

Oreochromis mossambicus					
	Site	**Body length (mm) ±SD**	**Body mass (g) ±SD**	**CSI (%) ±SD**	**CF ±SD**
Survey 2	**AD**	271.7 ± 18.9	333.0 ± 69.4	N/A	1.65 ± 0.22
	ND	290.0 ± 0.0	380.0 ± 0.0	N/A	1.56 ± 0.00
	XW	290.0 ± 34.7	438.0 ± 172.8	N/A	1.74 ± 0.24
Survey 3	**AD**	310.0 ± 62.3	422.2 ± 245.4	0.12 ± 0.03	1.34 ± 0.29
	ND	340.0 ± 30.4	670.0 ± 138.9	0.10 ± 0.01	1.70 ± 0.13
	XW	324.7 ± 25.6	608.2 ±116.4	0.11 ± 0.02	1.78 ± 0.24
Survey 4	**AD**	261.0 ± 12.1	293.8 ± 19.9	0.09 ± 0.01	1.66 ± 0.16
	ND	284.7 ± 44.8	395.8 ± 274.7	0.09 ± 0.01	1.58 ± 0.10
	XW	298.9 ± 39.4	424.0 ± 150.9	0.09 ± 0.02	1.57 ± 0.30
Clarias gariepinus					
	Site	**Body length (mm) ±SD**	**Body mass (g) ±SD**	**CSI (%) ±SD**	**CF ±SD**
Survey 2	**AD**	572.0 ± 49.6	1296.0 ± 340.2	N/A	0.68 ± 0.09
	ND	488.3 ± 72.2	953.0 ± 297.7	N/A	0.81 ± 0.09
	XW	533.6 ± 121.1	1259.0 ± 679.1	N/A	0.85 ± 0.37
Survey 3	**AD**	589.4 ± 98.9	1818.8 ± 1475.2	0.13 ± 0.04	0.98 ± 1.31
	ND	685.0 ± 54.4	2220.0 ± 637.4	0.12 ± 0.02	0.68 ± 0.07
	XW	592.2 ± 61.0	1655.6 ± 320.6	0.13 ± 0.03	0.81 ± 0.15
Survey 4	**AD**	550.9 ± 96.1	1232.0 ± 634.8	0.13± 0.03	0.75 ± 0.44
	ND	644.4 ± 167.5	1930.0 ± 1381.3	0.13 ± 0.04	0.65 ± 0.22
	XW	601.7 ± 68.4	1408.3 ± 405.5	0.12 ± 0.03	0.66 ± 0.23

CSI = cardiac somatic index; CF = condition factor; N/A = not applicable

9.3.2 Qualitative histological assessment

The heart of both species consists of two main compartments, a single atrium and ventricle (saccular in shape in *C. gariepinus* and pyramidal in shape in *O. mossambicus*). Blood enters the heart through the sinus venosus into the atrium, passes through the ventricle and exists via the bulbus arteriosus into the ventral aorta. The heart is located ventrally to the pharynx. In both species the relatively smaller atrium and larger ventricle are red to dark red in colour while the bulbus arteriosus have a distinct white appearance.

The cardiac histological structure of all specimens (n = 185) was found to be normal and mostly similar between the two species. The atrium is smaller than the ventricle but both compartments consist of an intermediate muscle layer or myocardium enclosed by an exterior layer, the epicardium, and lined with an internal layer, the endocardium. The myocardium is much thinner in the atrium compared to the ventricle and muscle fibres are

not as densely compacted. The cardiac muscle cells consist of a centrally located nucleus and flattened endothelial cell nuclei are visible on the periphery of most muscle cells.

The epicardium, a relatively thin layer, consists of flat mesothelium and connective tissue and also contains multiple coronary arteries that supply the heart tissue with blood. The epicardium of *C. gariepinus* specimens had high levels of adipose tissue. The endocardium consists of endothelial cells and is supported by connective tissue. The myocardium of the ventricle possesses a notably thicker compacted outer layer and an inner, less compacted trabecular layer in *C. gariepinus*. In *O. mossambicus*, the myocardium was identified to be exclusively trabecular in nature. The muscle fibres are organised in a mesh-like structure with fibres visible in transverse and longitudinal view. Blood enters the atrium via the sinus venosus through the sinoatrial valve and then passes to the ventricle through the atrioventricular valves and is finally pumped to the bulbus arteriosus through the semilunar valves. The various heart valves consist mainly of connective tissue lined by the endocardium.

The thick bulbus arteriosus wall consists of connective tissue, elastic fibres and smooth muscle and when viewed in transverse section, is arranged in a circular outer layer and a longitudinal inner layer. From the bulbus arteriosus blood is pumped into the ventral aorta. Apart from the normal histological structure, the qualitative histological assessment did, however, reveal an inflammatory response in a number of specimens from all three sites. Inflammatory responses were identified in the epicardium (epicarditis) (Figure 9.1), myocardium (myocarditis) (Figure 9.2) and to a lesser extent, the endocardium (endocarditis). The latter was mostly associated with the heart valves. In some cases the inflammatory response was accompanied by intercellular deposits and vacuolated cardiac tissue (Figure 9.3). Inflammation of the epicardium and the endocardium were restricted to focal areas whereas the inflammation of the myocardium was more diffuse. The inflamed areas were characterized by the infiltration of darkly-stained mono-nuclear leucocytes. In most specimens, lymphocytes were predominant, indicative of chronic inflammation. Large granulocytes (eosinophils) were also identified in the epicardium in close proximity of inflamed areas.

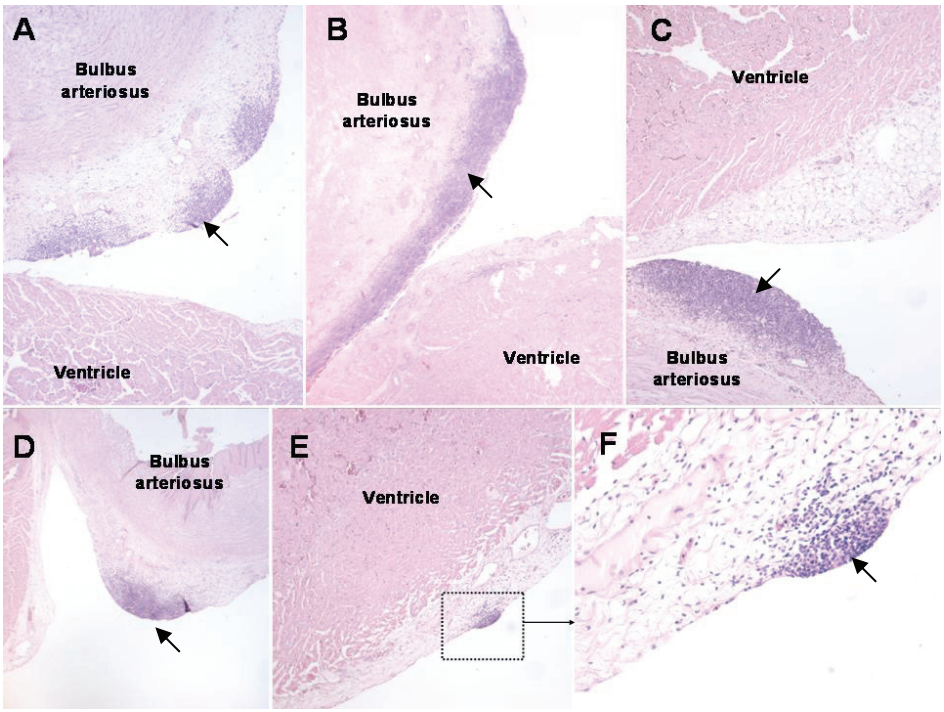


Figure 9.1: Focal areas of epicarditis (arrow) visible in the bulbus arteriosus (A,B,C & D) and ventricle (E & F) of *C. gariepinus* (H&E) x10.

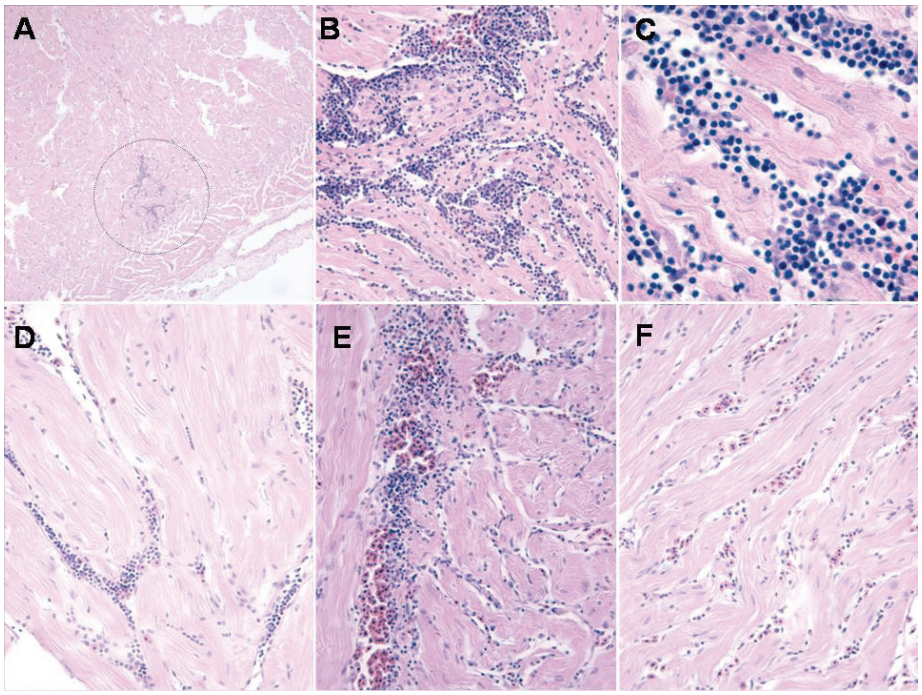


Figure 9.2: Focal (A, B (x20) & C (x40)) and diffuse (D,E & F) areas of myocarditis visible in the ventricle of *C. gariepinus* (H&E) x10.

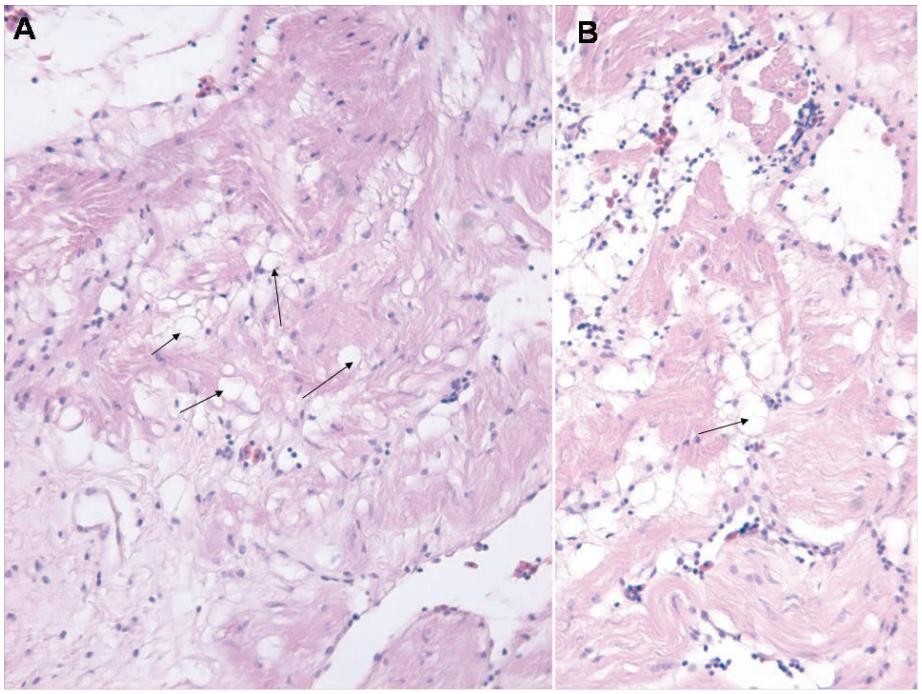


Figure 9.3: Vacuoles (arrow) visible between muscle and fibrous connective tissue in the atrium of *O. mossambicus* (H&E) x40.

9.3.3 Quantitative histological assessment

The quantitative histological assessment allowed an objective comparison of the histological results in terms of the severity of cardiac inflammation between the different sites, species and sampling seasons.

Table 9.3 summarizes the prevalence of the inflammatory response identified in fish collected over the three sampling periods. The number in each block represents the percentage (%) of fish from that specific sample group in which the specific inflammatory response, as stipulated in the first two columns, were observed. A cardiac inflammatory response was identified in a number of fish at all sites with the exception of *O. mossambicus* at ND during survey 2.

Table 9.3: The percentage (%) of fish with a cardiac inflammatory response.

Season		Survey 2						Survey 3						Survey 4					
Species		*Oreochromis mossambicus*			*Clarias Gariepinus*			*Oreochromis mossambicus*			*Clarias gariepinus*			*Oreochromis Mossambicus*			*Clarias gariepinus*		
Sampling sites		AD	ND	XW	AD	ND	XW	AD	ND	XW	AD	ND	XW	AD	ND	XW	AD	ND	XW
Sample size		8	1	20	15	3	7	9	3	11	16	10	9	8	12	20	20	7	6
Myo-carditis	Atr	38		5	60		43				50	100	78	25		10	75	86	50
	Ven			5	53	67	100	11	33	27	94	90	89		8		86	100	67
	BA																		
Epi-carditis	Atr					33						10		25					17
	Ven	13		5	7		14	11			19	30	11	50				14	33
	BA				40	100	43			9	31	70	67	25			35	71	
Endo-carditis	Atr																		
	Ven																		
	BA																		
	SLV				7						13	20	11				5	14	
	AVV										13	10	11				10		
	SAV																		

Atr = atrium; Ven = ventricle; BA = bulbus arteriosus; SLV = semilunar valve; AVV = atrioventricular valve; SAV = sinoatrial valve

The severity of the cardiac inflammatory responses was quantified using the quantitative protocol as proposed by Bernet et al. (1999). These results are presented in Table 9.4. Each value represents the mean cardiac inflammatory response for fish from the specific sampling site for each survey. The values presented in bold represent the mean inflammatory response for fish from the specific sampling site for all surveys.

Table 9.4: Mean Cardiac Inflammatory Index (I_{CII}) values with standard deviation calculated for both species (Median values are presented in brackets).

Species	*Oreochromis mossambicus*			*Clarias gariepinus*		
Site	AD	ND	XW	AD	ND	XW
Survey 2	2.00 ± 2.13 (2.00)	0.00 ± 0.00 (0.00)	1.00 ± 2.86 (0.00)	8.40 ± 5.35 (8.00)	10.67 ± 8.32 (8.00)	10.90 ± 6.81 (12.00)
Survey 3	0.89 ± 1.76 (0.00)	1.30 ± 2.30 (1.30)	1.45 ± 2.01 (1.50)	11.75 ± 9.17 (8.00)	20.00 ± 8.64 (20.00)	11.11 ± 5.57 (11.10)
Survey 4	8.00 ± 8.55 (4.00)	0.33 ± 1.15 (0.00)	0.40 ± 1.23 (0.00)	12.80 ± 7.95 (12.00)	17.14 ± 1.95 (16.00)	9.33 ± 9.00 (6.00)
Mean (I_{CII}) / site	**3.62 ± 3.82**	**0.54 ± 0.67**	**0.95 ± 0.52**	**10.90 ± 2.29**	**15.89 ± 4.77**	**10.44 ± 0.97**
Median (I_{CII}) / site	**2.00**	**0.00**	**0.00**	**8.00**	**16.00**	**11.00**

As the cardiac inflammatory responses were more severe in *C. gariepinus* specimens (Table 9.4), Pearson's Correlation Coefficient (r) was calculated to identify potential strong and/or similar linear relationships between the quantitative histological results of this species and related parameters measured including body mass, condition factor, haematocrit, leukocrit

and age, DDT levels in fat tissue and parasite infection. The r values calculated are presented Table 9.5.

Table 9.5: Correlation coefficients (r) calculated to determine possible linear relationships between the Cardiac Inflammatory Index results and related parameters measured in *C. gariepinus* specimens

Survey	Site	Cardiac somatic index	Body mass	Parasites	Condition factor	Haematocrit	Leucocrit	Age	Total DDT	*p,p'*- DDT	*o,p'*- DDT	*p,p'*-DDD	*o,p'*-DDD	*p,p'*- DDE	*o,p'*- DDE
Survey 3	**AD**	-0.44	0.49	0.34	0.36	0.01	0.11	-0.32	**0.70***	0.49	0.23	0.54	ND	**0.71***	ND
	ND	0.07	0.00	0.32	0.38	0.45	0.57	0.05	-0.33	0.01	0.42	-0.41	0.47	-0.34	0.69
	XW	-0.04	0.00	-0.15	0.02	0.65	-0.30	0.01	**0.74***	**0.93***	**0.91***	0.52	0.54	0.16	-0.43
Survey 4	**AD**	-0.28	-0.07	-0.18	0.17	0.03	0.24	-0.21	0.37	0.37	0.66	0.46	0.68	0.30	**0.87***
	ND	-0.38	0.25	-0.16	**0.91***	0.24	0.41	0.00	-0.58	-0.59	-0.54	-0.60	-0.54	-0.57	-0.56
	XW	0.38	0.32	-0.07	-0.49	-0.14	-0.53	0.50	0.44	-0.06	0.00	0.13	0.10	0.54	0.23

DDT, DDD & DDE values represent levels measured in fat tissue of sampled fish. * (significant positive association, $p < 0.05$)

9.4 Discussion

All hearts examined showed no macroscopic lesions. The histological results did, however, reveal inflammation of the heart muscle as well as vacuolated atrial muscle fibres in a number of specimens. An inflammatory reaction is the basic protective response to tissue damage in all vertebrates (Haaparanta et al*.,* 1993). According to Roberts (1989), if an acute inflammatory lesion does not resolve quickly, chronic inflammation follows, frequently with the formation of granulomas. Most inflamed tissue identified in the current study was chronic in nature as lymphocytes were predominant. A characteristic feature was the focal nature of the inflammation in the heart. This focal distribution was also noted by Haaparanta et al*.* (1993) in perch and roach from natural lakes in Finland.

In the current study, three types of inflammation were identified: myocarditis, epicarditis and to a lesser extent, endocarditis. The quantitative results (presented in Table 9.3) showed that myocarditis was only observed in ventricles and atria of affected fish. Epicarditis was confined to focal areas and occurred mostly in the bulbus arteriosus, to a lesser extent in the ventricle, and only in isolated cases in the atria of affected fish. Small focal areas of

endocarditis were observed in the semilunar and atrioventricular valves in only 7% of the 185 specimens sampled.

Due to the varied sample sizes of fish collected at the different sites throughout the various seasons, it was essential to only compare mean I_{CII} values between sample groups as the use of total values (sum of all individual index values per group as proposed by Bernet et al. (1999)) will compromise the accuracy of the results. Larger sample sizes will automatically results in a higher total index value for that specific group which does not necessarily reflect a more severe inflammatory response.

The I_{CII} values calculated (Table 9.4) showed that *C. gariepinus* specimens were more affected than *O. mossambicus* specimens from all three sites sampled during all three surveys. The results showed that *C. gariepinus* fish from ND were more affected than the same species from XW, but also that *C. gariepinus* specimens from the control site (AD) had a similar mean index value as fish from the XW. Comparing the Cardiac Inflammatory Index values of *O. mossambicus* specimens between the three sites, the specimens from the control site had the highest values followed by fish from the XW and the ND respectively.

When comparing inflammatory responses in fish between surveys, fish from ND and XW had higher values during survey 3 in both species compared to fish collected during the other two surveys, while fish from both species from the AD had higher values during survey 4 compared to the other two surveys. These results did not show a linear relationship with the DDT levels measured in water samples from the three sampling sites.

Without being able to identify the exact causative agent of the inflammatory response (whether it is chemical or pathogen related), the different results between the two species and the three sampling sites cannot be explained. Thus, further investigations regarding the aetiology of this response are essential.

As mentioned in the introduction, the presence of cardiac inflammation in feral fish has been described before in roach (Haaparanta et al., 1993), halibut (Johansen and Poppe, 2002) and salmon (Kongtorp et al., 2004; Ferguson et al., 2005; Kongtorp et al., 2006). Haaparanta et al. (1993) stated that initial studies on roach from four lakes in Finland showed inflammation of the myocardium and epicardium but no causative agents could be identified at that stage for this condition. An attempt to link the inflammatory condition exclusively with pulp and paper mill effluent exposure as well as eutrophication of the lakes was inconclusive. No other causative agents e.g. parasites or bacteria were identified. The authors stated that

since the majority of inflamed areas were chronic in nature, the initiating agent may no longer be present and thus complicates the identification of the specific causative agent (which might also be the case with the study in the Luvuvhu River). As also identified in *C. gariepinus* and *O. mossambicus*, a characteristic feature in the roach was the focal nature of most inflammations. It was subsequently concluded by the authors that the oligotrophic lake (selected control site) considered to be in a natural state by normal criteria, was no longer so, and that the fish in all the lakes were equally affected by pollutants.

In the current study it was also attempted to link environmental pollution, in this case DDT exposure, to the inflammatory response. The vacuolated muscle fibres identified in hearts with the inflammatory response, were mostly present between myocardial muscle fibres and only in the atria of the hearts. The vacuoles resemble lipid vacuoles, a common feature in fish liver, characterized by the nuclei being pushed towards the periphery of the cell. Adipose tissue was also a common feature in the epicardium of the ventricle of some fish. Johansen and Poppe (2002) found that inflamed epicardia of sturgeon showed high levels of fat with focal lesions of lymphocyte infiltrates. Ferguson et al. (2005) also showed changes in myocardial spongy layer of farmed salmon characterized by widespread vacuolation. Although the fat content of the vacuoles found in the current study could not be confirmed due to histological processing, the presence of fat in the heart may involve the bioaccumulation of lipophilic chemicals e.g. DDT and its metabolites, which are known to accumulate in fat tissue, thus making the atrial muscle fibres the primary target cells for these chemicals. However, the Correlation coefficients (Table 9.5) calculated for the DDT levels of fat samples showed no pattern of similar linear relationships between same DDT isomers comparing different seasons and sampling sites. Most relationships were weak negative or weak positive. Although cases of strong significant positive linear relationships were found in isolated sample groups ($r > 0.7$, $p < 0.05$) (e.g. cardiac inflammatory response and *o,p'*-DDT measured in a specimen from XW during survey 3), r values for the same variable (*o,p'*-DDT) calculated for the two other sampling sites during surveys 3 and 4 do not show a similar strong linear relationship.

However, it cannot be concluded that the inflammatory response is not related to exposure to pollutants as only DDT was investigated in the current study. The results highlighted that AD cannot be regarded as a control site for cardiac inflammation in the Luvuvhu River's fish population. Although AD can be regarded as a reference site for DDT exposure, the mixture of other pollutants in the dam could also have a negative effect on fish health and needs to be investigated.

In 2002, Johansen and Poppe identified pericarditis and myocarditis in Norwegian farmed Atlantic halibut and found that larger fish were more affected. In the study by Johansen and Poppe (2002), smears from the pericardial cavity of some fish resulted in the isolation of a mixed bacterial population while immunohistochemistry showed no evidence of pathogen infection. The authors stated that the selected sample group might have severe pathological changes in the heart and pericardial cavity without showing clinical signs of disease. According to these authors, the fact that larger fish were more affected indicated that dietary factors might play an important role. In the current study, no strong and/or similar correlation was found comparing the inflammatory response and the total body mass or related condition indices i.e. the condition factor or the CSI of the specimens.

Specifically concerning farmed fish, Guarda et al. (1997) stated that aspects of farming condition like low water level, sub-optimal nutrition, microbes or stress might also play a part in the development of pericarditis. These aspects are, however, more difficult to monitor in feral populations.

Apart from the nematodes present in the visceral cavity, only three *O. mossambicus* specimen had a cardiac nematode infection and none of these infected fish specimens showed evidence of a cardiac inflammatory response. In an attempt to correlate nematode infection of the visceral cavity in *C. gariepinus* to the presence of the cardiac inflammatory response, no strong and/or similar correlation was found comparing the different sampling groups. Correlation analyses of variations in blood parameters for *C. gariepinus* including Hct and Lct (surveys 3 and 4) to the inflammatory response was also inconclusive.

The different results identified in the two species might be attributed to the fact *C. gariepinus* is mostly a bottom dweller and is also omnivorous, thus more likely being exposed to infection by chemicals, pathogens and/or parasites present in the sediment (should these be related to the cardiac inflammatory response).

A more recent study by Kongtorp et al. (2004) identified heart and skeletal muscle inflammation (HSMI) syndrome in salmon. In this study, the authors demonstrated that HSMI is an infectious disease. Fish were injected with tissue homogenate from salmon previously diagnosed with HSMI. Injected fish and their cohabitants showed serious epicarditis and myocarditis six weeks post-challenge while control fish showed no lesions. However, further investigations were still proposed to make conclusions regarding the cause and pathogenesis of HSMI. In 2005, Ferguson et al. reemphasized that HMSI is an infectious

condition of undetermined cause and presented results on an outbreak of disease in salmon in Scotland that mirror those described for HSMI in salmon in Norway.

Kongtorp et al. (2006) states that the heart is the primary target organ in HSMI. Skeletal muscles of *C. gariepinus* and *O. mossambicus* were not included in the histological assessment in the current study, and a similar inflammatory response in skeletal muscle associated with HSMI cannot be concluded. However, the heart histological lesions identified correspond with the diagnosis for HSMI which is stipulated as the presence of epi-, endo- and myocarditis with mononuclear cell infiltration (Kongtorp et al., 2006).

Interestingly, Kongtorp et al. (2006) found that over time, the severity of cardiac lesions reduced, suggesting that the fish were able to control the infection and reduce tissue damage. Fish are able to regenerate myocardial cells (Poss, Wilson and Keating, 2002). Compensatory cellular hypertrophy may therefore be just one of the mechanisms helping the affected fish to survive serious myocardial insults (Ferguson et al., 1990). It is therefore possible that the fish from the Luvuvhu River might be infected with HSMI but are able to control the disease as the fish population do not visibly seem to exhibit any health or behavioural effects apart from the above mentioned histological lesions.

9.5 Conclusions

Three types of chronic cardiac inflammation were identified: myocarditis, epicarditis and to a lesser extent, endocarditis. *C. gariepinus* were more affected than *O. mossambicus* and fish from the control site were worse affected than fish in the two test sites. Future studies, focusing on associated skeletal muscle lesions of fish, with the inclusion of bacteriology and virology, are essential to further investigate the cause of the inflammatory response in the fish population in the Luvuvhu River.

CHAPTER 10

TESTICULAR APOPTOSIS IN TWO BIO-SENTINEL FISH SPECIES, INHABITING THE LUVUVHU RIVER

Patrick SM, Van Dyk JC and Bornman MS

10.1 Introduction

Endocrine disrupting chemicals (EDCs) have the ability to disrupt hormonally controlled processes, such as spermatogenesis. Spermatogenesis is a normal, tightly regulated process that is the maturation of germ cells into spermatozoa. Disruption of the process leads to inappropriate hormonal response, which may result in the production of inappropriate cell numbers, either too many or too few, or the production of malformed and damaged cells (Maag et al., 2003; Frigo et al., 2005). The process of apoptosis is one form of programmed cell death (PCD), in which the cell has the ability to activate an inherent suicide mechanism that ultimately eliminates the damaged or malformed cell/s in an ordered fashion (Bortner et al., 1995; Bellamy et al., 1995). During normal spermatogenesis, germ cell apoptosis can occur, but the degree of apoptosis within the testis could possibly be affected by exposure to EDCs. One tool which is used to quantify the extent of apoptotic activity is immunohistochemistry, using assays (Labat-Moleur et al., 1998). The use of caspase-3 and Terminal deoxynecleotidyl transferase-mediated dUTP Nick end-Labeling (TUNEL) assays for apoptosis, due to a host of insults has been investigated in various species (Frigo et al., 2005; McClusky, 2005; McClusky et al., 2008). A study (Bornman et al., 2007) investigated testicular apoptosis in *C. gariepinus* in an urban nature reserve in South Africa. This particular study used both caspase-3 and TUNEL assays, and concluded that both assays have utility in determining the types of cells affected, as well as quantification of apoptotic cells (McClusky et al., 2008).

10.2 Materials and methods

10.2.1 Fish collection procedure

Fish were collected during survey 3 (“low-flow” season, 22 October-2 November 2007), and survey 4 (“high-flow” survey 32 – 29 February 2008). Six gill nets were cast at various points in each of the three dams, increasing the probability of collecting fish. The fish were retained in two aerated containers filled with tap water, prior to dissection.

10.2.2 Tissue sampling

10.2.2.1 Testes preparation procedure

Each male specimen was weighed and the total length measurements were recorded (Table 10.1). The fish were subsequently sacrificed by severing the spinal cord posterior to the opercula, and the testes were harvested. The right and left testes lengths were measured and were weighed individually, to calculate the gonadosomatic index (GSI). The mid-piece of the excised testes was removed, and divided equally among three fixatives.

10.2.2.2 The Gonadosomatic index (GSI)

The gonadosomatic index (GSI) was calculated for each of the *C. gariepinus* and *O. mossambicus*. The GSI represents the total gonadal weight relative to the total body weight, expressed as a percentage:

$$GSI = \frac{\text{Total gonad weight}}{\text{Total body weight}} \times 100$$

The GSI, an indicator of gonadal growth and maintenance (Panther et al., 1998), has been used extensively in the past, as early as 1927, and currently still used as a histological tool. The establishment of a correlation, in male fish, between the inhibition of testicular growth and the potency of estrogenic compounds (De Vlamming, 1981), has lead to the utilization of GSI as a biomarker for the exposure of aquatic wildlife to environmental estrogens (De Vlamming, 1981; Panther et al., 1998).

Table 10.1: Length and total mass measurements of collected fish.

	n	Standard Length (mm)			Mass (g)		
		Mean	**± SD**	**Median**	**Mean**	**± SD**	**Median**
Survey 3							
O. mossambicus							
Albasini Dam	9	242	79	210	422	856	270
Nandoni Dam	0						
Xikundu Weir	10	270	27	267	591	107	605
C. gariepinus							
Albasini Dam	10	553	79	545	1762	856	1550
Nandoni Dam	3	630	66	655	2533	550	2500
Xikundu Weir	6	640	11	642	1666	280	1600
Survey 4							
O. mossambicus							
Albasini Dam	5	215	10	216	292	20	290
Nandoni Dam	10	236	39	226	405	301	329
Xikundu Weir	10	260	25	257	521	137	535
C. gariepinus							
Albasini Dam	10	512	83	537	1389	803	1360
Nandoni Dam	4	652	55	650	2850	1078	2850
Xikundu Weir	6	557	34	560	1408	405	1475

n = Number of fish

SD = Standard Deviation

10.2.2.3 Fixatives for histology and immunohistochemistry (IHC)

In order to establish an optimal fixative for both histological and immunohistochemical analysis, three different fixatives were employed to test their adequacy and efficacy. Following normal histological practices, Bouin's fluid (BF) and 10% neutrally buffered formalin (NBF), and the IHC fixative paraformaldehyde (PFA), were used for fixation. BF is

routinely used for histological analysis as it produces sections with great histological clarity and excellent cellular preservation.

Bouin's fluid (BF)

Testes were fixed in BF for 24 hours at room temperature. After 24 hours, samples were washed in tap water for 1 hour, and then dehydrated stepwise in 30% ethanol for 1 hour, followed by 1 hour in 50% ethanol, and finally stored in 70% ethanol until further tissue processing.

10 % Neutrally buffered formalin (NBF)

Testes were fixed for 24 hours in NBF at room temperature. After 24 hours, samples were washed in a buffer for 1 hour, then dehydrated stepwise in 30% ethanol for 1 hour, followed by 1 hour in 50% ethanol, and finally stored in 70% ethanol until tissue processing.

4% Paraformaldehyde (PFA)

Testes were fixed for 24 hours in PFA at 4°C. After 24 hours, the samples were washed in tap water for 1 hour, and then dehydrated stepwise in 30% ethanol for 1 hour, followed by 1 hour in 50% ethanol, and finally stored in 70% ethanol until tissue processing

10.2.3 Histological assessment

10.2.3.1 Haematoxylin and eosin staining technique

The sections were stained with haematoxylin and eosin for histological assessment under light microscopy. Haematoxylin is a chemical base, which stains acids, thus it binds to the DNA within the cell, and stains the nucleus blue. Eosin, which is an acidic substance, binds to bases and thus staining to the protein-rich cytoplasm pink (Kiernan, 1990). Based on the visualization of the various cells, it was possible to identify the gonadal reproductive stages.

10.2.3.2 Gonadal reproductive stages

The present investigation will utilize the classification system used by Pieterse (2004), which is based on the Goodbred et al. (1997) classification system. This classification system is explained in table 10.2.

Table 10.2: Histological characteristics of the testes.

Stage	Characteristics of testis
0	Immature Phase Sparse or no spermatogenic activity in the interstitial tissue; spermatocytes dominate; no spermatozoa present
1	Early Spermatogenic Phase Predominantly thin interstitial tissue with scattered spermatogenic activity; spermatocytes to spermatids dominate; few spermatozoa are observed
2	Mid-Spermatogenic Phase Moderate thickness of interstitial tissue; moderate spermatozoa proliferation, maturation and equal mix of spermatocytes; spermatids and spermatozoa present
3	Late Spermatogenic Phase Thick interstitial tissue; scattered spermatozoa proliferation and maturation regions; all stages of development observed; spermatozoa dominate

10.2.4 IHC assessment

10.2.4.1 Tissue preparation

The Department of Pathology at the Onderstepoort Veterinary Institute embedded blocks of *C. gariepinus* and *O. mossambicus* testes in paraffin wax, and prepared all the slides for histological and IHC analysis.

Sections of 4 µm thick were floated onto a distilled water bath, (45°C), and collected on Superfrost slides (Menzel-Glaser, Germany). Each slide comprised of three sections, obtained from the same specimen. Each section was fixed in a different fixative and placed in order, as indicated in figure 10.1.

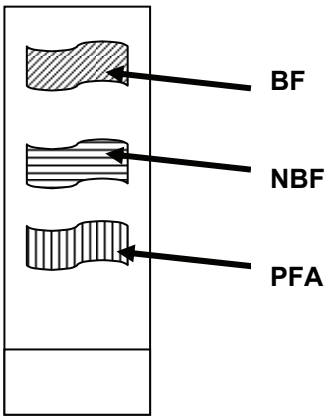


Figure 10.1: Layout of testicular section on slides for histological and IHC processing (BF=Bouin's fluid, NBF=neutrally buffered formalin, PFA=paraformaldehyde).

10.2.4.2 Caspase IHC

Active caspase-3 was studied in 4 µm sections from paraffin-embedded tissue from all fish. Sections were floated in a water bath (45°C) and collected onto Superfrost slides (Menzel-Glaser, Germany). After deparaffinization with Xylene, the sections were rehydrated through a series of graded ethanol (100 and 95 10 min each). Antigen retrieval was undertaken in Antigen Unmasking Solution (Vectorlabs, Burlingame, CA, USA) until boiling and then at 30% power for 10 min in a domestic microwave and then cooled for 30 min at room temperature (RT). Endogenous peroxidases were quenched with 3% hydrogen peroxide in distilled water in darkness for 10 min. Following a rinse in distilled water, the sections were placed in 0.1% (v/v) Tween 20 in phosphate buffered saline (PBS-A: 137 mM NaCl, 29 mM $NaH_2PO_4 \cdot H_2O$, 9 mM Na_2HPO_4, pH 7.4) for 5 min. Sections were incubated with blocking solution (5% normal goat serum in 0.1%) Tween 20-PBS-A) for 1 hour at RT. Then, slides were incubated with cleaved caspase-3 antibody (Asp175) (Cell Signalling Technology,

Beverly, MA, USA) at 1:200 dilution overnight at 4°C. After three washes in 0.1% Tween 20/PBS-A, a secondary antibody (biotinylated antirabbit IgG) was added and incubated at RT for 30min. Following a PBS-A wash, the sections were incubated with the Vectastain avidin-biotin-complex (Vectorlabs, Burlingame, CA, USA) for a further 30min at RT. After washing with PBS (2X), immunoreactions were detected by incubating the sections with a 3, 3´-diaminobenzidine (DAB) substrate kit. Counterstaining was done with Hematoxylin QS (Vectorlabs, Burlingame, CA, USA), a modification of Mayer's Hematoxylin, specifically developed for immunohistochemistry, with less than 45 seconds staining time. After staining slides were rinsed in running water, dehydrated in butanol, cleared in Xylene, and mounted with Entellan (Merck, Darmstadt, Germany).

10.2.4.3 Terminal deoxynecleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) IHC

Following deparaffinization and rehydration, permeabilization was achieved by incubation in 0.5% Triton X-100 for 10 min at RT before treatment with 3% hydrogen peroxide for 5 min to quench endogenous peroxidase. This was followed by two washes in phosphate buffered saline (PBS-B: 50 mM sodium phosphate, pH 7.4; 200 mM NaCl). Subsequent steps for TUNEL staining were carried out using the ApopTag-Peroxidase Kit according to the supplier's instructions (Chemicon, Temecula, CA). DNA fragmentation was detected by incubating with the TdT-mediated dUTP nick end-labelling reaction mixture in a humidified chamber at 37°C for 60 min. Thereafter, the sections were treated with the antidigoxigenin-peroxidase complex for 30 min at RT. After three PBS-B washes, immunoreactions were detected by incubating with 3,3´-diaminobenzidine (DAB) substrate kit. Counterstaining was done with Hematoxylin QS (Vectorlabs, Burlingame, CA, USA), a modification of Mayer's Hematoxylin, specifically developed for immunohistochemistry, with less than 45 s staining time. After staining, slides were rinsed in running water, dehydrated in butanol, cleared in xylene, and mounted with Entellan (Merck, Darmstadt, Germany).

10.2.5 Microscopic quantification

The entire section was evaluated and all the lobules in a given section were counted and scored as caspase-3-positive or caspase-3- negative (TUNEL-positive or TUNEL-negative). A positive cell is stained brown after the reaction with DAB. A lobule was designated as positive if it contained at least one cluster of three positively stained cells at a 20 x magnification, and expressed as a percentage. In each of the caspase-3-positive or TUNEL-

positive lobule, the lobule was scored for the number of caspase-3-positive or TUNEL-positive cells that the lobule contains. In addition the type of cell displaying positivity was indicated, i.e. spermatocytes, spermatids.

10.2.6 Analytical chemical analysis

The compounds selected for target chemical residue analysis, were the organochlorine pesticides (OCs) *o,p'*- and *p,p'*-DDT, -DDD and -DDE. The water and fish fat samples were analyzed in the Residue Laboratory of the Agricultural Research Commission (ARC) at Onderstepoort Veterinary Institute.

10.2.6.1 Tissue preparation for chemical analysis

When available, fat was collected for each specimen. The fat was wrapped in foil and stored at -20°C until processing for target chemical analysis in the laboratory (Barnhoorn et al., 2004).

10.2.6.2 Water collection

Water samples for water quality analysis were collected during survey 3 and 4 at a depth of 5-15 cm under the water surface, in glass bottles pre-washed with methanol. These samples were stored in a refrigerator until further analysis was conducted. In each of the three sampling sites, pH, conductivity (µS/cm), Total Dissolved Salts (TDS) (mg/L), water temperature (°C) and the oxygen percentage (%) was measured in both surveys. These parameters were measured using the pH Scan 2 pH meter (Eutech Instruments), the Cyberscan CON 110 conductivity/TDS/°C meter RS 232 (Eutech Instruments) and the Cyberscan DO 100 meter (for dissolved oxygen).

10.2.7 Statistical analysis

A Chi-squared (Goodness of Fit) Test was used to enable the comparison of the distribution between the samples in each study site, and to test associations between the fixatives, surveys, assays, and sites. Differences in selected water and tissue samples were calculated using the Microsoft software package, Excel.

10.3 Results

10.3.1 Water quality assessment

The water quality parameters recorded during both seasons are represented in Table 10.3. The measured pH values form AD and ND were above the suggested upper value of a pH of 8, for South African inland waters. The EC ranged from 139.2 in survey 3 to 825 in survey 4. Similarly, the TDS ranged from 69.50 in survey 3 to 413 in survey 4. The water temperature ranged from 18°C to 24.7°C during survey 3 and from 28°C to 29.5°C in survey 4. The percentage oxygen saturation ranged from 113.7 to 136.1 spanning the two surveys.

Table 10.3: Physical water quality data from the three sampling sites.

	AD		ND		XW		DWAF
Surveys	**3**	**4**	**3**	**4**	**3**	**4**	
pH	8.48	8.61	8.93	8.28	7.3	6.96	6.0-8.0
Electrical conductivity (EC)	825	305	424	150.5	182.3	139.2	**
Total Dissolved Salts (TDS)	413	153	214	75.2	-	69.50	**
Temperature (°C)	24.7	28	22.4	29.5	18	28.9	5-30
Oxygen (mg/L)	8.74	10	9.81	10.38	12	9.27	> 4*
Oxygen concentration (%)	114.9	127.3	113.7	136.1	135.2	123.3	> 40 % *

3 = 1 LF = Survey 3 Low Flow

4 = 2 HF = Survey 4 High Flow

* Lethal below this value

** Refer to DWAF (1996g) for guidelines

10.3.2 Chemical analysis of tissues

The chemicals that were selected for target analysis and measured in the fat of both *O. mossambicus* (Table 10.4) and *C. gariepinus* (Table 10.5) are represented as mean (µg/Kg) ± SD.

10.3.3 Immunohistochemical assessment

10.3.3.1 The Caspase-3 assay – *O. mossambicus* (Fig 10.2 A-G)

During survey 3, in XW, the fixative BF (n = 6745), yielded the greatest amount of positive cells as compared to PFA (n = 5245), and NBF (n = 3643). In survey 4, BF (n = 161) yielded the greatest amount of positive cells, followed by NBF (n = 39), but PFA had no positively stained caspase-3 cells. Interestingly, caspase-3 immunoreactivity was present in a cluster formation. In the AD, during survey 3, BF (n = 2673), yielded the greatest number of positive cells compared to NBF (n = 1790), and PFA (n = 934). During survey 4, a similar pattern is observed with BF (n = 65) yielding greater positive cells than PFA (n = 12). Interestingly, BF yielded no positively stained cells. Similar to XW, there is a greater amount of positive cells in survey 3, as compared to survey 4. Interestingly, the cluster formation is again observed in all three fixatives, as was observed in XW. In the ND, during survey 3, no *O. mossambicus* were sampled, despite numerous efforts. In contrast, in survey 4, similar to the situation in AD, BF (n = 2673), yields the greatest number of positive cells as compared to NBF (n = 277), and PFA (n = 254). It is noteworthy that the same cluster formation that was observed at XW and AD is also present in ND.

10.3.3.2 The Caspase-3 assay – *C. gariepinus* (Fig 10.2 H-L)

During survey 3, the “low flow” season, the fixative NBF (n = 733), yielded the greatest amount of positive cells as compared to PFA (n = 685), and BF (n = 31). In contrast, in survey 4, the “high flow” season, only NBF (n = 39) yielded positive cells, with BF and PFA having no positively stained caspase-3 cells in XW. In the AD, during survey 3, NBF (n = 108), yields the greatest number of positive cells as compared to PFA (n = 92), and BF (n = 23). In survey 4, a similar pattern is observed with NBF (n = 392) yielding greater positive cells than PFA (n = 387) and BF (n = 118). In the ND, during survey 3, PFA (n = 1926), yielded the greatest number of positive cells as compared to NBF (n = 453), and BF (n = 33). In contrast, in survey 4, similar to the situation in XW, only NBF (n = 20) yielded positive cells, with BF and PFA having no positively stained caspase-3 cells.

10.3.3.3 The TUNEL assay – *O. mossambicus* (Fig 10.3 A-F)

During survey 3, in XW, the fixative BF (n = 267), yielded the greatest amount of positive cells as compared to PFA (n = 266), and NBF (n = 200). In survey 4, NBF (n = 16) yielded

the greatest amount of positive cells, followed by BF (n = 8), but PFA having no positively stained caspase-3 cells. Interestingly, TUNEL immunoreactivity was present in a cluster formation. The fixative BF (n = 15712), yielded the greatest amount of positive cells as compared to PFA (n = 3519) and NBF (n = 2903). This was the site with the greatest number of positive cells observed in survey 3. In contrast, in survey 4, NBF (n = 45) yielded the greatest amount of positive cells, followed by PFA (n = 28) and BF yielding no positive cells. The cluster formation is again observed, as in the caspase-3 assay. In the ND, during survey 3, no *O. mossambicus* were sampled, despite numerous efforts. In survey 4, similar to the situation in XW, NBF (n = 53), yielded the greatest number of positive cells as compared to BF (n = 46), and PFA (n = 38).

Table 10.4: DDT and metabolites in *O. mossambicus* fat tissue (mean ± SD).

	Albasini Dam		Nandoni Dam		Xikundu Weir	
Survey	**3**	**4**	**3**	**4**	**3**	**4**
	(0 of n = 9)	**(1 of n = 5)**	**(0 of n = 0)**	**(10 of n = 10)**	**(0 of n = 10)**	**(9 of n = 10)**
DDT residues (µg/kg)						
o,p´-DDT	*	< 50	*	84.00 ± 16.00	*	340.00 ± 150.00
p,p´-DDT	*	< 50	*	445.00 ± 440.00	*	7000.00 ± 3300.00
o,p´-DDD	*	< 50	*	98.00 ± 54.00	*	190.00 ± 41.00
p,p´-DDD	*	352.00	*	4300.00 ± 5200.00	*	13000.00 ± 4100.00
o,p´-DDE	*	< 50	*	130.00 ± 85.00	*	270.00 ± 64.00
p,p´-DDE	*	< 50	*	1400.00 ± 1200.00	*	6300.00 ± 1700.00

- No fat available in collected fish

Table 10.5: DDT and metabolites in *C. gariepinus* fat tissue in (mean ± SD).

	Albasini Dam		Nandoni Dam		Xikundu Weir	
Survey	**3**	**4**	**3**	**4**	**3**	**4**
	(5 of n=10)	**(2 of n=10)**	**(3 of n=3)**	**(2 of n=4)**	**(3 of n=6)**	**(6 of n=6)**
DDT residues (µg/kg)						
o,p´-DDT	95.00 ± 16.00	62.00 ± 5.00	200.00 ± 42.00	77.00 ± 7.00	2300.00 ± 180.00	420.00 ± 260.00
p,p´-DDT	210.00 ± 100.00	75.00 ± 9.00	1400.00 ± 610.00	260.00 ± 39.00	18000.00 ± 1400.00	4500.00 ± 4300.00
o,p´-DDD	< 50	< 50	170.00 ± 43.00	100.00 ± 5.00	540.00 ± 190.00	280.00 ± 150.00
p,p´-DDD	290.00 ± 100.00	2400.00 ± 590.00	2200.00 ± 830.00	9400.00 ± 1600.00	8100.00 ± 960.00	35000.00 ± 27000.00
o,p´-DDE	< 50	< 50	< 50	93.00 ± 10.00	88.00 ± 31.00	570.00 ± 320.00
p,p´-DDE	2500.00 ± 2100.00	310.00 ± 100.00	9300.00 ± 5100	2200.00 ± 210.00	37000.00 ± 3700.00	8200.00 ± 3900.00

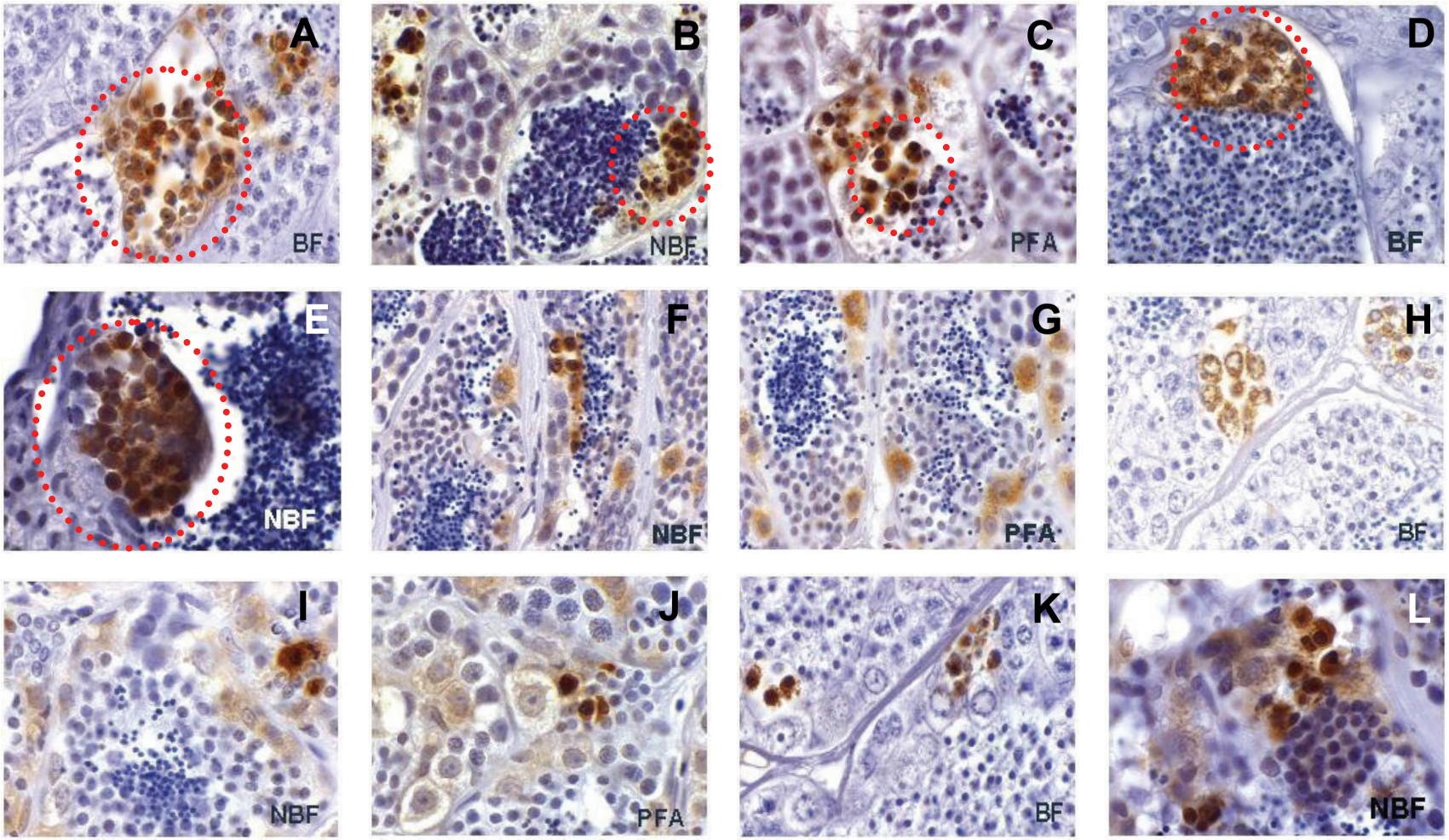


Figure 10.2: Caspase-3 immunoreactivity in *O. mossambicus* (A-G) and *C. gariepinus* (H-L). Note the large cluster formation in the *O. mossambicus*, not observed in *C. gariepinus*, indicated by dashed circle.

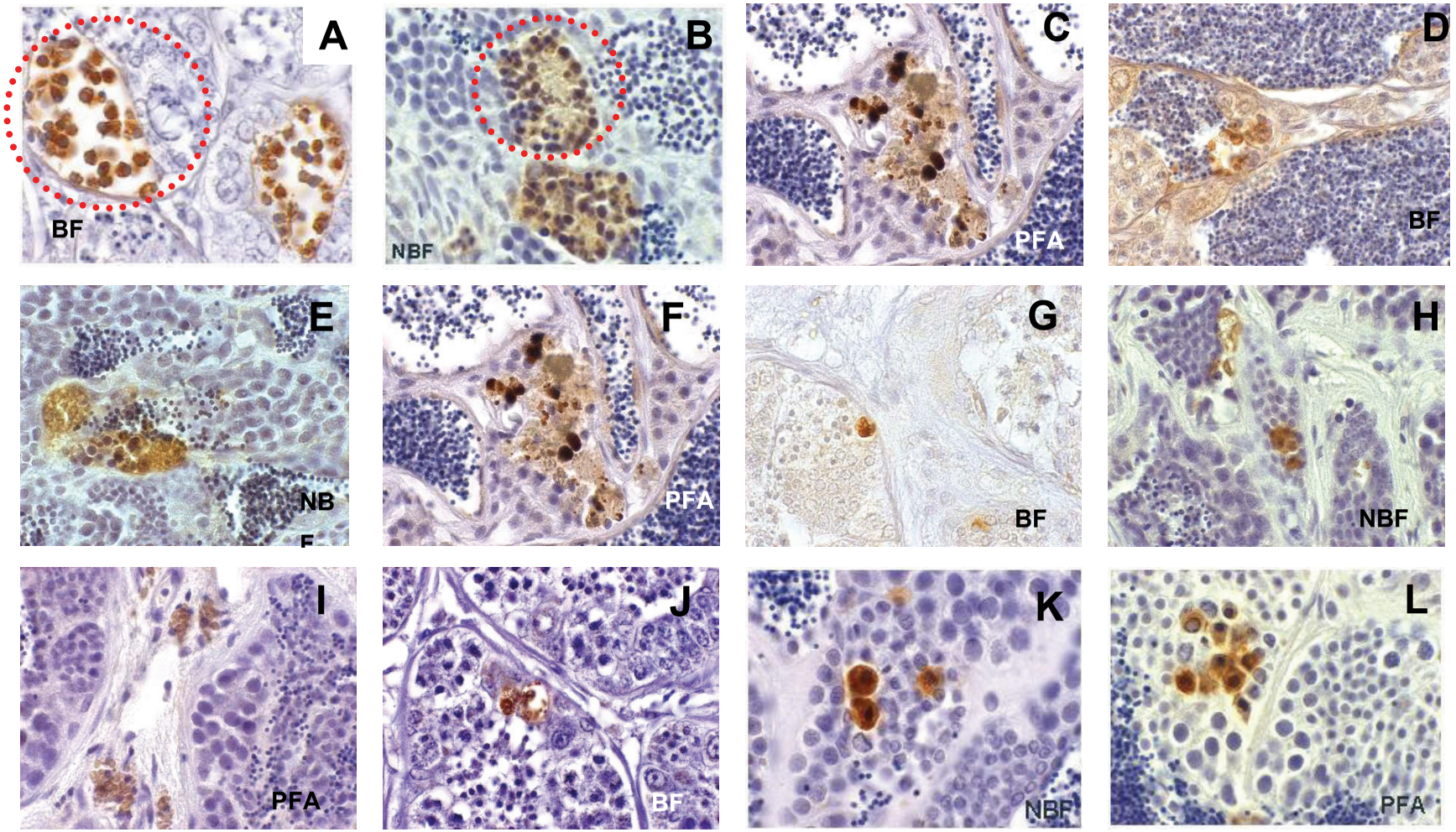


Figure 10.3: TUNEL immunoreactivity in *O. mossambicus* (A-F) and *C. gariepinus* (G-L). Note the large cluster formation in the *O. mossambicus*, indicated by dashed circle.

10.3.3.4 The TUNEL assay – *C. gariepinus* (Fig 10.3 G-L)

The TUNEL assay specifically labels the fragmentation of the DNA, indicating the death of the cell. During survey 3, the low flow season, the fixative NBF (n = 31), yielded the greatest amount of positive cells as compared to PFA (n = 14). The fixative BF yielded no positively stained cells. In contrast, in survey 4, the high flow season, only NBF (n = 50) yielded positive cells, with BF and PFA having no positively stained TUNEL cells. During survey 3, the fixative PFA (n = 488), yielded the greatest amount of positive cells as compared to NBF (n = 392) and BF (n = 246). In contrast, in survey 4, NBF (n = 50) yielded the greatest amount of positive cells, followed by PFA (n = 9) and BF (n = 6). During survey 3, the fixative NBF (n = 29), yielded the greatest amount of positive cells as compared to PFA (n = 26) and BF (n = 4). In contrast, in survey 4, all three fixatives yielded no observed TUNEL positive cells.

10.3.4 Analysis of quantitative results

10.3.4.1 Albasini Dam

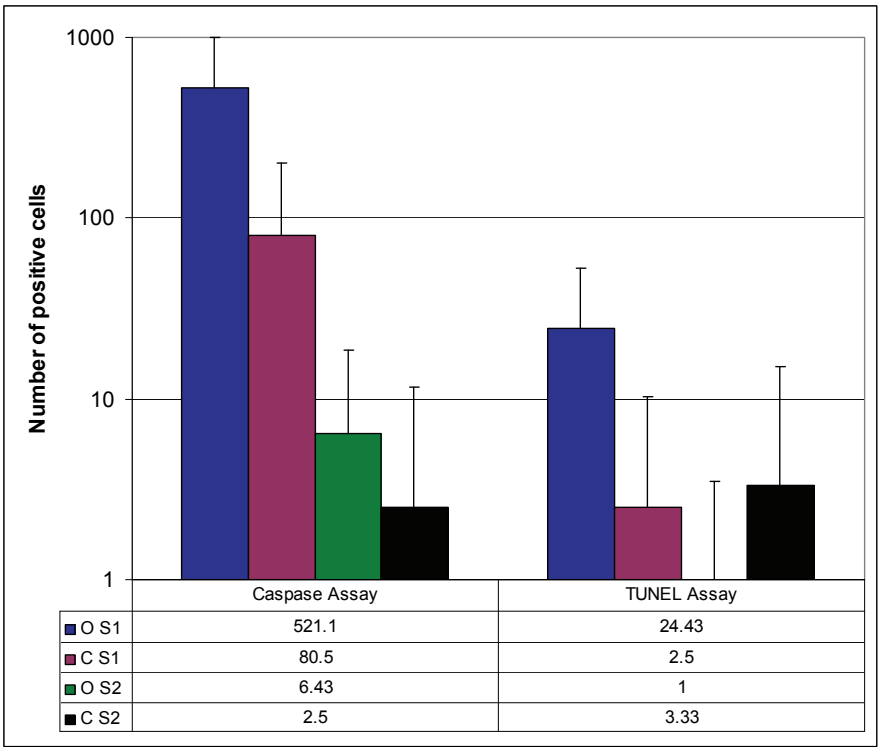
The comparison of the caspase-3 assay and the TUNEL assay in the efficacy of determining apoptotic death is reflected by the amount of positive cells (figs 10.2 and 10.3). In survey 4 in *O. mossambicus*, the amount of caspase-3 positive cells is much greater than in survey 3. The TUNEL positive cells, in *O. mossambicus* were however greater in survey 4, than in survey 3. In survey 3, the caspase-3 and TUNEL positive cells of *C. gariepinus* sections were greater than in survey 4 (Fig 10.4).

10.3.4.2 Nandoni Dam

In the ND, the amount of caspase-3 positive cells in *O. mossambicus* is the greatest in survey 3, when compared to AD. There were no *C. gariepinus* collected in survey 3, thus there is no recording on the graph (Fig 10.5). However, the amount of caspase-3 positive cells observed in *C. gariepinus* in survey 4 is the highest, when compared to XW and AD.

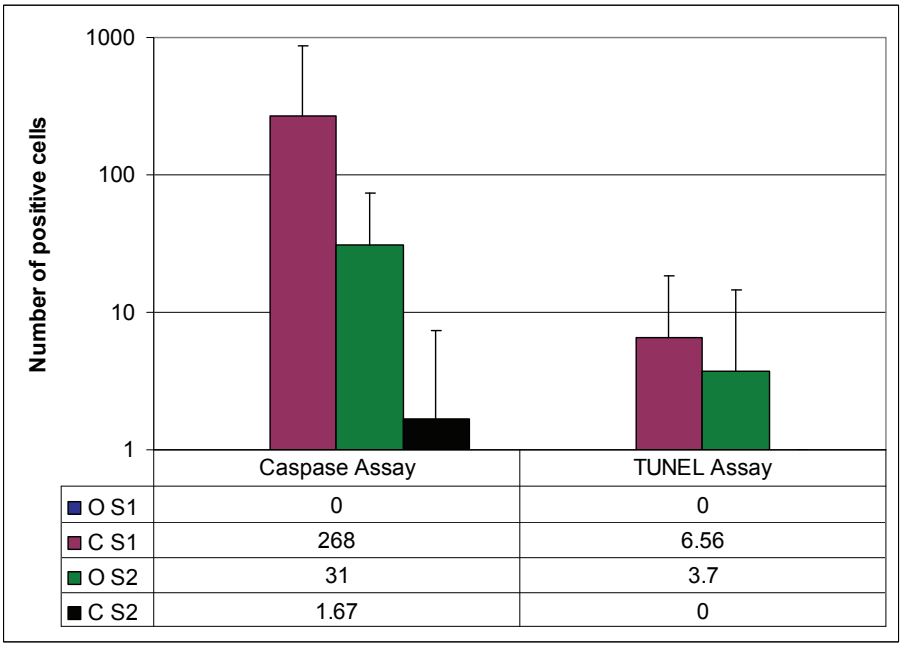
10.3.4.3 Xikundu Weir

In *O. mossambicus*, the amount of positive cells is much greater in survey 4, than in survey 3. The TUNEL positive cells, however, are greater in survey 3, than in season two. In survey 3, the caspase-3 and TUNEL positive cells of *C. gariepinus* sections were greater than in survey 4 (Fig 10.6).



	Caspase Assay	TUNEL Assay
O S1	521.1	24.43
C S1	80.5	2.5
O S2	6.43	1
C S2	2.5	3.33

Figure 10.4: Graphical representation of the total positive cells in both species in the AD (O S1 = *O. mossambicus* survey 3, C S1 = *C. gariepinus* survey 3, O S2 = *O. mossambicus* survey 4, C S2 = *C. gariepinus* survey 4).



	Caspase Assay	TUNEL Assay
O S1	0	0
C S1	268	6.56
O S2	31	3.7
C S2	1.67	0

Figure 10.5: Graphical representation of the total positive cells in both species in the ND (O S1 = *O. mossambicus* survey 3, C S1 = *C. gariepinus* survey 3, O S2 = *O. mossambicus* survey 4, C S2 = *C. gariepinus* survey 4).

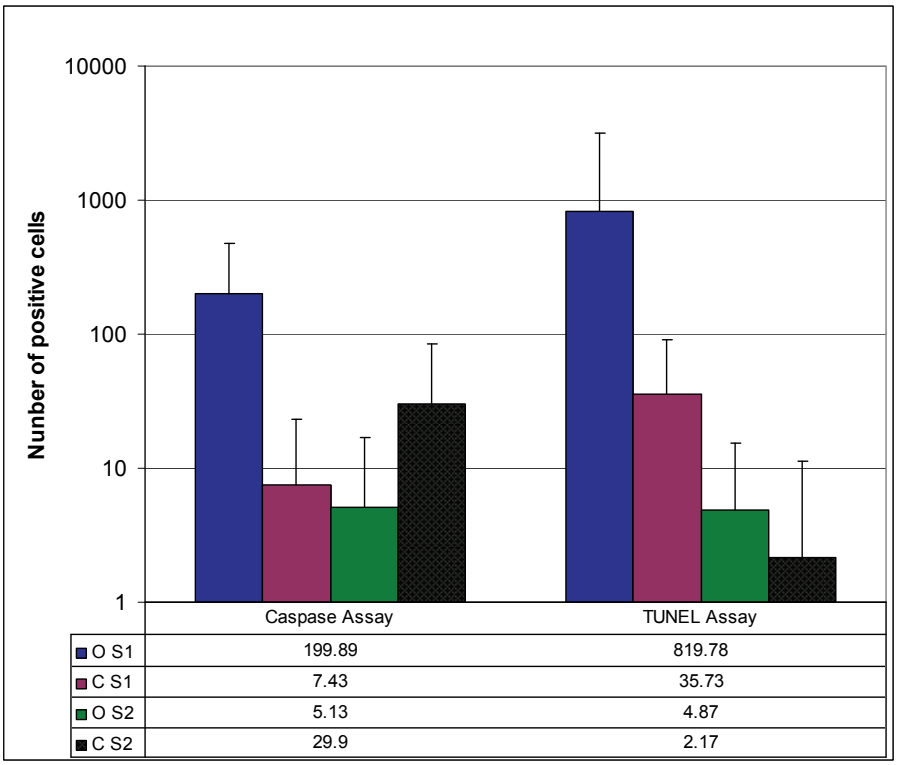


Figure 10.6: Graphical representation of the total positive cells in both species in the Xikundu Weir (O S1 = *O. mossambicus* survey 3, C S1 = *C. gariepinus* survey 3, O S2 = *O. mossambicus* survey 4, C S2 = *C. gariepinus* survey 4).

10.3.5 The Gonadosomatic Index (GSI)

The GSI, when compared between surveys 3 and 4, differs marginally, with the GSI from survey 4 being lower than that of survey 3, in both and *O. mossambicus* (Fig 10.7) *C. gariepinus* (Fig 10.8).

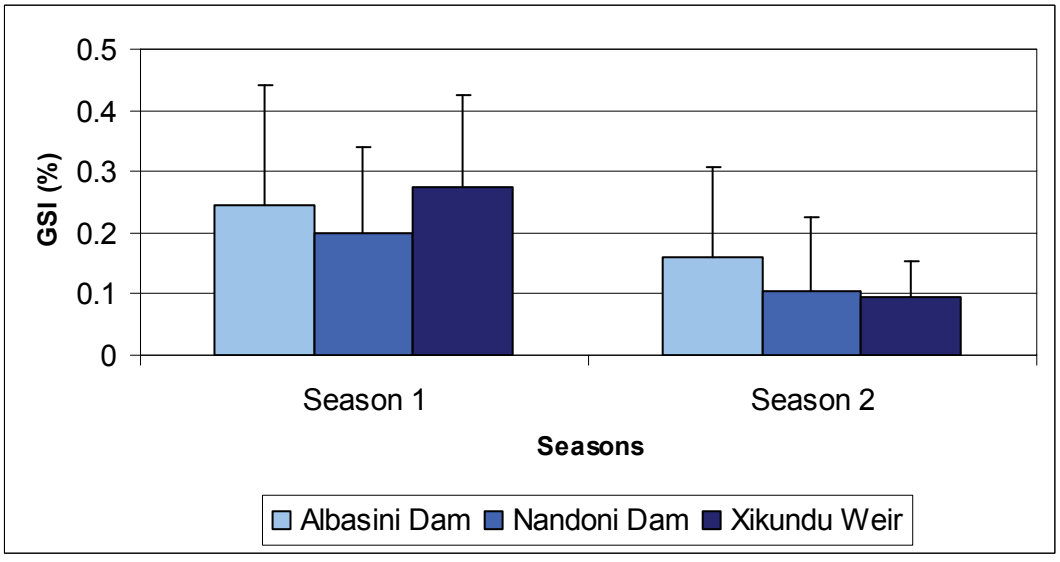


Figure 10.7: Comparison of GSI between survey 3 and 4 in *O. mossambicus*.

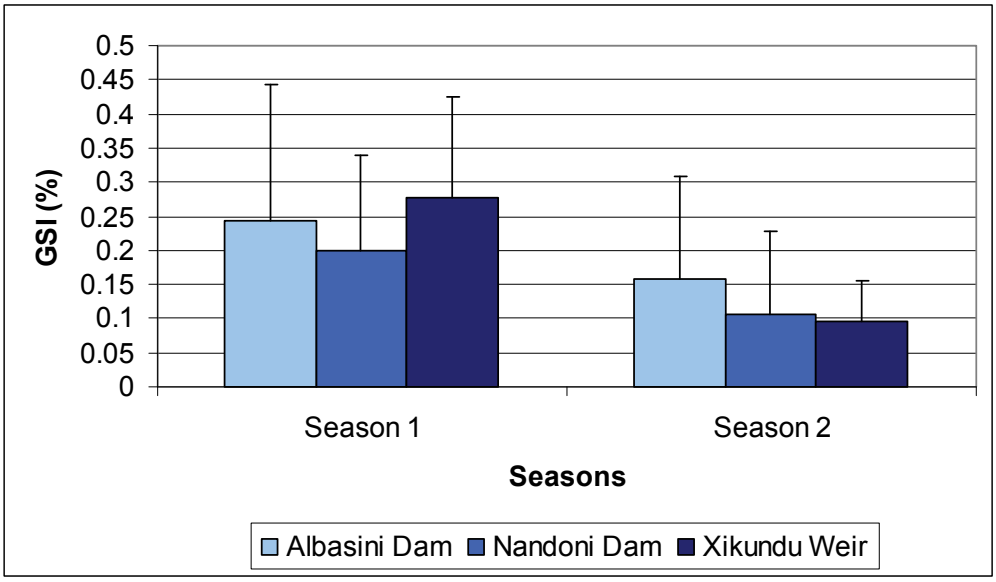


Figure 10.8: Comparison of GSI between survey 3 and 4 in *C. gariepinus.*

Statistically the surveys differ in both the GSI and caspase-3 and TUNEL positive cells. When taking into consideration that GSI is an indication of gonadal growth, the developmental stage of the collected fish is relevant. During survey 3, the majority of the collected specimens, were in the developed stage, with a few being in the developing stage. In contrast, in survey 4, the majority of the collected specimens were in mature stage of development. Thus, being reproductively active the amount of sperm and hence the gonadal weight will decrease. The decrease in the GSI in survey 4 may therefore be attributed to normal reproductive cycles, rather than abnormal behaviour.

10.4 Discussion

Even though the aquatic ecosystem is not the primary site of application of EDCs, such as pesticides, aquatic organisms are still affected by these hazardous compounds. The residues of these compounds that reach the water are either concentrated in certain parts of the ecosystem, or are in solution for prolonged periods of time, or some may be adsorbed into the particulate matter. The aquatic organisms possess the potential to absorb these residues and subsequently bioaccumulate these residues into the trophic chain. The persistence of a compound in the aquatic environment, as on the terrestrial environment, is influenced by numerous factors, such as the molecular chemistry of the compound, the physicochemical conditions, and the general health of the aquatic organisms (Murty, 1988).

10.4.1 Water quality

Although the physiochemical parameters that were measured were all within the recommended DWAF (1996g) ranges (Table 10.3), the pH showed a slight increase from the recommended 6-8 pH range, with values of 8.61 in AD survey 4, and 8.93 in ND in survey 3. Although these increases are slight, further increases may lead to the occurrence of algal blooms, which will adversely affect the water quality (Bornman et al*.,* 2004).

10.4.2 Chemical analysis of tissue

In aquatic ecosystems, a greater threat to organisms exists compared to terrestrial environments, as larger numbers of organisms are constantly in contact with the polluted environment. The residues of *p,p´*-DDT -DDD and -DDE were found in the highest levels in both *O. mossambicus* (Table 10.4) and *C. gariepinus* (Table 10.5), similar to the findings of Bornman et al*.* (2007). Seeing as DDT has a great affinity to lipid tissue, it is thus taken up rapidly from food and water sources and then metabolized slowly, and may then be stored for extended periods (Roberts, 1995). This gives rise to the conclusion that the fish were exposed to these particular EDCs at a point in their lifecycles, and subsequently bio-accumulated these chemicals in their lipid tissue.

Seeing as DDT is prevalent in the AD, ND and XW, and it being poorly water soluble, a greater route of intake of DDT exists. The high levels of DDT and its metabolites that are found in the fat of both *O. mossambicus* and *C. gariepinus*, supports the ability of the lipophilic compounds to accumulate in the fat tissue. It is however this interaction of fat breakdown and steroid hormone synthesis, that leads to disturbed cell function. If potent enough, this disturbance may result in the alteration of the functions of various processes (Roberts, 1995). In order to assess and visualize the cellular disturbances, it is of pivotal importance that the cellular integrity is maintained through optimal fixation (Montero, 2003).

10.4.3 Histological analysis

The fixative that is recommended for IHC assessment is PFA (Kiernan, 1990), and in some instances NBF. However, the usage of BF as a histological fixative has been demonstrated, but it has been suggested that it not be used for IHC, due to it being a stringent fixative, and over-fixing the antigens. This makes antigen revival difficult and results in a poor or no signal being obtained (Montero, 2003).

In the current study, BF showed utility as a fixative for IHC in *O. mossambicus*, in both assays, and yielding the best clarity when compared to NBF and PFA (Figs 10.2 and 10.3). In comparison, NBF yielded the weakest clarity in histological sections, making it difficult to distinguish cells in certain instances. The fixative, PFA, yielded a greater clarity than NBF, but still weaker than BF. As a histological tool, it is recommended that BF be used for both species. It should be noted that PFA yields clarity that, although not as great as BF, is still clear enough to distinguish cells, especially in *O. mossambicus*.

10.4.4 IHC analysis

Using all three fixatives, as mentioned in Chapter 3, sections of both *O. mossambicus* and *C. gariepinus* testes were stained for histological assessment, to assess the structure of the testes. In both season one and season two BF proved to produce the optimal clarity, in both *O. mossambicus* and *C. gariepinus* sections. It is noteworthy that in certain instances NBF is used for histological assessments. In this instance, NBF yielded the weakest clarity when compared to BF and PFA. There appears to be no difference in the clarity between the three sites and between the two surveys. Interestingly, PFA, which is recommended for IHC provided greater clarity of sections than NBF, but a weaker clarity compared to BF, in both *O. mossambicus* and *C. gariepinus*.

In the current study the utility of the caspase-3 as a marker for activation of apoptosis yielded positive results. The use of caspase-3 as a marker for the onset of apoptotic death is well documented (Maag et al, 2003). In fish however, apoptosis is a prolonged process (McClusky et al, 2008) and the caspase-3 assay may yield an inadequate result. There are no currently published data that utilizes the caspase-3 assay to detect testicular apoptosis in fish species inhabiting an area where there is ongoing DDT spraying.

The onset of apoptotic death is investigated by Arukwe (2008). The suggested notion is that, when there is a form of endocrine disruption due to various EDCs that the hormonal interference does not occur at the level of aromatization, but rather at the site of steroidogenesis, which involves the breakdown of lipid tissue. In all animal cells, there is a mitochondria that is the energy supplier of the cell, it is also consequently the site of hormonal production i.e. steroidogenesis. The testes has the ability to produce steroid hormones, thus the potential for hormonal disruption is possible. The rate-limiting step in the production of steroidal hormones is the movement of cholesterol across the mitochondrial membrane. This action is mediated through the action of the steroidogenic acute regulatory

(StAR) protein. The cholesterol is then converted through the cytochrome P450, and then steroidogenesis ensues. It has always been accepted that the site of endocrine disruption occurs after this P450 step, however it is possible that EDCs such as DDT may also act before this step.

The testes is a site of steroidogenesis any substance that can limit the action of StAR activation, will result in hormonal disruption. This hormonal disruption will lead to the alteration in the normal cell progression and consequently the cells functional maturation. Therefore the process of apoptosis, which may also be activated via the mitochondria, is activated to remove damaged cells

Following executionary caspase activation, the cell is dismantled, the DNA fragmented, and subsequently packaged into vesicles for phagocytosis (Bortner et al, 1995). The utility of TUNEL technique is accepted as the most widely used histochemical method for fragmentation detection (Bellamy et al, 1995). In the testes of *O. mossambicus* and *C. gariepinus* the TUNEL immunoreactivity is observed in survey 3 in both the AD and XW, and in *O. mossambicus* in survey 3 and 4 in the ND. Following the same pattern, as the caspase-3 immunostaining, season one produced a greater amount of positive cells. When all the results are taken together the AD and the XW appear to share similar immunostaining patterns, as well as similar numbers of positively stained cells. This leads to the conclusion that these two sites may possibly exert similar amounts of endocrine disruptive activity on the exposed fish.

10.4.5 Quantitative results

The immunoreactivity of the caspase assay in the *O. mossambicus* testes shows the same pattern as *C. gariepinus*, with there being a higher incidence of positive expression in the testes from the first season (survey 3). However, the results from the XW indicate the opposite, with a greater positive expression observed in the second season (survey 4). In support of this, the GSI values for *O. mossambicus* in the XW increased in survey 4. However the *O. mossambicus* testes in both the AD and XW showed a decrease in the GSI, and also a decrease in positivity in survey 4. The seasonal variations that have been observed are directly related to the season of application that occurs on land, the rate of transport to the aquatic environment, as well as the condition of the fish and stage of reproduction. During the first season, the fish were preparing for the next spawning season, thus there is an increase in the total lipid content of the fish (Murty, 1988).

The large cluster formation observed in *O. mossambicus* testes in both caspase-3 and TUNEL assays might be the result of the cytoplasmic bridges observed between the germ cells. The function of the cytoplasmic bridges in the murine model, are to allow the germ cells to communicate with each other as well as share nutrients (De Rooij and Russel, 2000), and therefore also pollutants. This notion leads to the conclusion that in *O. mossambicus*, EDCs and other pollutants may affect the germ cell interaction and communication more adversely compared to *C. gariepinus*.

Although this result is not observed in *C. gariepinus*, it may be due to the process of apoptosis being prolonged in fish (McClusky, 2005). Seeing that the process of apoptosis is prolonged in fish, a cell may have committed itself to undergo death, but may not fragment its nuclei until a much later time. A surprising low incidence of TUNEL-labelling as compared to caspase-3 labelling has been reported, resulting from inadequate DNA fragmentation. However, the reactivity was specific and abundant, suggesting that upstream events, such as caspase-3 activation, are prolonged.

Thus, McClusky et al. (2008) concluded that, the TUNEL assay greatly underestimates the apoptotic events occurring within the cell, and that caspase-3 immunostaining is superior in quantifying apoptosis. This is concomitant with the TUNEL assay being labelled as having limitations in its sensitivity and specificity (Labat-Moleur et al., 1998). Hence, it is advisable to use these two assays to determine the extent of apoptotic death.

The majority of the cells undergoing apoptosis are the spermatocytes, and some spermatogonia that have committed themselves to apoptotic death. In some human studies, the spermatogonia and spermatids are found to be the cells that generally undergo apoptosis (Print and Loveland, 2000). In the fish model very few spermatogonia were observed to undergo apoptotic cell death.

10.5 Conclusions

The use of the immunohistochemical assays could therefore be applied to *C. gariepinus* testicular sections, to investigate apoptosis, but these techniques have never before been applied to *O. mossambicus*. This is the first report on the use of the caspase-3 and TUNEL assays in quantifying the extent of apoptotic regulation in *O. mossambicus*. No direct link could be made between higher than normal expected apoptotic activity and chemical exposure. Further investigation may reveal links between the current results found and other possible environmental pollutants.

CHAPTER 11

SPERM MOTILITY PARAMETERS OF *O. MOSSAMBICUS* AND *C. GARIEPINUS*

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11.1 Introduction

Fish sperm is different to that of mammals since capacitation and hyperactivation are not processes involved in fertilization. Sperm are immotile until ejaculation when the milt is diluted with water, which immediately initiates motility for a very short period compared to human sperm. Fish sperm do not penetrate the oocyte wall, but enter through a channel known as a micropyle. For successful fertilization it is necessary for the male to produce motile, viable sperm that can locate the eggs and then the micropyle for entry into the oocyte. EDCs (e.g. DDT) affect the motility and morphology of fish sperm in various ways. The hormonal environment or function of the Sertoli cells may be disrupted, the seminal fluid or sperm maturation may be changed, or there may be cytological damage to the sperm itself and altered efficiency of mitochondrial energy production (Kime, 2001). The effects of DDT and its metabolites on the viability of spermatozoa of two fish species (*C. gariepinus* and *O. mossambicus*) from a reference site and two exposed sites in the Luvuvhu River, Limpopo Province, South Africa, were studied using computer assisted sperm motion analysis (CASA).

11.1.1 Computer assisted sperm analysis (CASA)

Methods for assessing sperm quality that are not dependent on eggs have a major advantage. Sperm is available over a longer period than eggs. Sperm collection and storage methods can be optimized by CASA without the use of females (Kime et al., 2001). The most common methodology used until recently to assess sperm quality has been to observe sperm, either directly on the microscope slide, or via video tape, and assess its movement in comparison to a rigid point scale (Kime et al*.,* 2001). Kime et al. (2001) suggests that neither method is clear as to what period of motility the scale refers to after initiation of motility and the methodology would be difficult to apply to an assessment over several short time intervals after induction of motility. Such scaling methods can give an estimated ranking of different sperm motilities but these methods are not amenable to statistical treatment, even if scores are made using several independent observers for each sample. In all cases the methodology is very time-consuming (Kime et al., 2001).

To improve the methodology of assessing sperm quality and to make it objective and quantitative, actual sperm velocity can be measured either manually or automatically. Such methods are time-consuming and there is an element of subjectivity in choosing which individual sperm to track. A major advance in the rapid and objective assessment of fish motility has come from using the sophisticated image analysis methodology for sperm measurement (CASA) used in human fertility clinics. A CASA system refers to the physical equipment used to visualize and digitize static and dynamic sperm images, and to the methods used to process and analyse them (Boyer et al*.,* 1989). The system quantifies different sperm motility parameters of interest, including those which are not observable manually.

Assessment of sperm motility using CASA methodology is rapid and allows for elaborated analysis of the components of that motility. However, temperature, the size and the concentration of sperm as well as the speed, may all affect the quality of results and must be taken into account during the calibration process (Rurangwa et al., 2004). It has required some adaptation before it could be used for fish sperm which is generally motile for less than two minutes compared to several hours for mammalian sperm (Kime et al., 2001). The use of a video camera allows the recording of sperm via a video microscope in the field or at a remote laboratory, followed by processing of the recording at any future time at a central or shared facility. Sperm trackers track sperm in real time and generate parameters of movement. The most commonly used parameters for fish sperm motility analyses can include curvilinear velocity (VCL, the actual velocity along the trajectory); straight line velocity (VSL, the straight line distance between the start and end points of the track divided by the time of the track); linearity (LIN, the straight line distance between the start and end points of the track divided by the sum of the incremental distances along the actual path); and % motile (% MOT, the number of motile sperm within the analysis field divided by the sum of the motile plus immotile sperm within the analysis field (Kime et al., 2001).

CASA has high repeatability and provides a more discriminating estimate of sperm motility than does the visual/microscopic subjective procedure. It permits a determination of sperm motility characteristics, improves reproducibility and facilitates the documentation of the results (Rurangwa et al., 2004). A novel CASA system based on open source software for characterization of sperm motility parameters was compiled and set up to suit the test species used in this study (Wilson-Leedy and Ingermann, 2007).

11.2 Materials and methods

11.2.1 Study area

The study areas concerned are located on the Luvuvhu River within the Limpopo Province. They are part of the Luvuvhu Catchment that forms part of the larger Limpopo system, which runs downstream into Mozambique. Both species (*O.* mossambicus and *C. gariepinus*) were sampled at a reference site, Albasini Dam (AD), (outside the DDT-sprayed area) and at two exposed site, Nandoni Dam (ND) and Xikundu Weir (XW), in the same river ± 70 km within the DDT-sprayed area, Limpopo Province, South Africa.

11.2.2 Sampling methods

Dam water and runoff water from roofs was collected from the AD and surrounding areas and from the ND and XW for DDT analysis. Physical water quality parameters were recorded for both sites including temperature, dissolved oxygen concentration, pH, conductivity and total dissolved salts (TDS). Gill nets were used to obtain a sample size of ten male fish per site for each species. Sampling was carried out in summer during two high flow seasons (survey 2 and 4) after rains, and during one low flow (survey 3) season before the rains. Surveys 2 and 4 were breeding season for both species. CASA was used to assess the motility parameters of sperm sampled from both species at all three sites. Uncontaminated milt collected directly from one testis of each fish was used for CASA. Once the testis was gently dissected from the body cavity, the surface was dried and blood remnants were removed. The length of the testis was lanced with a sharp scalpel blade and leaking sperm was collected using a positive displacement pipette. The collected sperm was diluted with extender (0.094 M NaCl, 0.027 M KCl, 0.05 M Glycine, 0.015 M TrisHydroxymethyl in distilled water, pH 7.5), described as an immobilizing medium for *C. gariepinus* (Volckaert et al., 1994). Collected tilapia spermatozoa were placed in an immobilization solution (Rana and Andrew, 1989; Rana, 1995) containing NaCl and KCl in distilled water (Linhart et al., 1999). This was further diluted with the same extender. No incubation time was allowed other than the time taken to follow the procedure. Immediately 0.5 µl of this dilution was pipetted into a Leja slide chamber (20 µm) already under a light microscope (Olympus BH-2). Water (1.5 µl) from where the fish were caught was added as an activator.

11.2.3 CASA

Immediately a recording was taken (using a Panasonic CCD video camera wv-F15E) for 50 seconds. A novel CASA system based on open source software for characterization of sperm motility parameters was adapted to suit the test species (Wilson-Leedy and Ingermann, 2007). This software was applied to analyze the video recordings of activated sperm. A minimum of 200 spermatozoa were tracked at 30 frames per second (fps) for 10 sec after initial activation, per sample. The 10 s of analyzed recordings were taken immediately as sperm became active. Four repeats per sample were carried out. Various motility parameters calculated during the 10-sec tracking period included percent motile sperm (MOT%, percent of sperm moving in a manner fitting motility determination parameters), velocity curvilinear (VCL μm/s, point to point velocity, total distance travelled, per second), progression (PROG, the average distance of the sperm from its origin on the average path during all frames analysed) and the number of tracked sperm. The software calculated an average over the 10 seconds for the above parameters. A low frame rate results in virtually no difference between VCL and VAP (velocity average path) parameters. Portions of the sperm's path are not recorded (primarily the side to side motion), thus the difference between VCL and VAP cannot be used to describe the degree of side to side motion exhibited by sperm. VAP and BCF (beat cross frequency) are dependent upon a higher frame rate (Wilson-Leedy and Ingermann, 2007).

11.2.4 Data analysis

The data obtained from the CASA software system was exported to Microsoft Excel for simplicity and ease of interpretation. Mean values and standard deviations were calculated. Statistical analysis was carried out for the data obtained from CASA. Univariate analysis of variance (ANOVA) and the Kruskal Wallis Test were used to compare between the three sites (AD, ND and XW) regarding the CASA data for each species combining the surveys on sperm motility parameters. Multiple comparisons were performed to identify specific differences between sites. Significant values were considered at $p < 0.05$.

11.3 Results

From all three sites < 0.10 μg/L of DDT, DDE and DDD were recorded in the river water samples for survey 2 and survey 3. The runoff water from roofs did however record 0.10 μg/L for AD (reference site) and between 0.12 μg/L and 0.27 μg/L for XW during survey 2. A month before survey 3, water from ND recorded 0.4 μg/L *p,p'*-DDE and water from XW

recorded 0.3 µg/L *p,p'*-DDE. A month after survey 3 water from AD recorded 0.95 µg/L *p,p'*-DDE, 0.50 µg/L *p,p'*-DDD and 0.30 µg/L and water from ND recorded 0.11 µg/L *p,p'*-DDE. Results from survey 4 revealed that water from AD and XW contained 0.6 µg/L *p,p'*-DDE, while water from ND contained < 0.5 µg/L *p,p'*-DDE. Table 11.1 shows the results of the physical water parameters.

Table 11.1: Physical water quality parameters for all sites and surveys.

Survey	Site	Temperature (°C)	Dissolved oxygen (mg/L)	pH	Conductivity (µS)	TDS (mg/L)
Survey 2	AD	24	5.4	7.62	299	149
	ND	27	6.09	7.41	164.4	82.2
	XW	28.2	6.1	7.65	142	72.1
Survey 3	AD	24.7	8.74	8.48	825	413
	ND	22.4	9.81	8.93	424	214
	XW	18	12	7.3	182.3	-
Survey 4	AD	28.00	10.00	8.61	305.00	153.00
	ND	29.50	10.38	8.28	150.50	75.20
	XW	28.90	9.27	6.96	139.20	69.50

Table 11.2 shows the sample size of both species obtained at each site combining the surveys together. From Table 11.2 it is evident that there are inconsistencies in sample size, with a low number of specimens for ND.

Table 11.2: Sample size obtained at each site combining all three surveys together.

Species	AD	ND	XW
O. mossambicus	20	10	34
C. gariepinus	27	4	15

The CASA results (Table 11.3) showed a general trend of a decrease in all parameters from the reference site (AD) to the exposed sites (ND and XW) for both species, except where VCL increased at ND for *O. mossambicus*. Comparisons for *O. mossambicus* yielded significant main effects of sites for VCL (F = 12.137, p = 0.000037) and PROG (Brown-Forsythe = 4.795, p = 0.012). Multiple comparisons indicated that ND reporting statistically significant higher VCL (M = 117.054; SD = 18.485) than both AD (M = 89.239; SD = 17.447) and XW (M = 82.490; SD = 20.877). AD (M = 2922.061; SD = 521.036) reported statistically significantly higher PROG than XW (M = 2431.517; SD = 874.424) but not ND (M = 2734.139; SD = 365.260). Statistical analysis resulted in no significant differences between the sites for *C. gariepinus*. In general the values for *C. gariepinus* were lower than those of *O. mossambicus* and the exposed sites showed slightly reduced sperm motility parameters as compared to the reference site.

Table 11.3: Results of mean CASA parameters for both species at each site combining all three surveys together.

O. mossambicus			
	Albasini Dam	Nandoni Dam	Xikundu Weir
Variable	(AD)	(ND)	(XW)
% MOT	69.873	65.92	56.622
ST DEV	23.131	10.548	25.649
VCL (µm/s)	89.239	117.054	82.49
ST DEV	17.447	18.485	20.877
PROG	2922.061	2734.139	2431.517
ST DEV	521.036	365.26	874.424
C. gariepinus			
Variable	AD	ND	XW
% MOT	39.995	20.25	39.1
ST DEV	28.338	11.428	32.905
VCL (µm/s)	99.928	80.72	88.608
ST DEV	22.018	31.388	21.646
PROG	2502.724	2340.894	1915.726
ST DEV	655.395	640.392	897.165

11.4 Discussion

The study sites were chosen for their applicability to the study concerned, being located within the same geographical region and suiting the distribution of both the chosen species. The reference site (AD) was close to the start of the river, outside the DDT sprayed area, while the exposed sites (ND and XW) were within the DDT sprayed area. AD was the best option for a reference site as downstream activity increased in terms of industry, settlements and DDT spraying. In retrospect AD may not have been an ideal reference site. Although the concentrations of DDT, and its metabolites, were between < 0.10 µg/L and 0.6 µg/L during sampling surveys, higher concentrations were recorded from runoff water from roofs and water samples collected periodically. DDT is known to drift for long distances (Rogan and Chen, 2005) and can be transported via air, rivers, and ocean currents (Bouwman, 2004). Nearby settlements and banana plantations could have lead to contamination of the water of the reference site.

The physical water parameters (Table 11.1) showed that surface water temperatures varied between 22.4°C and 29.5°C. Variation in temperature with respect to seasonal changes is expected and thermal characteristics of running water depend on various features of the

catchment area (DWAF, 1996g). For the most part the temperatures were within normal limits. The higher temperature recorded during survey 4 (February 2008) may have caused stress by reducing the solubility of dissolved oxygen and making it less available to the fish (DWAF, 1996g) and increasing the toxicity of trace metals (Cairns et al., 1975). Dissolved oxygen concentrations were slightly low during survey 3 (March 2007). Maintaining sufficient dissolved oxygen concentrations is vital for the survival and functioning of aquatic biota. Toxic chemicals have a magnified effect at low oxygen concentrations and levels below 5 mg/L are stressful to most aquatic organisms. A concern is that gametes and juveniles are often more sensitive to stress arising from oxygen depletion (DWAF, 1996g). The spermatozoa of fish contain mitochondria and inactive spermatozoa demonstrate oxygen consumption. A sufficiently oxygenated environment is necessary for activation and function (Alavi et al., 2007). Low oxygen concentrations may have had an impact on the sperm motility parameters.

The pH of natural waters is determined largely by geological and atmospheric influences. South Africa's fresh waters are relatively well buffered, with a pH range between 6 and 8. The pH values recorded in this study were within a normal to high range and where values above 8 were recorded may have acted as a stressor on fish by increasing O_2 diffusion distance. Lease et al. (2003) exposed Lost River suckers to elevated pH levels. Gill lamellar thickness and O_2 diffusion distance increased.

High concentrations of xenobiotic toxicants cause discomfort, stress, illness and mortality in exposed animals. According to Kime and Nash (1999), reproductive dysfunction at low concentrations can be caused either indirectly by modulation of the endocrine system so that gamete development occurs during an imbalance of the hormonal environment, or by directly acting on the gametes. Whether the action is at the hypothalamus, pituitary, liver, gonad, or on the gametes themselves, the overall result is an alteration in gamete quantity or quality. The ultimate goal of the reproductive system is to produce adequate numbers of viable gametes, making gamete quantity and quality the only important end points. Reproductive dysfunction may therefore be monitored by examination of gamete viability and show effects at concentrations less than those which completely inhibit gamete development (Kime, 1995; Kime and Nash, 1999).

Sperm development can be affected by changes in hormonal secretions and according to Kime and Nash (1999), many studies have shown that xenobiotics can act via these mechanisms to inhibit testicular growth and prevent development of mature sperm. Endocrine disruption can affect the quality and quantity of sperm produced along with many

other variables from changes in temperature, pH, osmolality (Alavi and Cosson, 2005, 2006) and storage time (Rurangwa et al., 2001), to the effects of pollutants (Kime et al., 1996; Rurangwa et al., 2002; Van Look and Kime, 2003; Runnalls et al., 2007). Sperm motility and velocity have been correlated to reproductive success in fish (Alavi and Cosson, 2005). CASA is the most objective and comprehensive quantification available for the analysis of these indicators of sperm quality (Cosson et al., 1992; Billard et al., 1995; Toth et al., 1995; Christ et al., 1996; Kime et al., 1996; Lahnsteiner et al., 1996, 1997; Dreanno et al., 1998; Alavi and Cosson, 2005; Wilson-Leedy and Ingermann, 2007).

Although this study cannot conclusively show that DDT was independently responsible for causing altered motility parameters due to the likelihood of there being a mixture of pollutants, it may be considered as a contributing factor.

Many studies have been carried out on the effect of hormonally active chemicals in the environment, such as DDT, being associated with declining human male reproductive health, especially semen quality (Carlsen et al., 1992; Sharpe and Skakkebaek, 1993). Numerous reproductive and hormonal endpoints have been examined in both men and women, and although associations have been determined, causal links have not been confirmed. In Chiapas, Mexico, where DDT was sprayed for malaria control, serum *p,p'*-DDE concentrations were inversely correlated with semen volume, sperm count, and bioavailable – total testosterone ratios in young men not occupationally exposed to DDT. Several sperm motion parameters decreased with higher *p,p*'-DDE, including the percentage of motile sperm (De Jager et al., 2006). Aneck-Hahn et al. (2007) found that mean CASA motility was lower with higher *p,p'*-DDE concentration. Results from another study of South African malaria workers found that although the semen quality in the study was less than normal, no strong evidence for a DDT effect was found. Eighty-four percent of morphology scores were however below the WHO or the Tygerberg criteria (Dalvie et al*.,* 2004). It has been reported that studies of populations with a much lower exposure than that seen in current malaria-endemic areas have shown only weak, inconsistent associations between DDE and testosterone amounts, semen quality, and sperm DNA damage (Rogan and Chen, 2005).

According to Duty et al. (2004) several researchers have explored the relationship between CASA parameters and chemical exposures in laboratory animals. The fact that both species showed primarily a decrease in parameters (Table 11.3) from the reference site (AD) to the exposed sites (ND and XW), suggests that the pollutants present in the water could have had an effect on the sperm motility parameters. Mansour et al. (2002) found that *C. gariepinus* had reference motility values between 70 and 90% and swimming velocities between 120-

140 μm s^{-1}. Low motility values for *C. gariepinus* (Table 11.3), other than being affected by pollutants, could be due to testes being in an immature state of development observed in some samples during dissection, along with incomplete sample sizes. The velocity of active sperm remains relevant even though results varied with increases and decreases between the reference site (AD) and the exposed sites (ND and XW) for each species. It is more apparent that VCL decreases with *C. gariepinus* at the exposed sites (ND and XW), while increases for *O. mossambicus* at the exposed site (ND). Selevan et al. (2000) examined the association between air pollution and CASA parameters. It was found that although sperm travelled faster at high air pollution levels, forward progression actually decreased. A similar result was seen for *O. mossambicus*, where the velocity is high and the progression is decreased. Betancourt et al. (2006) showed that insecticides and herbicides have an effect of different degree on the motility of porcine sperm, affecting in some way its ability to fertilize. A recent publication (Campagna et al., 2009) showed that mixtures of organochlorine insecticides affected sperm function in pigs. The possible mixture of pollutants present in the water of the study sites was unknown during this study. It is likely that a combination of pollutants was responsible for the effects to altered sperm motility parameters.

CASA has been used to determine a range of effects of toxic agents on the energy metabolism and motility of spermatozoa (Rurangwa et al., 2002; Duty et al., 2004; Betancourt et al., 2006). Herbicide-induced reduction in sperm motility may be caused by their action on the respiratory chain of mitochondria (Betancourt et al., 2006). It is necessary to study the possible interactions of pollutants with the energy producing systems of the sperm in the species used, to better understand the mechanisms by which spermatozoan motility is reduced by toxicants. In future, laboratory studies will be carried out in which potent effects of DDT on sperm motility will be determined by direct incubation of sperm with DDT. It is well known from other studies that ATP is essential for sperm motility and decreased ATP equates with decreased sperm movement (Christen et al., 1987; Perchec et al., 1995; Rurangwa et al., 2002). Incubation of catfish semen with tributyltin (TBT) caused a drop in intracellular ATP concentration. This depletion of ATP was harmful to the semen leading to poor motility (Rurangwa et al., 2002). TBT is also known to disturb the calcium homeostasis in cells, important in the initiation of sperm movement (Rurangwa et al., 2002). DDT may be affecting adenylate contents and enzyme activities of the spermatozoa of both species in this study, the sensitivity of which is unknown.

Due to extensive research carried out over the past few decades, a great deal is known about the unfavourable reproductive effects of EDCs (DDT) on a variety of organisms,

including humans. Based on this, it was of interest and importance to this study to determine the potential adverse effects likely to be recorded in the test organisms (*O. mossambicus* and *C. gariepinus*) as a result of exposure to DDT and its metabolites. Because multiple uses of organochlorines (DDT) has lead to an elevated level of environmental contamination and exposures, it is more essential than ever to develop sound means of biomonitoring these agents and their potential effects. CASA parameters may serve as a more sensitive marker of reproductive toxicity.

11.5 Conclusions

In fish, sperm motility and velocity are indicators of reproductive success (Alavi and Cosson, 2005). A computer aided sperm assessment, or CASA, is the most objective and comprehensive quantification available for the analysis of these indicators of sperm quality (Wilson-Leedy and Ingermann, 2007). Both fish species showed a decrease in motility and velocity in the exposed sites compared to the control site. It is likely that, if not solely attributable to DDT, a combination of pollutants was responsible for the damaging effects in terms of sperm motility parameters. It is also clear that CASA parameters may serve as a more sensitive marker of reproductive toxicity. Although on a cellular level the effects on the testis were slightly higher in the DDT-polluted area, it could not be correlated to DDT levels.

CHAPTER 12

A HISTOLOGY-BASED FISH HEALTH ASSESSMENT (HBFHA) IN A DDT SPRAYED AREA

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12.1 Introduction

The primary goal of environmental conservation and management of ecosystems is to prevent adverse biological and ecological effects caused by pollution. Therefore an urgent need has arisen for sensitive bio-monitoring tools to indicate the effect of pollution on fish health in aquatic ecosystems. Histopathological assessment of fish tissue allows for early warning signs of disease and detection of long term injury in cells, tissues or organs. Various biochemical and biological studies of fish have been used to assess the consequences of environmental toxicants on fish, but histology is able to enhance and add quality to the research carried out by describing cellular changes and to quantify the results. Lower level responses such as the prevalence of histopathological symptoms in fish is indicative of the general quality of the environment and can be related to contamination levels of pollutants such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), Polychlorinated biphenyls (PCBs) and DDT (Marchand et al., 2009). These pollutants induce pathological changes in fish. The histological assessment of fish tissue has relevance as a bio-assessment tool and serves as a method to determine preserved biochemical and physiological changes, caused by pollutants as they occur *in situ*.

The research question of the project was whether environmental levels of DDT and DDE may contribute to adverse health effects in aquatic animals. According to Short and Meyers (2001), histology can be used in fish health and can detect early signs of disease that is not easily recognised in gross examinations. A histology-based fish health assessment (HBFHA) was done on two fish species – the sharptooth catfish *Clarias gariepinus* and the *Oreochromis mossambicus* – in the DDT sprayed area by applying an adapted protocol of Bernet et al. (1999). The protocol included a qualitative and quantitative histological assessment of the three target organs: the gills, liver and gonads of the selected two fish species.

The aim of the study was to assess the health status of fish in an area where ongoing DDT-spraying occurs by means of the HBFHA assessment protocol. This bio-assessment tool

allows the quantification of organ damage, including extent and pathological importance of changes that occurred at organism level (Organism-Level Indicators) and at cellular level (Cellular and Histopathological Level Indicators).

12.2 Material and methods

12.2.1 Study area

The study areas are in the Limpopo Province and are part of the Luvuvhu Catchment, a part of the larger Limpopo system, which flows into Mozambique. Both species were sampled at a reference site, Albasini dam (AD), (outside the DDT-sprayed area) and at two exposed sites, Nandoni Dam (ND) and the Xikundu weir (XW) ± 70 km within the DDT-sprayed area.

12.2.2 Sampling methods

Gill nets were used to acquire a sample size of 20 fish per site for each species. Sampling was carried out during three surveys, Survey 2, Survey 3 and Survey 4.

Fish were weighed, measured and blood was drawn using vacutainers. The blood was kept on ice until laboratory analysis. All fish were externally examined to note any physical abnormalities. Once sacrificed, a macroscopic investigation was carried out using the Health Assessment Index (HAI). Fish were dissected to remove the gills, liver and gonads (testes or ovaries). The liver and gonads (left and right) were weighed and the testes lengths were recorded. Each organ was sampled at the same morphological positions to ensure comparable and sufficient tissue was available for histological analysis. The spleen was also removed and weighed for the purpose of calculating the splenosomatic index.

12.2.3 Organism-level indicators

12.2.3.1 Health assessment index (HAI)

A quantitative health assessment index (Adams et al., 1993) was used for rapid evaluation of fish condition in the field. All variables within the index were assigned a numerical value based on given field code designation. To calculate the HAI score for each fish, numerical values for all variables were summed. The HAI for a sample population was then calculated by the summation of all individual fish HAI.

Blood parameters

Haematocrit and Leukocrit

The blood samples were centrifuged at 3000-3.500rpm's for 15 minutes. The haematocrit (Hct) was measured and recorded (Hct = RBC/Total blood volume X 100). The leukocrit (Lct) was also measured following a standard method (Lct = WBC/Total blood volume X 100). The Hct and Lct were expressed as a percentage.

Total protein determination (TP)

For total plasma protein determination, blood was collected in 4mL heparin vacutainers for *O. mossambicus* and 4.5mL EDTA vacutainers for *C. gariepinus.* The samples were centrifuged at 30 000rpms for 15 minutes. Plasma was transferred to 2mL Eppendorf Safe-lock tubes and stored at -20°C. The samples were prepared using a Total Protein kit (Roche) and analysed in triplicate using a universal microplate reader (Marchand, 2007).

Condition factor

The condition factor is an organism-level response used to determine fish condition in the field (Adams et al., 1993).

The condition factor (CF) for each fish was calculated as follows (Carlander, 1969):

$$CF = \frac{\text{Mass (g)} \times 10^5}{\text{Length (mm)}^3}$$

This equation reflects the expected exponential gain in mass relative to length as fish grow.

Organosomatic indices

The organosomatic indices are used as indicators of the general health or well-being of individual organisms. Organosomatic indices are calculated as (organ weight/body mass)*100 (Busacker et al., 1990),

Hepatosomatic Index (HSI)

The HSI is an organosomatic index frequently used in various stress related studies. It works on a ratio of organ mass to body mass. Therefore, the liver mass together with the body mass was used to calculate the hepatosomatic index:

HSI = liver mass / body mass x 100

Gonadosomatic Index (GSI)

The GSI is an indicator of the state of gonadal development and maturity.

GSI = gonad mass / body mass x 100

Splenosomatic index (SSI)

Spleen size is considered a useful diagnostic factor because the spleen is a haematopoietic organ and dysfunction could have effects at the whole-organism level.

SSI = spleen mass / body mass x 100

12.2.4 Cellular and histopathological indicators

12.2.4.1 Qualitative Histological Assessment

Gill and liver samples were fixed immediately after the fish were sacrificed in 10% NBF formalin; gonads were fixed in Bouin's. The samples were rinsed in tap water followed by dehydration through a series of ethanol (Humason, 1962). The samples were sent to the Onderstepoort Veterinary Institute, University of Pretoria, for further processing and slide preparation.

The histologically prepared microscope slides of all tissue were studied using light microscopy for the identification of histopathological alterations. The adapted version of the quantitative histological assessment protocol described by Bernet et al. (1999) was used to quantify histopathological alterations observed in the selected target organs.

Each organ was assessed according to five reaction patterns and their associated histological alterations.

Reaction pattern 1 (rp_1): circulatory disturbances (CD)

These result from a pathological condition of blood and tissue fluid flow. The alterations included here are: 1) haemorrhage/ hyperaemia/ aneurysm and 2) intercellular oedema.

Reaction pattern 2 (rp_2): regressive changes (RC)

Such changes results in a functional reduction or loss of an organ including atrophy, degeneration (malformation or dysfunction of cellular structures as a result of cell damage) and necrosis. Possible alterations include: 1) architectural and structural alterations; 2) plasma alterations; 3) deposits; 4) nuclear alterations; 5) atrophy, and 6) necrosis.

Reaction pattern 3 (rp_3): progressive changes (PC)

Progressive changes are processes which lead to a heightened activity of cells or tissues. Characteristic lesions are: 10 hypertrophy, and 2) hyperplasia.

Reaction pattern 4 (rp_4): inflammation (I)

These changes are frequently linked to processes belonging to other reaction patterns (e.g. oedema). (Fluid content alterations in tissues related to inflammatory processes (e.g. exudates) are considered in reaction pattern 4). It is thus often not easy to attribute inflammatory changes to one solitary reaction pattern. Consequently, Bernet et al. (1999) used the term ‘inflammation’ in an extremely strict sense and the alterations are: 1) exudate; 2) activation of the reticuloendothelial system (RES) and 3) infiltration.

Reaction pattern 5 (rp_5): tumour (T) (neoplasm)

A tumour is an uninhibited cell and tissue proliferation (autonomous proliferation). Tumors are separated into two classes: 1) benign tumours and 2) malignant tumours. For the description of the alterations by the original author, refer to Bernet et al. (1999).

An importance factor (W) ranging from 1 to 3 was assigned to each alteration. The significance of a lesion depends on its pathological importance, i.e. how it affects organ function and essentially the capability of the fish to survive. A value of 1 is assigned when there is minimal pathological importance, the lesion is easily reversible as exposure to irritants ends; 2 is assigned when there is moderate pathological importance, the lesion is reversible in most cases if the stressor is neutralized; and 3 is assigned when there is marked pathological importance, the lesion is generally irreversible, leading to partial or total loss of the organ function (Bernet et al., 1999).

In addition, every alteration was assigned a score. The score ranged between 0 and 6 depending on the severity of the alteration where 0 = unchanged; 2 = mild occurrence; 4 = moderate occurrence; and 6 = severe occurrence (diffuse lesion).

Using the importance factors and score values, two indices were calculated:

(a) Organ Index ($I_{org.}$)

The organ index represents the extent of damage to an organ. It allows for comparison of the extent of damage of the same organ in different individuals and is calculated as follows:

$$\mathbf{I_{org.} = \sum_{rp} \sum_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})}$$

(b) Total index (Tot-I)

The Tot-I signifies a measure of the overall health status based on the histological lesions apparent. It is possible to compare individuals as the total index for each fish is calculated in the same way:

$$\mathbf{Tot\text{-}I = \sum_{org} \sum_{rp} \sum_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})}$$

This is calculated by adding all organ indices of an individual fish.

Where: org = organ (constant); rp = reaction pattern; alt = alteration; a = score value; w = importance factor.

A slight modification to the protocol of Bernet et al. (1999) was made when implementing the section of the assessment tool concerning gonads. Although Bernet et al. (1999) did not describe an assessment tool for gonads specifically; a similar protocol was constructed based on the layout of the already described organs. A sixth reaction pattern was added to

include the occurrence of intersex, considered an irreversible alteration to normal gonadal tissue.

12.3. Results

12.3.1 Organism-level indicators

12.3.1.1 Health assessment index (HAI)

The external examination of all the specimens showed that all the fish were in a state of good health in terms of the condition of the fins, eyes, mouth, scales, opercula as well as the general appearance. The internal examination showed structural abnormalities in a number of livers including darkening, discolouring and fat spots. Few external and internal parasitic infections were visible in both species from all the sampling sites.

The calculated HAI for *C. gariepinus* ranged from 10.00 (XW in survey 2)-38.00 (ND in survey 3). The HAI value for *C. gariepinus* in the reference site (AD) ranged from 32.50 to 35.80. In the DDT sprayed area the HAI ranged from 18.57 to 38 (ND) and in XW it ranged from 10.0 to 28.89. The HAI values were higher than 30 (34.67, 32.50 and 35.50) for all three surveys.

The results of the HAI for the two fish species *C. gariepinus* and *O. mossambicus* for the sampling trips in surveys 2, 3 and 4 for the reference site (AD) as well as for the two DDT sprayed areas (ND and XW) are given in Figure 12.1.

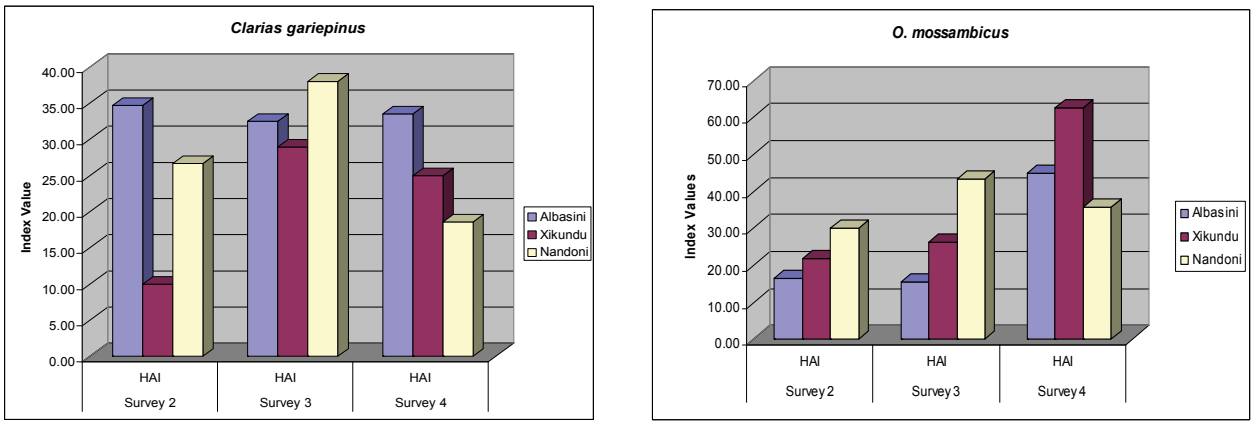

Figure 12.1: HAI for all sampling periods for *C. gariepinus* and *O. mossambicus*.

The calculated HAI for *O. mossambicus* ranged between 15.56 (AD in survey 3)-62.5 (XW in survey 4). In the reference site (AD), the values ranged from 15.56 to 45.0. In the DDT sprayed areas, the HAI ranged between 30.00-43.33 and in XW it ranged from 22.0 to 62.50. The lowest HAI values (16.67 and 15.56) were calculated for *O. mossambicus* in surveys 2 and 3 in the reference site (AD).

Blood parameters

Figure 12.2 shows the mean values for the blood parameters, namely Hct, Lct and TP for *C. gariepinus* and *O. mossambicus*. There were no notable differences between the different surveys.

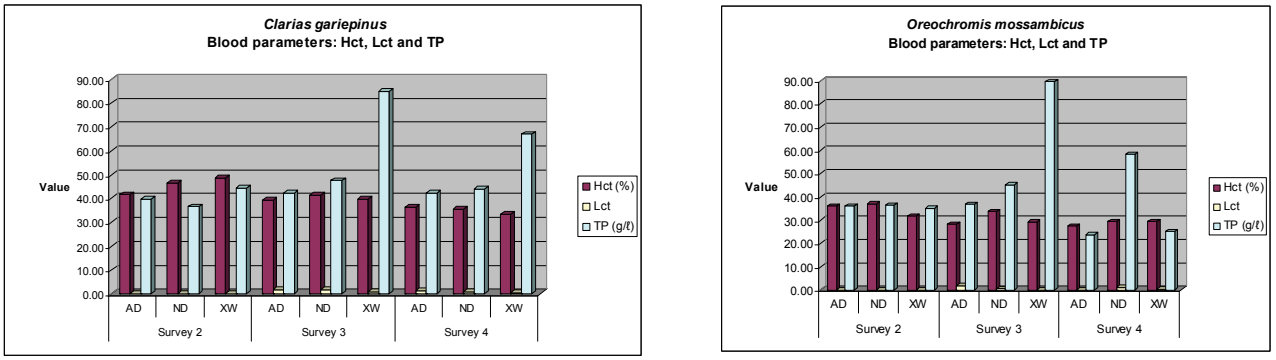

Figure 12.2: Mean values for the blood parameters, Hct, Lct and TP between *C. gariepinus* and *O. mossambicus.*

XW (surveys 3 and 4) had the highest means for TP in blood. The Hct and Lct values were within a normal range (Figure 12.2). It should be noted that there were no large differences between the means of each variable.

Condition factor (CF)

Figure 12.3 shows the mean values for the Condition Factor *C. gariepinus* and *O. mossambicus*. There were no notable differences between the two groups

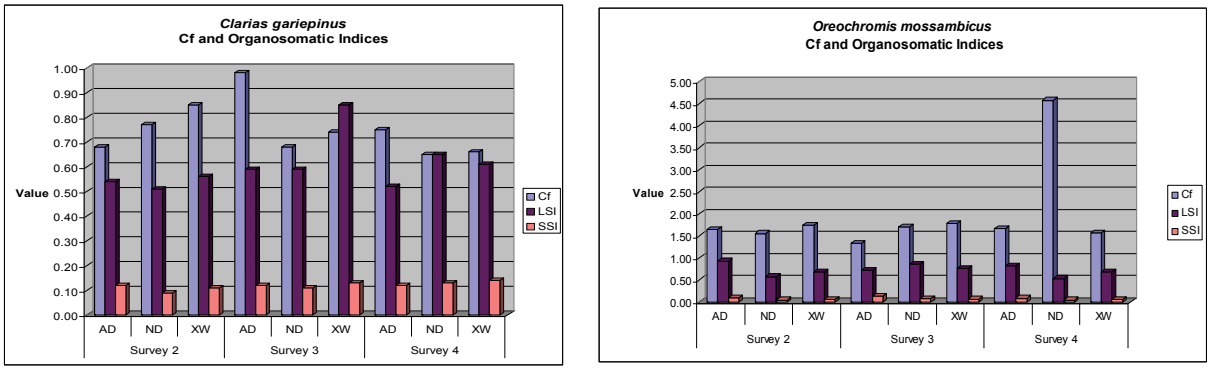

Figure 12.3: Mean values for the CF and the Organosomatic Indices (liver and spleen) between *C. gariepinus* and *O. mossambicus*.

O. mossambicus ND (survey 4) had the highest mean for CF.

Organosomatic Indices

The Organosomatic Indices reflect the status of organ systems (liver and spleen), which may change in size due to environmental factors more rapidly than changes in organism mass or length. We found no large differences between the means of this and SSI for both species (Figure 12 .3).

12.3.2 Cellular and histopathological indicators

12.3.2.1 Qualitative histological assessment

All 93 specimens of both *C. gariepinus* and *O. mossambicus* were histologically assessed. This adequate sample size allowed thorough histological assessment of the gills, liver and gonads. The following section reports on the histological observations noted for each organ.

Gill alterations

The following histological alterations were observed:

- circulatory disturbances including telangiectasia and oedema of secondary lamellae;
- structural alterations in the form of fusion and branching of primary and secondary lamellae; and
- plasma alterations with the presence of vacuolation; hyperplasia of the epithelium and infiltration of leucocytes.

During the assessment, hyperplasia in the gills of *C. gariepinus* was noted in 16 specimens from AD during survey 3 and intercellular deposits were noted in 21 specimens of *O. mossambicus* in XW from survey 2. Figure 12.5 shows structural alterations that were frequently observed at all three sampling sites. These structural alterations include epithelial lifting and hyperplasia of the secondary lamellae.

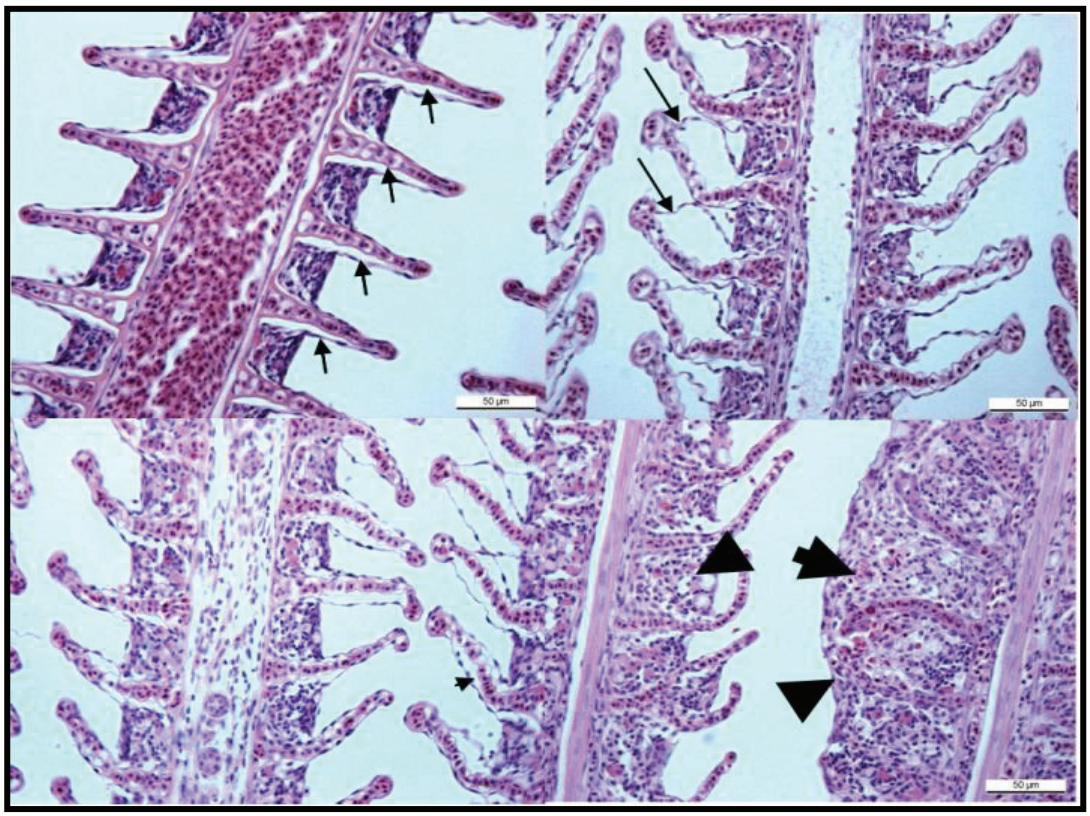

Figure 12.4: Histological alterations identified in the gills (H&E). Structural alterations that were most frequently observed at all three sampling sites were epithelial lifting (arrow) and hyperplasia of the secondary lamellae (Arrow head).

Liver alterations

The following histological alterations were observed:

- Cord disarray;
- plasma alterations including granular degeneration of hepatocytes and inter/intra cellular deposits as well fatty degeneration;
- an increase in the presence of melanomacrophage centers;
- nuclear alterations;
- necrosis of hepatic tissue;

- hypertrophy of hepatocytes, an increase in connective tissue surrounding central veins, infiltration of mononuclear leukocytes; and,
- alterations to the bile ducts including wall proliferation, structural alterations, granular degeneration and nuclear alterations.

Figure 12 .5 shows the melanomacrophage centres in the liver and leukocyte infiltration in the liver.

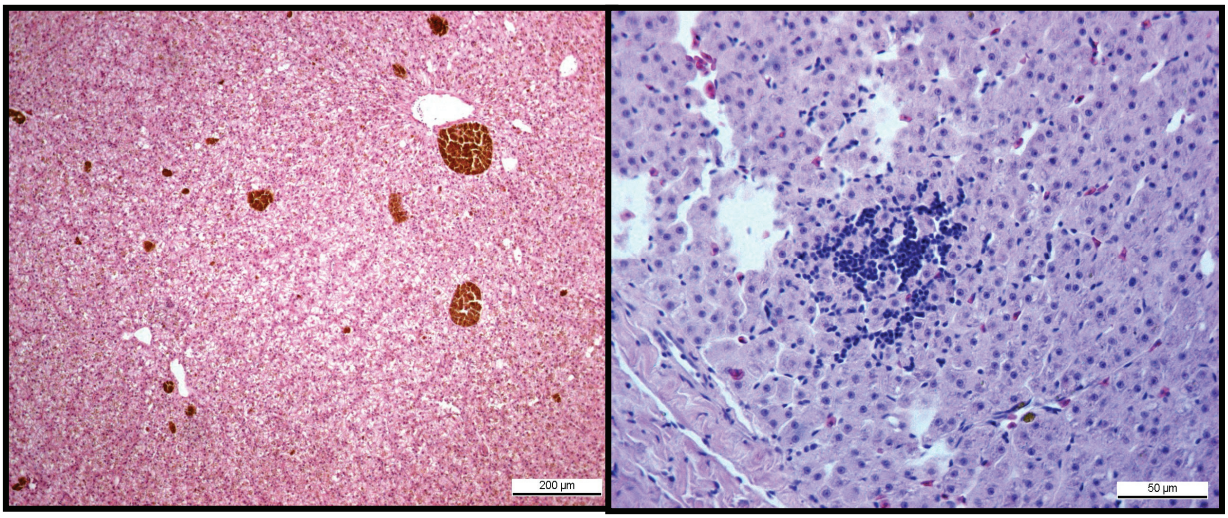

Figure 12.5: The melanomacrophage centres and infiltration of leukocytes in the liver.

Gonad alterations

Ovaries

- The alterations that were observed in the ovaries of both species were melanomacrophage centres (MMC) and infiltration of leucocytes in both species.

Testes

- Detachment of the basal membrane was observed in *C. gariepinus* from all sampling sites during survey 3, and in AD during survey 4.
- Interstitial deposits were observed in *C. gariepinus* in AD, ND and in XW during survey 4 and also in AD and XW during survey 2 and 3.
- Vacuolation was observed AD (survey 3) and XW (survey 4).

No cases of intersex were noted in the testes of *C. gariepinus* but in *O. mossambicus* intersex were present at all sampling sites. Table 12.1 shows the occurrence of intersex in male *O. mossambicus* during the three sampling surveys.

Table 12.1: The occurrence of intersex in male *O. mossambicus.*

***Oreochromis mossambicus* (Testes)**			
	Albasini	**Nandoni**	**Xikundu**
Male	18	10	31
Female	8	6	20
Total	26	16	51
Intersex	11	6	18
% intersex in Males	61	60	58
% intersex in Fish	42	38	35

No cases of intersex were noted in the testes of *C. gariepinus* but in *O. mossambicus* intersex were present in all sampling sites. Figure 12.6 shows the presence of testicular oocytes (intersex) in the testes of *O. mossambicus.*

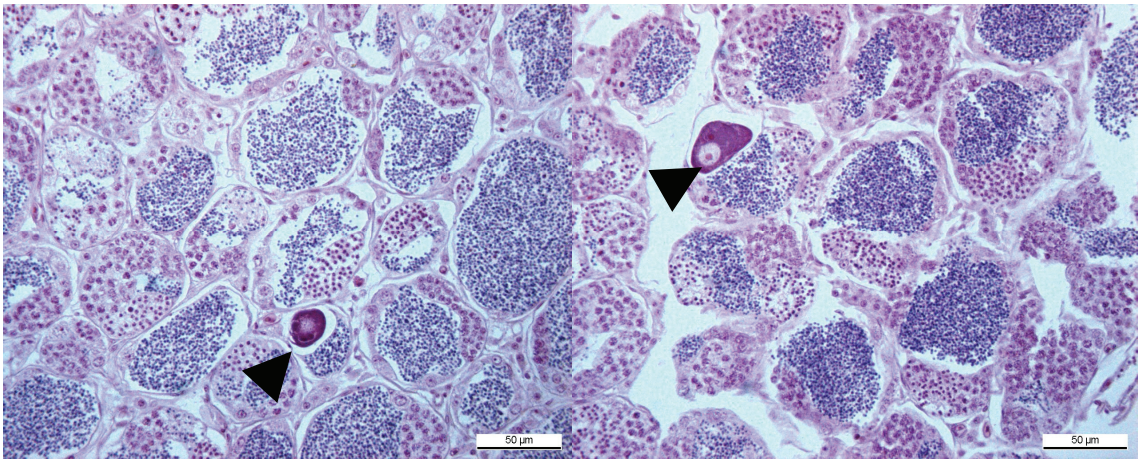

Figure 12.6: Intersex in the testes of *O.* mossambicus in AD, ND and XW.

The most severe changes that were observed in the gonads were the occurrence of intersex in the testes of *O. mossambicus* (Figure 12.6). Intersex was observed in all sampling sites in *O. mossambicus* for all the sampling periods. There were no cases of intersex observed in any of the male *C. gariepinus.*

12.3.2.2 Quantitative histological assessment

During the assessment, certain histological alterations were identified in the selected target organs. The alterations identified in *O. mossambicus* are presented in Table 12.2 and the changes observed in the *C. gariepinus* specimens are presented in Table 12.3. Table 12.2 list the various alterations identified per target organ and specifies the number of specimens in which these alterations were present (also as percentage).

Table 12.2: The histological alterations found in *O. mossambicus* during all surveys.

All Surveys										
Sampling Site		AD			ND			XW		
Organ	Alterations identified	Number of fish	Sample group	Percent (%)	Number of fish	Sample group	Percent (%)	Number of fish	Sample group	Percent (%)
Gills	Aneurysm/Hyperaemia/Haemorrhage	19	26	73	12	15	80	21	54	39
	Intercellular oedema	13		50	4		27	33		61
	Plasma alterations-epithelium	1		4	2		13	0		0
	Intercellular deposits	9		35	0		0	21		39
	Rupture of pilar cells	5		19	12		80	17		31
	Structural alterations			0	0		0	8		15
	Plasma alterations-supporting tissue	1		4	0		0	0		0
	Necrosis	6		23	3		20	0		0
	Hyperplasia-epithelium	26		100	10		67	38		70
	Hyperplasia-supporting tissue	5		19	1		7	2		4
	Infiltration	10		38	7		47	18		33
Liver	Aneurysm/Hyperaemia/Haemorrhage	3	26	**12**	0	16	**0**	2	54	**4**
	Structural -cord disarray	4		**15**	0		**0**	0		**0**
	Hepatocytes-Granular degeneration / intra cellular deposits	22		**85**	3		**20**	35		**65**
	Fatty degeneration (e.g. fatty change)	0		**0**	2		**13**			**0**
	Glycogen vacuoles	2		**8**	2		**13**	8		**15**
	Increase MMC	17		**65**	10		**67**	33		**61**
	Nuclear alterations	0		**0**	10		**67**	35		**65**
	Necrosis - hepatocytes	0		**0**	0		**0**	7		**13**
	Structural alterations - bile duct	1		**4**	0		**0**	1		**2**
	Wall proliferations - Bile ducts	2		**8**	0		**0**	2		**4**
	Hypertrophy-IT	0		**0**	0		**0**	0		**0**
	Hyperplasia-IT	1		**4**	0		**0**	0		**0**
	Hypertrophy of bile ducts	0		**0**	0		**0**	0		**0**
	Infiltration	5		**19**	10		**67**	23		**43**
Testes	Detachment of basal membrane	10	20	38	2	10	13	11		20
	Plasma alteration	0		0	1		7	0		0
	Deposits	0		0	0		0	1		2
	Intersex	11		42	6		40	18		33
Ovary	Intersellular deposits - MMC	4	**6**	67	0	**4**	0	16	**17**	94
	Infiltration	2		33	1		25	1		6

Table 12.3: The histological alterations found in *C. gariepinus* during all surveys.

All Surveys										
Sampling Site		AD			ND			XW		
Organ	Alterations identified	Number of fish	Sample group	Percent (%)	Number of fish	Sample group	Percent (%)	Number of fish	Sample group	Percent (%)
Gills	Aneurysm/Hyperaemia/Haemorrhage	29	51	57	16	20	80	8	22	36
	Intercellular oedema	4		8	2		10	0		0
	Structural alterations-epithelium	4		8	0		0	5		23
	Rupture of pilar cells	30		59	13		65	9		41
	Structural alterations-supporting tissue	0		0	1		5	2		9
	Hypertrophy-epithelium	3		0	0		0	0		0
	Hyperplasia-epithelium	42		82	16		80	18		82
	Hyperplasia-supporting tissue	13		25	2		10	2		9
	Infiltration	3		6	0		0	0		0
Liver	Aneurysm/Hyperaemia/Haemorrhage	6	51	**12**	5	20	**25**	0	22	**0**
	Structural -cord disarray	2		**4**	2		**10**	2		**9**
	Hepatocytes-Granular degeneration / intra cellular deposits	39		**76**	12		**60**	20		**91**
	Fatty degeneration (e.g. fatty change)	3		**6**	1		**5**	2		**9**
	Glycogen vacuoles	12		**24**	11		**55**	3		**14**
	Increase MMC	35		**69**	12		**60**	16		**73**
	Nuclear alterations	14		**27**	0		**0**	1		**5**
	Wall proliferations - Bile ducts	4		**8**	1		**5**	1		**5**
	Wall proliferations - Hepatocytes	10		**20**	10		**50**	5		**23**
	Hypertrophy-Bile ducts	0		**0**	1		**5**	0		**0**
	Hyperplasia-Hepatocytes	0		**0**	0		**0**	1		**5**
	Infiltration	31		**61**	13		**65**	12		**55**
Testes	Detachment of basal membrane	4	29	14	1	8	13	2	15	13
	Plasma alteration	0		0	0		0	0		0
	Deposits	9		31	1		13	4		27
	Vacuolation-spermatocytes	3		10	0		0	0		0
	Vacuolation-spermatogonia	0		0	0		0	1		7
Ovary	Intersellular deposits - MMC	17	**22**	77	1	**12**	8	4	**7**	57

Figure 12.7 presents the Organ Index Values (I_{org}) for both *O. mossambicus* and *C. gariepinus* for the three sampling surveys. The I_{org} means obtained for *O. mossambicus* was also higher than the values obtained for *C. gariepinus.* The I_{org} values in both species were higher in the testes than in the ovaries.

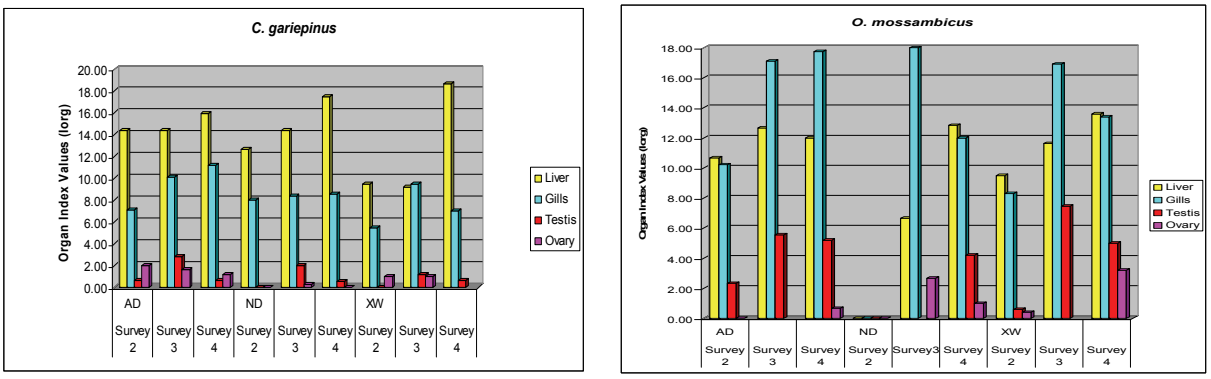

Figure 12.7: Organ Index (I_{org}) means for the liver, gills, testes and ovaries for *C. gariepinus* and *O. mossambicus.*

The Total Organ Index (Tot-I) means which determines the cumulative degree of damage to all the organs assessed were compared between fish from the three sampling sites, namely AD, ND and XW. The results are presented in Figure 12.8.

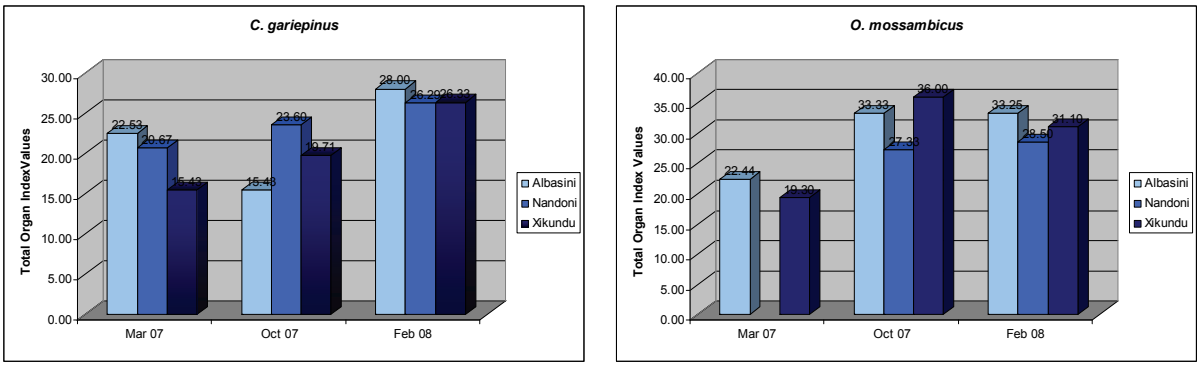

Figure 12.8: The Total Organ Index (Tot-I) values for *C. gariepinus* and *O. mossambicus* in the AD, ND and XW.

In *C. gariepinus,* the highest Tot-I means were recorded during survey 4 for all the sampling sites. The highest Tot-I values for *O. mossambicus* were recorded in survey 3 in XW. In *O. mossambicus* the reference site AD had the lowest Tot-I values.

12.4 Discussion

12.4.1 Organism-level indicators

12.4.1.1 Necropsy-based Health Assessment Index (HAI)

Adams et al. (1993) developed a systematic fish health/condition or necropsy-based procedure for use by fisheries personnel at field level. It was developed to use minimal equipment to provide rapid, relatively inexpensive method in order to detect trends in health and condition of fish populations. This method is a robust and sensitive indicator of fish health as it includes both internal and external observations and also provides numeric ratings to the observations. The HAI is not intended to be diagnostic but rather to alert investigators to possible problems that should be investigated with more diagnostic (specific) tests. This method is not a biomarker, but rather a protocol for documenting lesions or change that have advanced to the point of being grossly visible. It further provides a systematic method for documenting lesions and to compare incidences of gross-observable lesions between sites.

Index variables are assigned numerical values based on the degree of severity or damage incurred by an organ or tissue from environmental stressors. Figure 12.1 shows that high and low index means were obtained for *C. gariepinus* in the reference site (AD) and low index values for the reference site in *O. mossambicus.* According to Adams et al. (1993), the HAI was successfully applied by the Tennessee Valley Authority (TVA). The range of reservoir

HAI values was 17 (best) for a relatively pristine system, to 79 (worst) for a river that receives contaminants from numerous sources, the most notable being a river into which effluents from a pulp and paper plant were discharged. The HAI has also been applied to assess the effects on fish health exposed to polychlorinated biphenyls (PCBs) in Hartwell reservoir (Adams et al. 1993). The reference site HAI value was 42, while the most contaminated site had an HAI value of 74. HAI values for Pigeon River receiving pulp and paper effluents ranged from 21 (reference site) to 60 (contaminated site) (Adams et al*.,* 1993). These results confirm that fish exposed to toxicants have a higher HAI score indicating more alterations and poorer fish health. The results indicate that all the fish were in good health.

Blood parameters

Measurement of blood components can yield useful information about the health of fish (Goede & Barton, 1990). Hct is the most readily determined, commonly measured and widely employed haematological variable (Houston, 1997). A high Hct can result from acute stress (Barton et al*.,* 1985); a low haematocrit may indicate disease (Cardwell & Smith, 1971). Depending on the stressor involved, haematocrit can increase or decrease, resulting in the 'normal range value' (30-45%) employed with the HAI (Adams et al., 1993). According to Barnhart (1969), 'normal Hct values' are not definitive, since published haematological standards for fish have wide amplitudes, techniques and methods are not standardized, and biological variations appear significant. However, the results of this study showed the mean Hct values for all three sites (Figure 12.1) fell within the normal range as in the HAI.

Lct is a gross measure of white blood cell abundance, and plasma protein values. Leukocytes are involved in the immune system and according to Hopson and Wessels (1990), rise rapidly in response to infection or disease. The 'normal range', as defined in the HAI classification (Adams et al., 1993), is < 4%. All values were within normal limits for this study.

TP concentration may vary with respect to the sex, size, and state of maturity of the fish, and it is influenced by environmental factors such as temperature or food availability (Miller et al*.,* 1983). Fish exposed to pollutants have been found to have significantly lower TP concentrations than control fish (MacKenzie et al*.,* 1995). However, all values for this study were within the 'normal range' of 3-6 g/dl as defined in the HAI (Adams et al*.,* 1993). XW had higher values for the TP in species during survey 3 (Figure 12.2)

Condition factor

According to Goede and Barton (1990), the most common types of condition indices are ratios between morphological and anatomical features of fish. Condition factor, expressed as mass(g) x 10^5/length^3(cm) described by Carlander (1969) is determined in many fishery studies because length and mass are routinely measured (Anderson & Gutreuter, 1983). Bolger and Connoly (1989) stated that when fish condition is studied on the basis of length-mass analysis, it is assumed the heavier fish will be in better condition. However, many factors influence fish mass such as food availability, metabolic rate as dependent on temperature and seasonal changes in terms of breeding activity. The values obtained in this study varied in *C. gariepinus* between 0.65 (ND) in survey 4 and 0.98 (AD) in survey 3. In *O. mossambicus* it varied between 1.34 (AD) to 4.58 (ND) in Survey 4 (Figure 12.3). Crafford (2000) and Watson (2001) obtained similar results. The values obtained in this study (except for *O. mossambicus* in ND, 4.58), could be considered to fall within the normal range of ± 1 (Adams et al., 1993).

Organosomatic indices

Hepatosomatic index (HSI)

The HSI is the one often associated with contaminant exposure (Adam and Mc Lean 1985). An increase in the HSI could indicate hyperplasia (increase in cell number), hypertrophy (increase in cell size) or atrophy (decrease in cell size) (Van Dyk, 2003). Several investigators have suggested that liver enlargement in fish indicates exposure to environmental carcinogens or other toxic chemicals. Increased HSI has been reported in brown bullheads (*Americanus nebulosus)* from sites polluted with polycyclic aromatic hydrocarbons (PAHs) and PCB's (Fabacher and Baumann 1985; Gallagher and Di Guilio 1989). The values recorded in this study fell within the normal range. This was not the case as although the means were within a normal range, 10.5% of individuals suffered from hypertrophy of hepatocytes (Figure 12.3).

Splenosomatic index (SSI)

Spleen size is considered a useful diagnostic factor because the spleen is a haematopoietic organ (Anderson 1990) and dysfunction could have effects at the whole organisms' level. Spleen size is not as thoroughly investigated as the HIS, but certain endogenous and exogenous factors are known to affect it.

The SSI has not been as thoroughly investigated as the HSI, but certain endogenous and exogenous factors are known to affect it (Schmitt and Dethloff, 2000). Enlargement or swelling of the spleen is considered to be indicative of disease or immune system problems (Goede and Barton, 1990). There were no significant differences in the different sampling sites for the two fish species (Figure 12.3).

Gonadosomatic index (GSI)

The GSI and gonadal histopathology fall in a category of indicators that provide structural rather than functional information about gonadal health and maturational stage. The GSI is one of several organosomatic indices, including the HSI and SSI, which establishes a relationship between organ and the entire body. There is substantial evidence that most species undergo reproductive cycling, and, frequently, dramatic variation in gonadal size is observed throughout the cycle (De Vlamming et al., 1981). Consequently, gonadal mass as a percentage of body mass has routinely been used to determine reproductive maturity, as well as to assess gonadal changes in response to environmental dynamics (e.g. seasonal changes) or exogenous stresses (e.g. contaminant exposure).

Gonadal histopathology is often utilised alone or in conjunction with the GSI to confirm gonadal phenotype, determine the state of sexual development, and investigate reproductive impairment. Although gonadal histopathology is routinely used to detect higher level responses expressed as morphological abnormalities such as the presence of ovotestes or multinuclear ova, this method is capable of providing information at multiple levels of biological organisation (i.e. distribution of molecules; distribution of number, volume morphology of organelles, cells and organs. The means recorded in this study fell within the normal range.

12.4.2 Cellular and histopathological indicators

12.4.2.1 Qualitative histological assessment

Histopathology allows the examination of organs from fish of any size, age or type to be examined. Sectioning of these fixed tissues allowed retention of *in vivo* relationships. More than one tissue may be studied simultaneously to determine biological effects of toxicants not only in localised portions of certain organs, but consequential derangements in tissues or cells at other locations. This often allows for diagnoses of changes observed grossly as well as indications of mechanisms of toxicity. Macroscopic signs of toxicity are almost always preceded by changes at the tissue, cellular or molecular levels (Segner and Braunbeck

1990). When cell injury or death of cells without death of the organisms occurs, this is followed by cellular reactions and / or host responses that can be described and sometimes be diagnostic of cause.

12.4.2.2 Quantitative histological assessment

All target organs were assessed in terms of a quantitative histological assessment protocol. During the assessment, certain histological alterations were identified in the selected target organs. The alterations identified in the gills, liver and testes of *C. gariepinus* and *O. mossambicus* specimens are shown in Figure 12.4, 12.5 and 12.6 respectively. Various alterations were identified per target organ. Table 12.2 and 12.3 shows the total number of histological alterations and the number of specimens in which these alterations were present. The results show that in both species the gills and liver were more affected than the gonads (testes and ovaries). *O. mossambicus* were also more affected (showing higher Tot-I values). *O. mossambicus* showed higher Index values for the gonads, which could be due to the occurrence of intersex in the testes in all the sampling sites. Intersex was not observed in the testes of *C. gariepinus.*

12.5 Conclusion

Fish histology was used as a tool to monitor the health status in an area where ongoing DDT spraying occurs by implementing qualitative and quantitative histological assessment methods. The results obtained from the reference site (AD) showed that fish are equally affected by the overall water quality and pollutants present. No specific causative agent has been identified.

Based on the HAI, the fish from surveys 2, 3 and 4 in all three sampling sites were in a state of good health. Histopathological alterations of *C. gariepinus* and *O. mossambicus* were identified in the gills, liver and gonads. All the values were higher than the values obtained for a reference group for both species that were bred in toxicant free water. Histopathological alterations in fish tissue can be used as a tool to monitor the health status of ecosystems as they provide a more comprehensive knowledge of the effects of toxicants on fish health. Histopathology gives an indication of the serious effects of toxicants, known to be in the water, on fish health. There was no proof that fish from ND and XW was more severely affected than AD. It should be noted that intersex was present in the testes of *O. mossambicus* during all surveys in ND, XW and AD. However, the analysis of the Tot-I from the quantitative histological assessment did not provide outright evidence that ND and XW

was in a more severe state than AD, based on overall histopathological alterations in fish tissue.

CHAPTER 13 – *IN VIVO* TOXICOLOGICAL TESTING

13A CHRONIC PARTIAL LIFE-CYCLE EXPOSURES OF FEMALE AND MALE *C. GARIEPINUS* TO DDT CONCENTRATIONS

Brink KA, Van Vuren JHJ and Bornman MS

13A.1 Introduction

As has been previously mentioned, DDT is a highly persistent, lipophilic, pesticide that has numerous toxicological effects documented by many journals and governmental organisation (EXTOXNET, 1996; ASDTR, 2002). In spite of the large amount of research supporting both DDT's lethal and sub-lethal effects on humans and wildlife, no data is available on the chronic effects induced in South African indigenous species. Therefore the objective was to identify reproductive effects caused by chronic, low dose DDT exposure on the indigenous fish species, *C. gariepinus*. The reproductive effects that were assessed included abnormal vitellogenin (VTG) levels in males due to the estrogen mimicking action of DDT, reduced gonad condition (GSI) and condition factor (CF), the presence of intersex in male gonads and the reduced hatchability and survival of juveniles.

One of the most extensively reviewed mechanisms of action of DDT in fish is the mimicking of the female hormone, estrogen (Cheek et al., 2001). Under normal conditions, estrogen stimulates the production of VTG in the liver, which is transported to the ovary where it enters the mature female's oocyte to function as an essential protein in egg development (Marin and Matozzo, 2004). However, in the presence of DDT, male fish may produce excess VTG, which can be used as a biological indicator of exposure to estrogenic compounds (Lomax et al., 1998). Therefore the aim of the present study was to identify if there is in fact increased VTG levels in male fish at lower concentrations of DDT exposure. Many assays for measuring VTG concentrations directly in fish plasma have been developed. For example the enzyme-linked immunosorbent assay (ELISA), radio-immunoassays (RIA), western blot analyses, and VTG mRNA determination by DNA hybridization strategies (Marin and Matozzo, 2004). Undoubtedly the most favoured of these is the ELISA due to it being a relatively rapid and simple tool for monitoring EDC contamination. However, this method is disadvantaged in that it is limited to only a few species and not one is commercially available for any South African indigenous fish species (Verslycke et al., 2002). A rapid, cost effective alternative to the ELISA is the alkali-labile phosphate (ALP) assay. This assay has been

shown to successfully estimate the amount of VTG present in plasma by colorimetrically measuring the amount of phosphate groups bound to VTG. Similarly, concentrations of calcium and magnesium can be used as an indirect measure of VTG since these are increased significantly in the presence of VTG.

The GSI and CF were then selected in the present study due to their ability to measure the general fish condition (Huang et al., 2003). The GSI is specifically utilised to measure the gonad condition, which is calculated as the percentage of gonad weight to total body weight. This index is based on the broad assumption that proportionally larger gonads indicate greater development and because reduction in relative gonad mass can occur in response to increased DDT exposure, GSI can be used to estimate the degree of EDC effects on reproduction (Schweer, 2002). Although measurements of the GSI are a successful index to monitor reproductive effects, care should be taken when interpreting results. According to the author of the Verband der Chemischen Industrie (VCI) position paper on “endocrine active substances” (2005), GSI may be influenced by many different factors which may occur alone or in combination, including age, temperature, health, fish density, time of year or inter-individual variation in gonad weight during spawning season. Similar abiotic and biotic factors can influence the CF, which assesses the fish health by correlating the fish body mass to its length. In fact this index is often used in aquaculture, to monitor feeding intensity, age and growth rates in fish (Anene, 2005). Within an ecotoxicological context however the CF is based on the fact that there is usually depletion in the energy resources available, as these resources are utilised to cope with the increased stresses posed by a toxicant. An astounding number of ecotoxicological based studies, including those measuring DDT, have incorporated this index and is particularly popular due to its simplistic and cost effective nature. To measure this CF, there are many different approaches available and are reviewed by Stevenson et al. (2006). In the current study a more meaningful approach was used where a standardised mass was predicted on the basis of a statistical formula for a specified test species and then compared against the actual mass for each of the respective individuals.

According to Kime (1998), early life stages of fish are very sensitive to pollution. This is however not surprising as there are so many modes of action in the fish reproductive system for toxicants to affect, especially if all life stages are exposed. Hence, toxicity test on the survival of the juveniles are essential to identify the possible risk that toxicants pose on fish populations within their natural environment (Ankley et al., 2004). A similar approach is thus used in the present study, which identifies the hatchability and survival of juveniles in the presence of environmentally relevant concentrations of DDT.

13A.2 Materials and methods

13A.2.1 Experimental design

13A.2.1.1 DDT

As previously mentioned, the technical grade DDT, which is currently being sprayed for malarial control in South Africa, is predominantly made up of *p,p'*-DDT (65-85%). Hence the effects of this isomer were analysed in the present study.

13A.2.1.2 Selection of test species

Fish as test organisms have long been seen as suitable bioindicators in aquatic toxicity testing. The general consensus is that fish are able to satisfy a broad range of criteria required for toxicity testing. These may include their cost effectiveness to collect and identify, their wide availability, their sensitivity to stressors (i.e. respond accordingly), and their ability to apply many different methods. In fact, by satisfying this latter criterion, Chovanec et al. (2003) suggested that no other aquatic organism is as suitable for the evaluation of toxic impacts as fish are.

For the purposes of the present study the fish species, *C. gariepinus* was primarily selected because it is an indigenous species present within the Luvuvhu River. Furthermore this species is advantageous since it allows for ample plasma sampling due to their relative sizes; it has a large database of culturing techniques as it is a relatively important economical species; they have relatively high tolerances and fertilization rates and above all they can easily adapt to laboratory conditions. Nevertheless, the utilization of *C. gariepinus* in toxicity testing does pose some problems. For instance they are relatively large and thus require more space for culturing and testing and their life cycles are relatively long.

13A.2.1.3 Culture and handling of test species

For an extensive review on the culture, handling and general biology of *C. gariepinus* refer to Tucker (1985), Viljoen (1999) and De Graaf and Janssen (1996).

13A.2.1.4 Chronic exposure

Since pollution can affect lifetime fecundity, survival of the offspring to adulthood, and the fertility of these offspring, a holistic approach incorporating all these endpoints, through whole life-cycle studies, is highly recommended for reliably evaluating the chronic effects of

EDCs on fish (Kime, 1998). However, few assessments have utilised this holistic approach due to the difficulty in maintaining long-term exposure systems and the cost of running these studies (Schweer, 2002). In order to overcome these disadvantages either an early life-stage or a partial life-cycle study has been suggested. Although according to Dionne and Kiamos (1994), early life studies should be avoided as toxicity of approximately 20% of the chemicals and metals tested in full life-cycle studies in fish could not be predicted from the results of only an early life-stage exposure. Therefore, the partial life-cycle exposures should rather be utilized.

In partial life-cycle exposures, sexually mature adults (optimally weighing between 300 and 800 g) are exposed to at least two or three widely spaced treatment levels for a period of time prior to and during spawning, followed by a short-term exposure of F1 embryos and juvenile fish. According to Ankley et al. (2001), a minimum of four replication tanks should be used per treatment in order to obtain a statistically significant difference between control and exposure groups. Following this exposure a number of adverse effects are then assessed in order to identified reproductive disruption. Although a number of fish species including the fathead minnow, medaka and zebrafish have successfully demonstrated this, no such studies have utilized the commercially important African catfish, *C. gariepinus* (Hutchinson et al., 2003).

According to Schmitz (1996), three basic groups of bioassays exist; including static, flow-through and the renewal test. However, for the purposes of this study a flow-through system, where a test solution is continuously fed into the test vessel, was selected due to a number of factors. This test, in contrast with the other two tests, is particularly useful for chronic toxicity testing. It minimizes toxicant properties such as volatilization, precipitation absorption and oxygen demand and it eliminates the build-up of waste products and excess food.

13A.2.2 Partial life-cycle exposures

13A.2.2.1 Test conditions

Four replicate flow-through exposure systems per treatment were used. For each treatment of DDT there were 16 tanks, 8 solvent-containing control tanks and 8 DDT-containing exposure tanks. A 50 mg/L stock solution of DDT was prepared by dissolving DDT powder in the solvent, ethanol, ensuring that the concentration of solvent does not exceed 20 µl/L (Hutchinson et al., 2000). The volume of stock added to 900 L of aged tap water in stock tanks was dependent on the nominal concentrations of DDT to be tested. These concentrations were determined using the medians, maximum, 20% of the maximum DDT

concentrations in water from Luvuvhu River. These concentrations were 0.659 µg/L, 1.36 µg/L, 2 µg/L and 2.724 µg/L. They were pumped into the respective exposure tanks and exchanged every 48 hours.

13A.2.3 Adult exposure

Mature adult female and male *C. gariepinus* were obtained from a broodstock fish bred at Bushveld catfish aquaculture farm. The broodstock were safely transported to the aquarium facilities at the University of Johannesburg, where they were left to acclimate between one and three months in holding tanks. The 8 females and 8 males were then transferred into separate exposure tanks; 4 females and 4 males in DDT exposure tanks, and 4 females and 4 males in control tanks. They were left to acclimate for a further six weeks under summer photoperiod and a constant water temperature of 27°C. This was done to ensure that adult fish induce mature gonads for breeding (De Graaf and Janssen, 1996).

The catfish were then chronically exposed for 21 days in these tanks, to the three respective DDT concentrations via the water. 12 hours prior to the completion of the 21 days, a single injection of 0.5 ml/kg Aquaspawn (a decapeptide, GnRH, which stimulates the release of pituitary hormones) was injected into the dorsal muscle of the male and female in order to induce spermiation and ovulation. The female catfish were striped after 12 hour incubation and the eggs collected whilst the male catfish were sacrificed before sperm could be collected from testes.

Sample collection

The total length (cm) and total wet weights (g) were recorded. Then plasma samples from males were collected from the caudal aorta into ethylenediaminetetraacetic acid (EDTA) coated-, heparinised-(with aprotinin added) vacutainers kept on ice, centrifuged at 3 600 rpm for 10 minutes at 4°C and resulting plasma stored at -20°C until analysed for ALP, and metals. Then the females were stripped of their eggs before ethically severing the vertebrae. The male catfish were first sacrificed before their gonads could be dissected, weighed and used for histology and fertilisation. Then the pectoral spines were sampled for age determination.

Sample analyses

a Age

The pectoral spines were collected and prepared according to Staples (1970). Sections of about 2 mm were cut perpendicular to the basal recess of the pectoral spine, using a hacksaw blade. The thin sections were then examined in ethanol using a dissecting microscope (x 10).

b Alkali-labile phosphate

Protein-bound phosphate was extracted from 10µL of plasma according to the procedure described by Brasfield et al. (2002), with minor modifications. To the plasma, 1.5mL of 10% Trichloroacetic acid (TCA) (w/v) was added before it was precipitated for 15 hours at 4°C. Thereafter the sample was centrifuged at to obtain a pellet. The resulting pellet was then re-suspended in 1ml ice cold 5% TCA (w/v) and incubated at 50°C for 30 minutes and again centrifuged at 7 000 rpm for 10 minutes. The pellets were subsequently washed with 1 ml of 100% ethanol (incubated for 1minute at 80°C); chloroform: ether: ethanol (1:2:2) solution; 100% acetone; and 100% ether. The ether formed pellets that were allowed to dry for 10 minutes before 250 µL 2 M sodium hydroxide was added. The sample was then incubated at 100°C for 15 minutes and allowed to cool before the sample was neutralised with 250 µL 2 M hydrogen chloride. Extracts were assayed spectrophotometrically using a modified method derived by Stanton (1968). Extracts were initially diluted 4 x using a 1:1 2 M HCl: 2 M NaOH. Then 100 µL 2.5% molybdate solution (2.5% ammonium molybdate tetrahydrated in 2.5 M sulphuric acid) and 25 µL Fiske-Subbarow solution (1g in 6.3 mL distilled water) were added to diluted extracts, incubated for 10 minutes and measured at 660 nm. A standard solution of 10mg phosphate/mL was prepared with 4.39 g KH_2PO_4 in 100 mL distilled water and used to obtain a standard curve between 0 and 10 µg phosphate/ml. The results were then expressed as µg phosphate/ mg protein, using a bovine serum albumin (BSA) standard.

c Plasma metal analysis

Plasma was thawed and 500 µL was added to an acid solution made of 8 mL 65% nitric acid and 2 mL 30% hydrogen peroxide. The samples was then placed in a Milestone ETHOS microwave and digested for 26 minutes at temperatures varying from 85-230°C. The digested samples were then diluted (10 x) in 1% nitric acid and metal concentrations were determined using a Varian Ultra Mass 700 inductive coupled plasma mass spectrophotometer (ICP-MS). All samples were analysed for calcium (Ca) and magnesium (Mg). Indium was used as an internal standard to correct for interferences from high-dissolved solids. All concentrations were validated with reference materials. Certified values and recoveries for the reference materials are presented in Table 13A.1.

Table 13A.1: Certified values and recoveries for the metal concentration reference materials.

Element	DOLT-3 (µg/g)	Recovery (µg/g)
Arsenic	10.2	10.77
Cadmium	19.4	21.81
Copper	31.2	37.95
Iron	1484	1786.9
Lead	0.32	0.281
Zinc	86.6	60.58

d Condition factor and GSI (gonadosomatic index)

The condition factor was calculated using the formula $K = W/(aL^b)$ according to Hagenaars et al. (2008). Where W is the total body mass (g) and L is the total length (cm). The parameters a and b were determined from the length-mass relationship ($W = aL^b$) of total number of fish. The GSI was then calculated as the percentage of gonad mass (g) to total body mass.

e Histology

The gonads that were not used for fertilisation were fixed in Bouin's solution made up of 225mL picric acid, 75mL 40% formaldehyde and 15mL glacial acetic acid. After 48 hours, the gonads were then dehydrated in graded ethanol until within 70% ethanol. The gonads were then taken to the Pathology Department at Onderstepoort for further dehydration, embedding in paraffin wax, section cutting and staining with hematoxylin and eosin. The resulting slides were examined for intersex using light microscopy with a range of magnifications between 20 x and 100 x.

13A.2.4 Juvenile exposure

Within 10 minutes of collection, an equal ratio of eggs and semen collected from 2 females were mixed with semen from 4 males, respectively and then fertilized using an equal volume of water. Fertilized eggs were then gently stirred and dispensed evenly on incubation sieves with a 1mm mesh size. The incubation sieves were suspended vertically in the 8 flow-through exposure tanks and 8 controls for each of the DDT concentrations. After about 48-72 hours, the eggs hatched and the numbers of live embryos were counted. Thereafter the survival of free embryos (about 120 hours after fertilization) and resulting juveniles were monitored every 24 hours for 4 days. The amount of free and dead embryos was counted. The hatching success was calculated using the number of inseminated ova divided by the number of embryos and multiplied by 100. The percentage of juveniles that survived after

120 hours were calculated by dividing the number of surviving embryos with the total number of hatched embryos and multiplying that by 100 to get a percentage.

13A.2.5 Descriptive statistics

The Graphpad v4 software was utilised to obtain all the graphs represented in this report. The significant variations in the variables between the various exposure concentrations and controls were tested by one-way analysis of variance (ANOVA). Data were tested for homogeneity of variance using Levene's test, prior to applying post-hoc comparisons. Post-hoc comparisons were made using the Scheffé's test for homogeneous or Dunnett'sT3 test for non-homogenous data. The use of either one of the two tests resulted in the determination of significant differences ($p < 0.05$) between variables. In order to identify significant relationship between the variables a two-tailed bivariate Pearson's correlation was done. All these statistical tests were performed using SPSS V14.

13A.3 Results

The results of experiments in which male *C. gariepinus* were exposed to DDT are shown in Figure 13A.1 and 13A.2. The GSI, in Figure 13A.1a, showed no significant differences between any of the control or DDT exposure groups. Nevertheless, it was evident that the gonad condition was slightly better in all of the control groups except for those of 0.6 µg/L DDT exposures. This latter exposure group did however have a better gonad condition compared to the fish exposed to higher DDT concentrations. The general condition factor shown in Figure 13A.1b, also had very little deviation between the exposure groups, with control fish in 1.3 and 2 µg/L DDT having a lower condition than in the exposed fish.

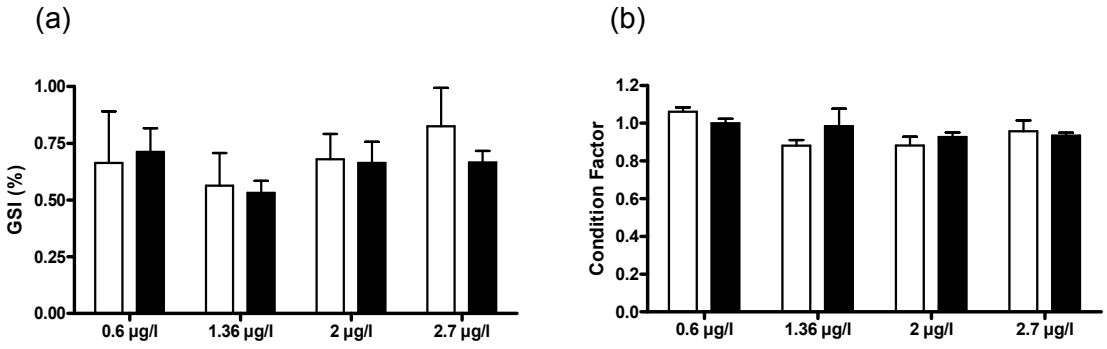

Figure 13A.1: The gonad condition (GSI) and condition factor (CF) in male catfish exposed to 0.6, 1.36, 2 and 2.7 µg/L DDT in the ambient water (represented by black bars) and the associated solvent controls represented by white bars. Values are medians ± SE.

In Figure 13A.2, plasma VTG content expressed as calcium and magnesium showed no significant differences between control and exposed fish, although there was a general tendency of a higher metal concentration in the plasma of exposed males. The ALP concentrations in Figure 13A.2c were only measured in plasma of males exposed to 1.36, 2 and 2.7 µg/L DDT and their respective controls. A significantly lower ALP concentration ($p < 0.05$) in the control males of the 2.7 µg/L DDT exposure grouping was found, with a general trend of an increase in ALP with an increased DDT exposure. In contrast, intersex in both control and exposed male gonads showed no trends between the different concentration groups as none of the samples had intersex.

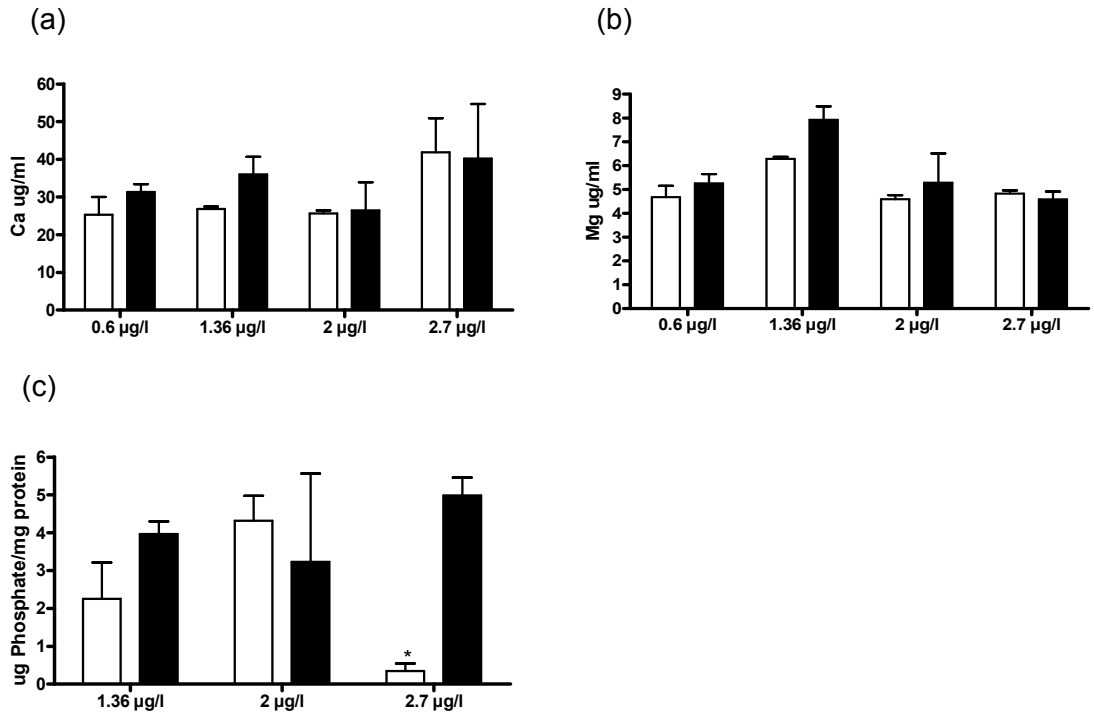

Figure 13A.2: Plasma VTG content expressed as calcium, magnesium and alkali-labile phosphate of male catfish (details described in Figure 13A.1).

In order to identify if any of the indicators are influenced by the respective DDT concentration exposures as a whole, a Pearson's correlation was performed. As can be seen in Table 13A.3, none of the parameters were significantly correlated ($p < 0.05$) with the different DDT exposure concentrations, although plasma ALP and calcium concentrations did exhibit the highest correlations with a significance of $p = 0.16$ and $p = 0.13$, respectively. Age, another possible contributing factor to effects of the fishes was also correlated, but also no relationships were found. In fact the only relationship that did show a significant ($p = 0.03$) positive correlation was that found between ALP and calcium.

Table 13A.2: Pearson's correlation between indicators of environmentally available DDT concentrations. Only those variables showing a significant correlation were represented in bold ($p < 0.05$).

	ALP	Ca	Mg	GSI	AGE	CF	DDT
Ca	**0.41**	1.00					
Mg	**0.59**	**0.56**	1.00				
GSI	0.09	0.27	0.09	1.00			
AGE	-0.20	0.07	-0.24	-0.07	1.00		
CF	-0.14	0.07	0.09	0.21	0.07	1.00	
DDT	0.28	0.26	0.19	0.00	0.11	0.07	1.00

As shown in Table 13A.3, most of the control fish had successfully produced an F1 generation in this study, except for male 3 and 4 from the 1.36 µg/L DDT exposures. The 2 µg/L DDT groupings were not represented at all in the table as none of the control adults successfully spawned juveniles suggesting an inherent problem in fish, perhaps a result of handling stress. In all the exposures, the only parents that yielded a successful F1 generation were those exposed to 0.6 µg/L DDT. However as shown in Table 13A.4, the hatching success was not great and after 72 hours there were no survivors.

Table 13A.3: The success of the artificial reproduction of 2 exposed and control females with respectively 4 exposed and control males (M1-M4) to different DDT concentrations.

DDT concentrations	EXPOSURE		CONTROL	
	FEMALE 1	FEMALE 2	FEMALE 1	FEMALE 2
0.6 µg/l	**M2**	**M3**	**M1**	-
	M3	-	-	-
	-	-	-	-
	-	-	-	-
1.36 µg/l	M1	M1	**M1**	**M1**
	M2	M2	**M2**	**M2**
	M3	M3	M3	M3
	M4	M4	M4	M4
2.7 µg/l	M1	M1	**M1**	**M1**
	M2	M2	**M2**	**M2**
	M3	M3	**M3**	**M3**
	M4	M4	**M4**	**M4**

Bold: parents that successfully produced an F1 generation. Hyphen: parents not exposed.

Table 13A.4: The median percentage hatching success and survival of juveniles exposed to different concentrations from parents exposed to the same concentrations.

DDT concentration	Hatching success	Survival of hatchlings (72 hrs)	Survival of embryos (120 hrs)
Control	28	69	9
0.6 µg/L	4.25	0	0
1.36 µg/L	0	0	0
2.7 µg/L	0	0	0

13A.4 Discussion

Numerous studies have successfully used GSI to monitor the reproductive effects of DDT exposure in fish (Fiest et al., 2005; Mills et al., 2001; Schweer, 2002). In the present study, no significant reflection of DDT impact was observed for any of the fish groupings, with the

least changes in the gonad condition found in fish exposed to the lowest DDT concentration, 0.6 µg/L. This suggests that the DDT concentration of 0.6 µg/L in the water had no influence on gonad condition after 21 days of exposure, with a very slight reduction in testicular condition upon exposure to 1.36, 2 and 2.7 µg/L DDT. These weak associations could have perhaps been due to the DDT concentrations being too low to show any significant changes in the gonad condition for the time exposed. A study done by Tricklebank et al. (2002) also showed weak associations between gonad condition and tissue DDT concentrations in damselfish. Although no data was available on the concentrations in the water of this study, the residues present in the damselfish were relatively lower. Other fish species have however been more responsive to lower concentrations of DDT for example *O. mossambicus* showed structural changes in the gonads exposed to DDT concentrations in the water as low as 1µg/l (Bhattacharya and Pandey, 1989), unfortunately the GSI was not measured so exact comparisons could not be made.

The CF is commonly utilised as an indicator of the general 'well being' of individual fish, which relates the total body weight to the body length. The condition of the fish during the present study exposures remained relatively constant for the various exposure groupings. Again suggesting that exposure to DDT in this study did not induce an effect in the body weight of the individual fish. This is consistent with other studies that have also shown the insensitive nature of the condition factor to lower pollutant concentrations (Bervoets and Blust, 2003; Pait and Nelson, 2002).

Plasma VTG is often used as an indicator of the presence of estrogen mimicking chemicals in males (Kime, 1998). In order to determine the influence of DDT on the activation of VTG levels in the present study, calcium, magnesium and ALP concentrations were measured. The results showed no significant dose-dependent increase in either of the parameters in the plasma of the male *C. gariepinus*. Perhaps the reason for this lack of significance was due to the DDT concentrations or exposure time being too low. As shown in Chinese loaches by Lv et al. (2006), the vitellogenic responses in male fish are significantly time- and dose-dependent. Nevertheless there was a positive response of calcium and ALP, albeit not significant, to the DDT concentrations with good correlations (Table 13A.2), and is illustrated in graphs a and c in Figure 13A.2. Both calcium and ALP concentrations in the male fish plasma increased as the DDT exposure rose from 0.6 µg/L to 1.6 µg/L to 2.7 µg/L. Since both of these have successfully demonstrated significant correlations with VTG levels in the fish plasma (Bjornsson and Haux, 1985; Gillespie and De Peyster, 2004; Verslycke et al.,

2002) it can be concluded that there was an increase (not significant) in endocrine disruption due to DDT exposure in *C. gariepinus* at these environmentally relevant concentrations.

Intersex however was not observed at any dose of *p,p-'* DDT in *C. gariepinus* of the present study, which was perhaps due to the low DDT exposure doses. As was established by Papoulias et al. (2003) in medaka exposed to *o,p'*-DDE concentrations between 0.005 and 0.5 mg/L, intersex is usually (with a few exceptions) only induced at higher DDT isomer concentrations. For instance according to Metcalfe et al. (2000) in the medaka fish the nominal concentration of 5 µg/L *o,p'*-DDT is the lowest level required for the induction of testis-ova. Another study using medaka exposed for 2 weeks showed that intersex was only induced at concentrations above 4.32 µg/L DDT (Cheek et al., 2001), whilst Edmunds et al. (2000) identified an entire sex reversal of medaka when exposed to 227 mg/kg. Calson et al. (2000) however showed in trout and salmon no intersex when exposed to concentrations between 1 and 80 mg/kg.

As shown in Table 13A.3, the only exposed adult fish that successfully produced an F1 generation were those exposed to 0.6 µg/L DDT. In these exposed juveniles, the hatching success and survival percentage was lower than in the control juveniles. These results along with the lack of hatching success from adults exposed to 1.3 and 2.7 µg/L DDT were similar to those found by Cheek et al. (2001). In their study, there was a significant reduction in the reproductive success of Medaka exposed to concentrations of 0.23, 1.37 and 4.32 µg/L DDT. Therefore it is probable that DDT concentrations utilised in the present study does have a major influence on the reproductive success of *C. gariepinus*, perhaps largely due to the accumulative effects of DDT on both the male and female parents and the juveniles (Kime, 1998).

However, it must be said that this conclusion must be viewed with caution considering that there were influencing factors affecting these results. Firstly, the percentage of juveniles hatched, under control conditions, was much lower than what was expected. According to the results obtained by Viljoen (1999), (who utilised a similar approach) the control percentages in hatching success should have been 99%. Secondly, final oocyte maturation in females was not always a 100% successful. It was noted that in some of the females of the unhatched juveniles, there were oocytes that were macroscopically more round than those female oocytes producing progeny. According to De Graaf and Janssen (1996), oocytes are only round before hypophysation (artificially induced spawning) where upon the pituitary hormone is activated and utilised to complete the final oocyte maturation that yields a more translucent and flat oocyte. However in the present study, the final maturation of

some of the oocytes did not occur due to the inactivation of the pituitary hormone but probably as a result of the inefficiency of the GnRH hormone (Aquaspawn) injected into the females to activate the pituitary hormone involved in ovulation. The exact cause for this is unknown as there were no consistent deviations in the materials and methods involved in this procedure. Thirdly, many other inherent variables can influence the successful spawning of juveniles in captivity even under natural conditions, which are discussed in detail in De Graaf and Janssen (2001).

13A.5 Conclusion

The GSI and CF showed no changes in the growth of catfish after exposure to the different DDT concentrations. Calcium and ALP concentrations showed subtle dose-dependent increases in exposed male catfish, while the overall reproductive success of the control juveniles indicated that an escalating effect of the increasing DDT concentrations (albeit the extent unknown) on the spawning success of adults may have occurred.

In conclusion, the results showed that no severe effects were found in adult *C. gariepinus* exposed to 0.6, 1.3, 2 or 2.7 µg/L DDT concentrations; however a possibility of a dose-response relationship was evident suggesting that prolonged duration and increased concentration of DDT exposure may lead to irreversible long term negative effects in *C. gariepinus*. The juvenile exposures highlighted the accumulative effects of DDT on the reproductive success of *C. gariepinus*, again showing that early life stages of these fish are more sensitive to toxicants than adult fish.

13B THE USE OF *OREOCHROMIS MOSSAMBICUS* AS A SENTINEL SPECIES OF THE POSSIBLE EFFECTS ON HEALTH AND REPRODUCTION OF DDE *IN VIVO* EXPOSURE

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13B.1 Introduction

Organisms under natural field conditions may respond quite differently to pollutant exposures, under laboratory conditions (Shugart, 1996). Laboratory studies generally lack ecological realism, because of many environmental factors that can influence organism responses at all levels of biological organization (Adams, 2001). Laboratory exposed organisms can exhibit an increased sensitivity towards environmental pollutants that are generally absent in many artificial culture media. Therefore, acclimation (tolerance) and adaptation (genetic) are factors related to organisms under field conditions that cannot be neglected when extrapolating laboratory data to field conditions (Muyssen et al., 2002).

There is a growing need for more studies to demonstrate that biological responses to toxicants in the laboratory are comparable to field data. Exposures under laboratory conditions could help establish cause and effect relationships, as it is only in such an environment, that organisms can be exposed to pollutants under specific controlled conditions (Kruger, 2002). Under these controlled laboratory conditions it was possible to establish a dose-response relationship between concentrations of pollutants and between biomarker responses (Walker, 1998; Hyne and Maher, 2003), thus further contributing toward the validity of the use of biomarker responses in field monitoring. However, a number of biotic and abiotic factors can influence the extrapolation of individual biomarkers to the field monitoring of pollutant effects (Lagadic et al., 1994), thus making the comparison of field data and laboratory data difficult.

A sub-lethal exposure study was undertaken in addition to the field exposure study and the use of the *O. mossambicus* as a biomonitor was tested under laboratory conditions. Predicting the potential effect of known amounts of a pollutant requires a task in which biomarkers might be employed. Biological responses may link the bioavailability of compounds with their concentrations at target organs and intrinsic toxicity (Van der Oost et al., 2003). Certain responses established for one species, however, are not necessarily valid for other species (Van der Oost et al., 2003). The determination of toxicity of a chemical, its by-products and its degradation products to aquatic organisms is important because any

compound that is used and manufactured in considerable quantities is likely to become a contaminant of water sources (Nussey, 1994). To evaluate the effects that various concentrations of the chemical may have on aquatic organisms, toxicity tests are used. The information gained from various toxicity tests can be of use in the management of pollution for the purpose of either the prediction of environmental effects of a waste product, a comparison of toxicants and even test conditions (Nussey, 1994). Available data for DDT and its metabolites indicate that acute toxicity to freshwater aquatic life may occur at concentrations as low as 11,600 µg/L and would occur at lower concentrations among species that are more sensitive than those tested. No definitive data are available concerning the chronic toxicity of these chemicals to sensitive freshwater aquatic life (EPA, 1986).

Toxicity tests are designed to evaluate the relative toxicity of a test material or the level of toxicity of an agent to a selected group of aquatic organisms. They can be undertaken through injection of the test material into the organism or by inclusion in the food, however, most of these tests are carried out by exposing groups of organisms to several controlled environments in which different concentrations of the test material are mixed in water. The exposure is expected to produce a specific effect in the exposed test organisms over a specific period of time, for example, 24, 48 or 96 hours (Nussey, 1994).

Many factors can affect or modify the degree of sensitivity shown by an organism to different toxicants, for example the season of the year, specific diet and water quality variables such as hardness, pH and temperature. Since fish are ectothermic (poikilothermic), temperature is the most important environmental factor controlling rates of biological processes, and thus, would be expected to influence tissue uptake of various substances as well as the toxicity tolerance in fish. An increase in water temperature will increase the rate of chemical reactions in fish, as predicted by the Arrhenius theory. This theory predicts that the toxicity of a toxicant also increases with a rise in temperature (Nussey, 1994). The calcium content (hardness) of water acts as a modifying factor with an increase in metal toxicity in water with a low calcium content. The toxicity of pollutants may be increased by a reduction in dissolved oxygen whilst the hydrogen ion concentration (pH) can affect the ionization and the solubility of the toxicant (Nussey, 1994).

The objective of this section of the project was to conduct sublethal toxicity exposures using varying concentrations of DDE in a controlled environment for adult *O. mossambicus*, which are widely distributed in South Africa (Skelton, 2001).

13B.2 Materials and methods

Mature *O. mossambicus* males, all of comparable size and age, were obtained from Aquaculture Innovations (bred in captivity), Grahamstown, South Africa.

13B.2.1 General holding system and laboratory conditions

Five exposure experiments were done i.e. control, solvent control and 0.1 µg/L, 0.4 µg/L, 0.7 µg/L of DDE derived from a series of stock solution dilutions at 24 ± 1°C for a period of 96 hours with a 12 hour day-night cycle. These exposure concentrations were calculated from values obtained in a previous study. The mean of the lowest (0.4 µg/L) and highest (0.7 µg/L) values determined and a value between the 2 values (0.4 µg/L) were selected. Eight healthy, males were used per concentration per exposure and were placed in a flow through system consisting of four exposure tanks and a stock tank (Figure 13B.1).

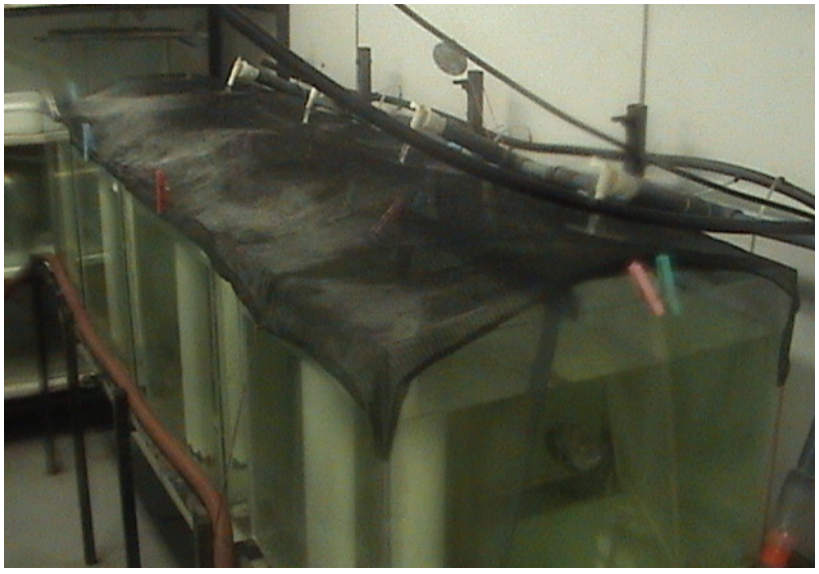

Figure 13B.1: Test organisms exposed to DDE concentrations in a continuous flow through system.

Fish were allowed to acclimatize for a period of two weeks and were fed commercial trout pellets (50 percent protein) every second day. The stock tank solution was made up using aged tap water and DDE in a 0.02% ethanol solution.

13B.2.2 Water quality

The physical water quality variables were monitored in each of the stock tanks at the following intervals: 1 and 96 hours (Table 13B2). These included the pH, dissolved oxygen,

temperature, conductivity and TDS. Temperature and dissolved oxygen content were measured with a Eutech Cyberscan DO 310 meter, pH was taken with a Eutech Cyberscan pH 310 meter, while total dissolved salts (TDS) and conductivity were measured with a Eutech Cyberscan CON 400 meter.

13B.2.3 Sample preparation

After exposure, fish were removed from the tanks, blood was drawn from each fish and each fish was weighed and measured. The ventrally opened fish was examined macroscopically for abnormalities, the testes were removed, weighed and measured and were then stored in Bouin's. The gonadosomatic index (GSI) was calculated using gonadal weight/ (total body weight – gonadal weight) and expressed as a percentage (for screening purposes). Sections of liver, gills and muscle were collected for biomarker analysis and stored at -80°C until further analysis.

13B.2.4 Biomarkers

The biomarker responses of the test organisms were determined, after the 96 hour exposure period. Selected tissues were used for each of the biomarker analyses. Tissues were thawed and homogenised on ice, in the appropriate homogenising buffer. The biomarkers assayed were: EROD, CAT activity and CEA. All samples were analysed in triplicate. All glassware used in the analyses was washed before use. Glassware was washed and placed in a soap bath containing a 2% Contrad TM (Merck Chemicals) solution for 24 hours. Thereafter, it was rinsed with dH_2O and placed in a 1 M hydrochloric acid bath (Merck Chemicals) for another 24 hours. It was then rinsed again with dH_2O and allowed to dry

13B.2.4.1 EROD (7-ethoxy resorufin O-deethylase)

EROD was determined by method of Burke and Mayer (1978), with some modifications. Liver samples were homogenized in 0.25 M sucrose – 0.05 M Tris (pH 7.4) on ice at a ratio of 1:3 and then centrifuged at 13 500 rpm for 20 minutes at 4°C. To the supernatant of each sample was added a general reaction mixture of 0.1 M potassium phosphate buffer (pH 7.8) and 0.05 M Ethoxyresorufin in 1.25% Tween 80. A 0.05 M aliquot of NADPH was stirred into the mixture to start the reaction and the progressive increase in fluorescence, as ethoxyresorufin was deethylated to resorufin. A baseline of fluorescence was recorded at an excitation wavelength of 544 nm and an emission wavelength of 590 nm with a fluorimeter and using resorufin as a standard. The excitation emissions were then recorded another six

times at one minute intervals measuring the progressive increase in resorufin. Ethoxyresorufin and resorufin are both light sensitive compounds and the solutions of these compounds were thus prepared and stored in the dark and the metabolic reaction was then initiated in a room with dim infrared lighting.

13B.2.4.2 Catalase activity

Catalase activity was determined by method of Cohen et al. (1969). Gill samples were homogenized with 0.01 M phosphate buffer (pH 7.0) on ice at a ratio of 1:2 and then centrifuged at 10 000 rpm for 10 minutes at 4°C. To an aliquot of the supernatant was then added cold 6 mM H_2O_2 to start an enzyme catalyzed decomposition reaction of the H_2O_2. After an incubation period of 3 minutes, the reaction was stopped with the addition of 6 N H_2SO_4. The remaining H_2O_2 was measured by allowing it to react with a standard excess of 2 mM $KMnO_4$ and then measuring the residual $KMnO_4$ spectrophotometrically at a wavelength of 490 nm within 30 to 60 seconds of the addition of the $KMnO_4$. The absorbance was read using an Elx-800 universal microplate reader (Bio-tek instruments, USA). The protein content was determined using the method of Bradford (1976) and the CAT activity was expressed as mol H_2O_2/mg protein.min.

13B.2.4.3 Cellular Energy Allocation (CEA)

Available energy reserves (E_a)

The Cellular Energy Allocations were determined by method of De Coen and Janssen (1997). The samples were homogenised on ice in 1 mL of ice-cold deionised water. The energy reserves determined were: carbohydrate, lipid and 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 560 nm using an automated microplate reader. Total lipids were extracted following the method of Bligh and Dyer (1959), and the absorbance was read at 340 nm using tripalmitin as a standard. The protein content was determined using Bradford's reagent (Bradford, 1976). The absorbance was measured at 630 nm using bovine serum albumin (BSA) as a standard.

Energy consumption (E_c)

The energy consumption or cellular respiration rate was determined by measuring the electron transport activity (ETS). Each sample was homogenized in 1 mL homogenising buffer (0.1 M Tris-HCl pH 8.5, 15 percent (w/v) Poly Vinyl Pyrrolidone, 153 µM MgSO4 and 0.2% (w/v) Triton X-100). After centrifugation (at 3000 rpm for 10 minutes at 4ºC), 25 µL

supernatant was added to 75 µL buffered substrate solution (0.13 mM Tris-HCl and Triton X-100, pH 8.5) and 25 µL NAD(P)H solution (1.7 mM NADH and 250 µM NADPH). The reaction was started by adding 100 µL INT (*p*-IodoNitroTetrazolium) and the absorbance was measured kinetically at 490 nm for 10 minutes at 20°C. The amount of formazan formed was calculated using e = 15900 / M.cm.

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: 17 500 mJ/mg glycogen, 39 500 mJ/mg lipid and 24 000 mJ/mg protein. The cellular respiration rate (E_c) was determined; using the theoretical stoichiometrical relationship that for each 2 µmol of formazan formed, 1 µmol oxygen was consumed in the ETS system. The amount of oxygen was transformed into energetic equivalents using an average oxyenthalpic equivalent of 484 kJ/mol O2. The total energy budget was calculated using the following equation:

$CEA = E_a - E_c$

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

$E_c = E_{ETS}$

Table 13B.1: A summary of the methods and apparatus used in the biomarker analysis.

Biomarker		**Apparatus**	**Absorbency Wavelength (nm)**	**Method**
EROD		Perkin-Elmer 650-40 Fluorescence Spectrophotometer	Excitation: 544 Emission: 590	Burke and Mayer (1978)
CAT		Elx800 Universal Microplate Reader	490	Cohen et al. (1969)
CEA	Lipids	Elx800 Universal Microplate Reader	340	Bligh and Dyer (1959)
	Protein		630	Bradford (1976)
	Glucose		560	Glucose test kit
	ETS		490	De Coen and Janssen (1997)

13B.2.5 Statistical analyses

Statistical analyses were performed using the program, SPSS 14.0. The Levene's test was used to test all data for homogeneity of variance. Differences among the biomarker responses following exposure to the five differing DDE concentrations were determined using one-way ANOVA. The Scheffé's multiple comparison tests (for homogenous data) as well as Dunnett's-T3 test (for non-homogenous data) were applied as post-hoc criterion, when significant differences of greater than 0.05 were found amongst the variables.

13B.3 Results and discussion

Fish respond to pollutants either by a rapid death or sublethal effects, or when a pollutant concentration is very low there may even be no visible effects.

13B.3.1 Water quality

Table 13B.2 shows the water quality variables measured for the water in each of the stock tanks at both the 1 and 96 hour intervals of the exposure periods. All the physical water quality parameters remained relatively constant throughout the exposure period.

13B.3.1.1 pH

In closed recirculating systems, pH generally decreases with time since acid is produced during nitrification of ammonia to nitrate (DWAF, 1996g). This correlates with the pH values for both exposure systems in the current study (Table 13B.2), however, the pH values remained relatively constant throughout each of the exposures. In poorly buffered systems, the pH may decrease to 6 or less. Increased pH in recirculating systems is often indicative of poor biological filtration (DWAF, 1996g), which was not the case here.

13B.3.1.2 Dissolved oxygen

Dissolved oxygen did not vary much over the life-time of each of the exposures, as a result of oxygen being constantly added to the exposure tanks by means of an air-stone throughout the experiment.

13B.3.1.3 Temperature

The temperature for the duration of both exposures did not vary much as temperature fluctuations were carefully controlled throughout.

Table 13B.2: Selected water quality variables of aged tap water used during the DDE toxicity tests.

Water Quality Variables	DDE Concentration (µg/L)	1 hr		96 hrs	
		1st Exposure	2nd Exposure	1st Exposure	2nd Exposure
pH	Control	7.0	7.0	6.9	6.9
	Solvent control	7.3	7.1	7.0	7.0
	0.1	7.1	7.0	6.9	6.8
	0.4	7.1	7.0	6.8	6.8
	0.7	7	6.9	6.9	6.9
Dissolved oxygen (%)	Control	32.6	31.5	39.8	40.6
	Solvent control	38.8	38.8	45.0	35.0
	0.1	30.1	33.9	35.4	32.8
	0.4	43.3	31.8	31.1	37.2
	0.7	29.5	36.4	36.8	39.2
Dissolved oxygen (mg/L)	Control	3.3	2.8	3.7	3.5
	Solvent control	3.4	3.4	3.9	2.9
	0.1	3.6	3.0	3.2	2.8
	0.4	4.0	3.8	2.7	3.2
	0.7	2.6	3.3	3.4	3.5
Temperature (°C)	Control	22.5	22.5	22.6	22.8
	Solvent control	22.5	22.5	22.6	22.7
	0.1	22.5	22.5	22.6	22.7
	0.4	22.4	22.4	22.5	22.6
	0.7	22.5	22.4	22.5	22.6
TDS (mg/L)	Control	194.0	194.0	190.0	190.0
	Solvent control	138.0	140.0	135.0	137.0
	0.1	138.0	139.0	129.0	93.0
	0.4	139.0	139.0	95.0	93.0
	0.7	141.0	139.0	96.0	95.0
Conductivity (mS m^{-1})	Control	285.0	285.0	281.0	281.0
	Solvent control	276.0	279.0	269.0	273.0
	0.1	275.0	277.0	258.0	185.0
	0.4	277.0	279.0	191.0	186.0
	0.7	280.0	277.0	192.0	189.0

13B.3.1.4 TDS (Total Dissolved Solids) and Conductivity

The conductivity and TDS values for both exposures showed only very slight decreases in both the control and the solvent control. However, in both exposures, with the exception of the 0.1 µg/L of the first exposure, the values showed significant and similar decreases in the 0.1 µg/L, 0.4 µg/L and 0.7 µg/L exposure concentrations, respectively. As previously mentioned, DDE is a lipophilic substance and binds to organic particles in nature. The reduced conductivity and TDS values over time during the exposure periods suggest that the DDE in the exposure concentrations are binding to free ions and dissolved organic particles and then either precipitating out of solution or being released into the atmosphere due to its semi-volatile nature. All of the water quality values obtained was within the acceptable range with the South African Water Quality Guidelines for Aquaculture DWAF, 1996f).

13B.3.2 Biomarkers

13B.3.2.1 EROD

Looking at table 13B.3, EROD enzyme activity was clearly affected with clear induction at each of the exposure concentrations in both of the exposures. In the first exposure, the 0.1µg/L concentration showed a significant induction when compared statistically with the control and a clear trend of increasing EROD induction can be seen with increasing concentrations of DDE. In the second exposure, the 0.7 µg/L concentration shows a significant EROD induction when compared statistically with both the control and the solvent control, thus further showing the effect of EROD induction in aquatic organisms on exposure to DDE despite slightly more erratic results obtained for EROD enzyme activity in the second exposure.

It is well known that exposure to organic chemicals/xenobiotics causes the induction of EROD synthesis. Both ethoxyresorufin and resorufin are intensely fluorescent. The excitation and emission maxima for both compounds lie in the visible spectrum. However, there is a distinct separation in the excitation maxima of the two respective compounds and this allows for easy differentiation between the two (Besselink et al., 1996). Increases in fluorescence at 590 nm with excitation set at 544 nm, suggests that the O-deethylation of ethoxyresorufin to resorufin was being observed. According to Joubert (2000), inhibition of cytochrome p450 1A mediated catalytic activity occurs when fish are exposed to high levels of polychlorinated biphenyl congeners. It is therefore not surprising that EROD enzyme activity was highly induced in the fish exposed to increasing concentrations of DDE. Evident EROD induction occurred at even the lowest DDE concentration in both exposures, thus indicating a high

level of sensitivity with regard for use of this assay in measuring DDE exposure in *O. mossambicus* even at low environmentally relevant concentrations. Additionally, according to Besselink et al. (1996) it must not be neglected the effectivity of EROD induction for indicating specific dose-related induction of the total cytochrome P450 content in hepatic microsomes, thus, making EROD an effective measure of increased concentrations of organic pollutants not only in laboratory procedures, but also in field-related environments.

Table 13B.3: Average biomarker levels and activities determined during each of the DDE exposures.

Exposure No.			1					2				
Concentration (µg/L)			Control	Solvent Control	0.1	0.4	0.7	Control	Solvent Control	0.1	0.4	0.7
EROD Activity (nM/min.mg protein)		Mean	9.05	13.11	25.08	36.96	54.86	26.48	28.98	87.42	81.21	198.99
		Std. Dev (±)	4.77	6.65	8.41	22.71	52.19	10.20	12.41	52.53	44.83	93.50
Catalase Activity		Mean	16.94	16.97	18.37	18.5	18.39	28.27	32.97	25.24	30.63	36.82
		Std. Dev (±)	3.00	3.76	5.11	4.97	1.77	2.28	1.48	3.34	2.34	15.70
CEA	Glucose	Mean	45.59	46.73	46.03	33.27	31.06	53.44	56.13	55.77	54.59	54.21
		Std. Dev (±)	1.93	1.58	2.00	8.27	3.50	6.69	1.01	1.69	1.98	1.38
	Protein	Mean	31.45	31.89	31.69	31.97	31.11	38.44	39.75	38.76	33.66	31.29
		Std. Dev (±)	0.48	1.75	0.84	0.70	1.47	1.30	1.15	1.53	3.07	1.42
	Lipids	Mean	100.60	133.09	134.33	144.38	141.12	205.56	162.62	164.51	160.86	150.54
		Std. Dev (±)	16.37	48.62	39.25	22.35	29.62	44.93	22.3	31.86	26.3	28.55
	Ea	Mean	162.77	198.33	212.05	223	218.16	297.44	258.5	259.05	249.11	236.03
		Std. Dev (±)	17.30	45.51	37.73	24.35	30.99	50.49	22.07	31.26	26.62	29.66
	Ec	Mean	0.92	0.79	0.66	0.55	0.61	1.90	1.75	1.56	1.26	1.32
		Std. Dev (±)	0.39	0.22	0.32	0.32	0.26	0.53	0.27	0.44	0.56	0.46
	CEA	Mean	217.24	222.21	211.39	197.79	162.16	295.54	256.75	257.49	247.85	234.71
		Std. Dev (±)	30.79	24.23	37.87	45.60	17.08	50.42	22.21	30.95	26.55	29.47

13B.3.2.2 Catalase (CAT) activity

Depending on the duration and intensity of the pollutant to which an organism is exposed, antioxidants may only be induced during the first phase of the response, while in other conditions organisms can exhibit no variations or transitory responses before adaptive mechanisms occur (Regoli et al., 2002). According to Wu et al. (2005), there is ample evidence that suggests that if an exposure is prolonged, the induced response may decline, or even return to background levels. It is due to these adaptive mechanisms, that the exposure periods were kept at no longer than 96 hours each. Significant catalase activity was noted in the 0.1 µg/L, 0.4 µg/L and 0.7 µg/L exposure concentrations in the first exposure (Table 13B.3), with little induction being noted in both the control and the solvent control. However, there was no significant difference obtained between the different DDE exposure concentrations. Values obtained in the second exposure were erratic, with no significant effects being noted between either the controls or the three DDE exposure concentrations. Therefore, although several studies have found that an increase in CAT activity does occur in aquatic organisms due to exposure to organic pollutants (Wenning et al., 1988; Nasci et al., 1999), due to the erratic nature of the results from the second exposure, no obvious explanation can be given to relate the observed CAT activity to environmental conditions during the exposures in this study. However, it may be that due to its general nature as a bioindicator of stress, CAT activity may be less sensitive to specific exposure studies as was the case in this study.

13B.3.2.3 Cellular Energy Allocation (CEA)

The effects of the different DDE concentrations on the available energy reserves and the energy consumption of *O. mossambicus* in each of the exposures can be seen in table 13B.3. Significant reductions in the energy values for the glucose content were observed between the control and the 0.4 µg/L and the 0.7 µg/L DDE concentrations respectively in the first exposure, while no significant differences were observed in the glucose energy reserves of fish exposed to DDE in the second exposure. However, when noting the results obtained for the available protein energy reserves in fish from each of the exposures, no significant differences were observed in the available protein energy reserves from the fish exposed to the different DDE concentrations in the first exposure, while significant reductions in the protein values for the glucose were observed between the control and the 0.4µg/L and the 0.7 µg/L DDE concentrations, respectively, from fish in the second exposure. Significant reductions were observed in the available lipid energy reserves of the first exposure when comparing the values obtained for the controls and the 0.7 µg/L DDE concentration with a

significant difference seen between the lipid energy reserves in the 0.7 µg/L DDE concentration and that obtained for the solvent control. A significant difference was also noted in the lipid energy reserves between fish in the control and the 0.7 µg/L DDE concentration in the second exposure. The total energy reserves (Ea) gave an integrated overview of the temporal changes of the available energy reserves of *O. mossambicus*, after exposure to the different DDE concentrations during each of the exposures. With increased DDE exposure in each of the exposures, the total energy reserves were significantly reduced, when compared to the control. The cellular respiration rate (Ec) decreased significantly during both of the exposures periods, when compared to the control. However, no significant differences in ETS (quantification and energy consumption) activity were observed in either of the exposures, with comparison to the control. In the first exposure, a significant decrease in CEA was observed between the two controls and the 0.7 µg/L DDE concentration. However, although a decreasing trend in CEA can be seen in the second exposure, no significant differences were observed. Looking at the results as a whole and their individual contributions, it can be said that both the total energy reserves and the respiratory enzyme activity exhibited the same trend as the CEA values and clearly contributed to the decrease in CEA in both exposures.

The rationale for the use of energy-related measures in exposure studies is due to the costly requirements of the biological responses of organisms in terms of metabolic demands (Beyers et al., 1999). Additional costs like, restoring cellular damage from pollutant exposures, requires the relocation of an organism's energy expenditures, to repair and/or maintain their physiological integrity. Biological responses that are costly should increase the metabolic rate with increasing levels of toxicants until irreversible pathological effects impair metabolism itself (Koehn, 1989; Calow, 1991). Thus, the energy budget will reflect how energy availability and consumption changes within an organism under exposure conditions. It can be said with all surety that changes in the glucose, lipid and protein reserves contributed to the changes in the Ea and CEA. The differential effect on the glucose, lipid and protein reserves for the two exposures is more difficult to explain. While the results observed for the first exposure appear to correlate with existing literature in that the toxicant stress preferentially causes a depletion of the more readily available glucose and lipid reserves instead of protein (Smolders et al., 2003b), the protein energy reserves in the second exposure seemed to be more affected than the glucose reserves. However, this is not entirely unusual, as protein is also considered a prominent source of energy in organisms such as fish (Verslycke et al., 2003) and as such, also has the potential to indicate metabolic disruptions caused by exposure to toxicants in various organisms. According to Muyssen and

Janssen (2001) lipids are known as more efficient energy-storage products than either proteins or glucose and consequently, lipid reserves can be the first energy source to be consumed under conditions of toxic stress. This was observed in the results obtained in both exposures. The observed reduction in Ea values is either a result of a depletion of the energy reserves caused by increased metabolic activity or a decrease in energy (food) uptake. According to De Coen (1999), when no increase in Ec is observed, as was the case in both the exposures in this study, decreases in Ea must be a result of reduced energy uptake. A lower respiration rate would result in a lower metabolic fuel demand, in turn, resulting in a lower CEA value.

13B.4 Conclusion

In vivo toxicological exposure of *O. mossambicus* and biomarker responses were used to characterize the extent of the impact of environmental contamination. During the controlled aquarium exposures, *O. mossambicus* reflected differences in the various biomarker responses. The biomarker assay, EROD, appeared to be the most rapid and sensitive indicator of the physiological status of the organism. The CEA biomarker was also a relatively rapid and sensitive indicator. It reflected the health of the organism and probably compensated for the energetic costs required by the other biomarker responses for combating environmental stresses. The results also indicate the need to understand the biochemical changes induced/inhibited in organisms, by factors that are independent of environmental concentrations of pollutants. Good dose-related responses were noted in each of the controlled aquarium exposures. *O. mossambicus* did, however, successfully identify biological effects of environmental stress in general.

CHAPTER 14

HUMAN HEALTH RISK ASSESSMENT OF AN AREA WHERE ONGOING DDT SPRAYING OCCURS

Genthe B

14.1 Introduction

This section of the report deals with the human health risk assessment of DDT in the study sites.

14.2 Summary of health effects

(The following is from the US EPA's Integrated Risk Information System (IRIS))

Numerous studies have been conducted on the effects of DDT and related compounds in a variety of animal species. Most of the information on health effects in humans comes from occupational studies where exposure to DDT occurs as a result of work. Epidemiological studies of the general population are also available.

DDT, an organochlorine pesticide, is a **neurotoxin** and can cause impairment of nerve impulse conduction. Effects of DDT on the nervous system have been observed in both humans and animals and can vary from mild altered sensations to tremors and convulsions. Humans have been reported to tolerate doses as high as 285 mg/kg without fatal result, although because vomiting occurred, the absorbed dose is not known.

In addition to being a neurotoxicant, DDT is capable of inducing marked alterations on **reproduction and development in animals**. This is attributed to hormone-altering actions of DDT isomers and/or metabolites. Of all the DDT-related compounds, the *o,p'*-DDT isomer has the strongest estrogenic properties, although it is still several orders of magnitude less potent than the natural hormone, 17β-estradiol. *p,p'*-DDE, a metabolite of DDT and a persistent environmental pollutant, has anti-androgenic properties and has been shown to alter the development of reproductive organs when administered perinatally to rats. There have been studies in humans suggesting that high DDT/DDE burdens may be associated with alterations in end points that are controlled by hormonal function such as duration of lactation, maintenance of pregnancy, and fertility.

14.3 Exposure assessment

People may 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 (World Health Organization (WHO), 2003; United States Environmental Protection Agency (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) (International Programme on Chemical Safety (IPCS), 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.

14.4 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.

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 (US EPA, 1998):

$$LADD = ADD \times \left(\frac{ED}{Lft} \right)$$

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 calculation was used:

Home-grown vegetables

Water to root uptake equations (US EPA, 1997)

$$C(r) = RCF(w) \times C(w)$$

$$\log(RCF(w) - 0.82) = (0.77 \times \log(Kow)) - 1.52$$

C(r) Concentration in root

C(w) concentration in water

Proportion in Root_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ 100.00%

RCF = Root Concentration Factor

Kow = Octanol-water partition coefficient

Dermal absorption calculations (USEPA, 1992)

The following equations were used to calculate the absorbed dose of chemical per square centimetre 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:

$$DA_e = 2 \times Kp \times Cw \times \left(\left(6 \times t \times t_e \right) / p \right)^{\frac{1}{2}}$$

for $t_e < t^*$

or

$$DA_e = Kp \times Cw \times \left(\left(t_e / \left(1 + B \right) \right) + \left(2 \times t \times \left(\left(1 + 3B \right) / \left(1 + B \right) \right) \right) \right)$$

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:

$$Log\left(K \right) = 2.72 + \left(0.71 \times \log\left(Kow \right) \right) - \left(0.0061 \times MW \right)$$

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 ß:

If ß < 0.1, then t* = 2.4

If 0.1 < ß < 1.17, then t* = (8.4 + 6 log ß) τ

If ß > 1.17, then t* = 6 (b - (b-c)) τ

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.

Deriving fish concentration from water concentration (US EPA, 1986)

The following equation is used to calculate chemical concentration in the fatty tissues of fish from the concentration in water:

Cf = (Cw/Γ BCF')

where

Cf is the chemical concentration in fish.

Cw is the chemical concentration in water.

Γ is the density of water, 1 kg/l by definition.

BCF is. the bioconcentration factor.

BCF' is the BCF (actual fat content/assumed fat content).

The BCF is either obtained from databases or calculated such that

Log (BCF) = (0.79 Log (K)) - 0.40

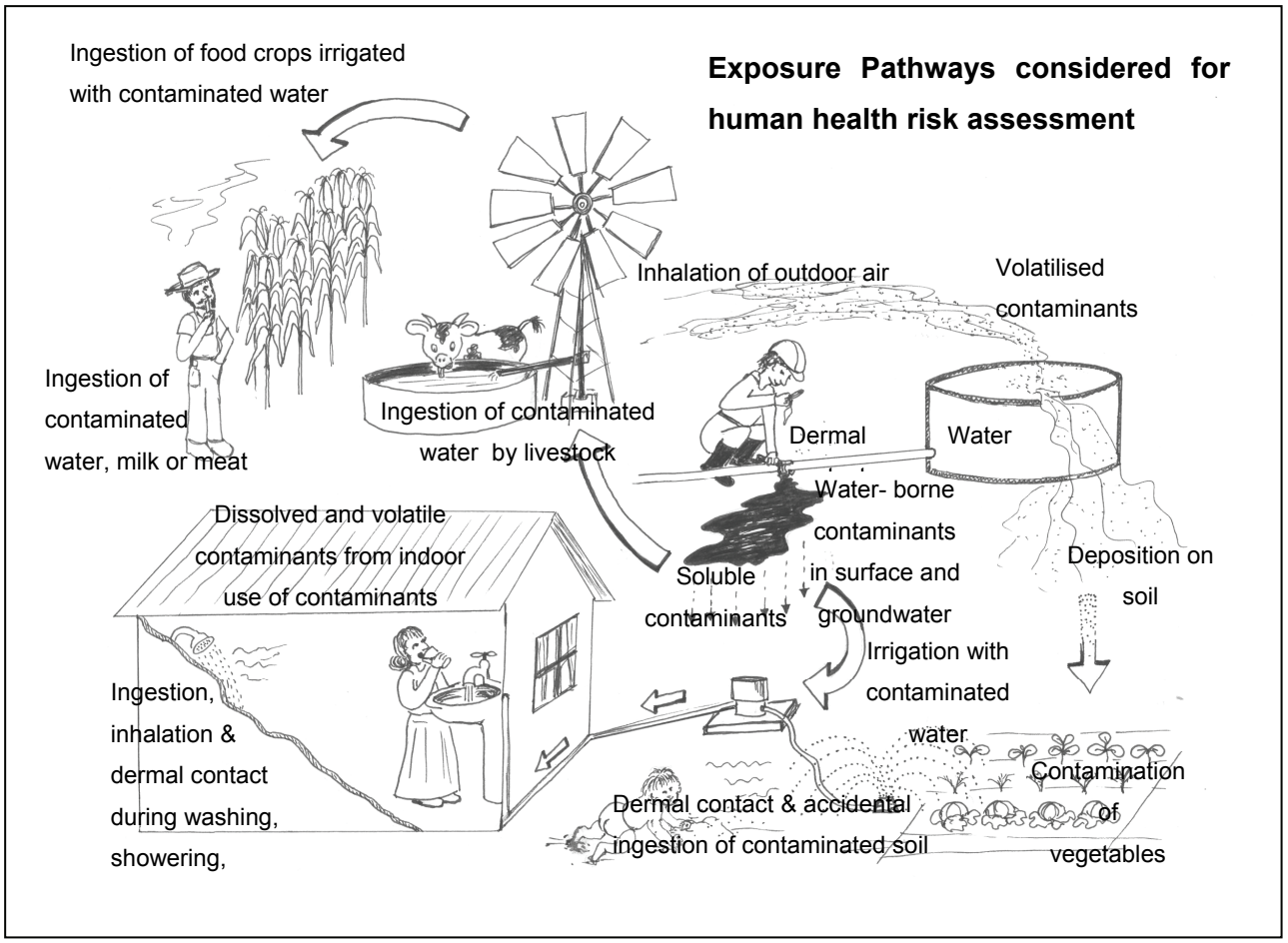

Figure 14.1: Possible exposure pathways to consider for human health risk assessment (illustration by Lisa Cavé).

14.5 Risk characterisation

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; Centre for Infectious Disease Control Netherlands (RIVM), 1997).

14.5.1 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).

14.5.2 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 (2006) 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.

Table 14.1: Health effects of DDT and metabolites (ATSDR, 2002).

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 oestrogenic 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.

14.6 DDT in the study site

The concentrations of DDT found in various media such as water food and soil are presented in Table 14.2. Mean or average concentrations were used to conduct the human health risk calculations.

Table 14.2: Concentrations of total DDT used in risk assessment.

Study site	Water µg/L		Fish µg/kg		Soil µg/kg		Vegetables µg/kg		Chicken meat µg/kg		Chicken 15% fat µg/kg	
	Median	max	Median	max	Median	max	Median	max	Median	max	Median	max
Control village					14	93	*5* ##	*9* ##	311	2110	172	2126
Albasini control dam	0.3	1.8	2.8	3.5								
Test village					23	59	42.5	150	0 Ave 285	1376	**141932**	290889
Nandoni test dam	0.1	0.3	61	110								
Xikundu test site	0.2	1.8	61	115								

hypothetical values were used as all samples were below the detection limit of 10ug/kg. A value of half the detection limit was used for the average and 9ug/kg as a maximum value as one just below the detection limit.

The US EPA methodology (1998) was used to calculate both toxic and carcinogenic risks to humans in the health risk assessment. In addition to being toxic in their own right, they are also known to have additive effects on endocrine disrupting chemicals (Kortenkamp, 2007).

As people may be exposed to chemicals via more than one carrier medium or exposure pathway this health risk assessment dealt with exposure to DDT via multiple routes, namely ingestion of either water or food, directly and indirectly through trans-media transfer, as well as via dermal exposure as a result of swimming or bathing. 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) (IPCS, 1994). Exposure parameters as relevant to the study site as possible were used when specific information was available. When this data was not available default values derived from the US EPA's exposure factors handbook were used (US EPA, 1989).

The following table (Table 14.3) summarises the exposure parameters used in this health risk assessment.

Table 14.3: Exposure parameters used in risk assessment and risk calculations.

EXPOSURE INFORMATION	UNITS	VALUES
Body weight (Bw)	**Kilogram (kg)**	**65 *(SA data)***
Life expectancy (Lft)	**Days (d)**	**70 (70 x 365)**
Exposure duration (ED)	**Days (d)**	**365 per year *(SA specific)***
Drinking water intake rate (IR)	**Litres (L)**	**1.2 *(SA specific)***
Dermal exposure rate	**Hours (h)**	**0.2 h (12min) per day based on 23000 cm^2 skin surface area**
Ingestion of soil/dust	**mg**	**100 for adult ingestion**
Vegetable exposure rate	**kg**	**Daily 40% fraction contaminated and 0.15 kg per event**
Fish and chicken ingestion rate	**kg**	**Daily 0.15kg per event per 365 days per year #**

 # Based on an averaging factor of 0.15kg fish eaten per day but with conversion of chemical content in fat to amount of fat eaten. This is based on freshwater fish having 1% fat content. For chicken, according to the literature, a 15% fat content was used.

Ingestion of drinking water was based on South African studies examining the liquid consumption of different cultural groups in South Africa (Bourne and Coetzee,1983). The remaining values were based on the US EPA Exposure Factors Handbook (1997).

The quantity of DDT and metabolites used in the exposure assessment was calculated in two ways for ingestion of vegetables and fish. The first method made use of modelled and predicted concentrations based on the concentration in water, and trans-media formulae. The second method was calculated via the consumption of vegetables or fish collected and measured for DDT content directly.

14.7 Quantitative human health risk assessment

Risks calculated for the various assumptions for the test sites are presented in the following tables (Tables 14.4 & 5) and figures (Figures 14.2, 3, 5 & 6). The figures for meat/ chicken ingestion are separated from the other assumptions to allow the distinction between these scenarios to be clearly seen.

Table 14.4: Cancer risks resulting from DDT exposure.

	Control average	Control max	Test average	Test max
Ingestion of drinking water 1.2 L	8 X10 $^{-7}$	5 X10^{-6}	3 X10^{-7}	5 X10^{-6}
Cancer risk vegetables via watering calculated using trans-media equations and Testing vegetables direct ‡	8 X10^{-5} 4 X10^{-6}	**5 X10^{-4}** 1.2 X 10^{-5}‡	3 X10^{-5} 4 X10^{-6}	**5 X10^{-4}** 1.2 X10^{-5}‡
Cancer risk – soil or dust ingestion	3 X10^{-6}	2 X10^{-5}	1 X10^{-5}	3 X10^{-5}
Cancer risk fish ingestion via calculated via trans-media equations via water and direct calculation from fish ingested	**8 X10^{-4}** 3 X10^{-6}	**5 X10^{-3}** (6 X10-^{6})‡	3 X10^{-5} 3 X10^{-6}	**5 X10^{-3}** (6 X10^{-6})‡
Cancer risk chicken meat	**4 X10 $^{-4}$**	**5 X10^{-4}**	7 X10^{-5}	**3 X10^{-4}**
cancer risk chicken fat	8 X10^{-6}	**1 X10^{-4}**	**7 X10^{-4}**	**1 X10^{-2}**
Chicken fat 80% reduction	2 X10^{-6}	2 X10^{-5}	**1 X10^{-4}**	**3 X10^{-3}**
Cancer swimming dermal absorption	3 X10^{-5}	**2 X10^{-4}**	1 X10^{-5}	**2 X10^{-4}**

‡ Risks were calculated in two ways for ingestion of vegetables and fish. The first method made use of modelled and predicted concentrations based on the concentration in water, and trans-media formulae. The second method was calculated via the consumption of vegetables or fish collected and measured for DDT content directly.

Table 14.5: Hazard indices for toxic effects of DDT.

	Control average	Control max	Test average	Test max
Drinking water 1.2 L	0.0	0.0	0.0	0.0
Vegetables via watering and calculated using trans-media equations and testing vegetables direct ‡	1.1 0.01	**6.7** 0.02	0.0 0.1	**6.7** 0.4
Soil or dust ingestion	<0.001	<0.001	<0.001	<0.001
Fish ingestion calculated via trans-media equations from water and direct calculation from fish ingested	**1.5**	**63** **6.6**	**3.5**	**63** **6.6**
HI chicken meat	1.4E+00	**7.3**	1.3	**6**
HI chicken fat	1.10E-01	1.4	**94**	**193**
Chicken fat 80% reduction	2.X10-02	0.3	**19**	**39**
Swimming dermal absorption	<0.001	**2.4**	<0.001	**2.4**

‡ The quantity of DDT and metabolites used in the exposure assessment was calculated in two ways for ingestion of vegetables and fish. The first method made use of modelled and predicted concentrations based on the concentration in water, and trans-media formulae. The second method was calculated via the consumption of vegetables or fish collected and measured for DDT content directly.

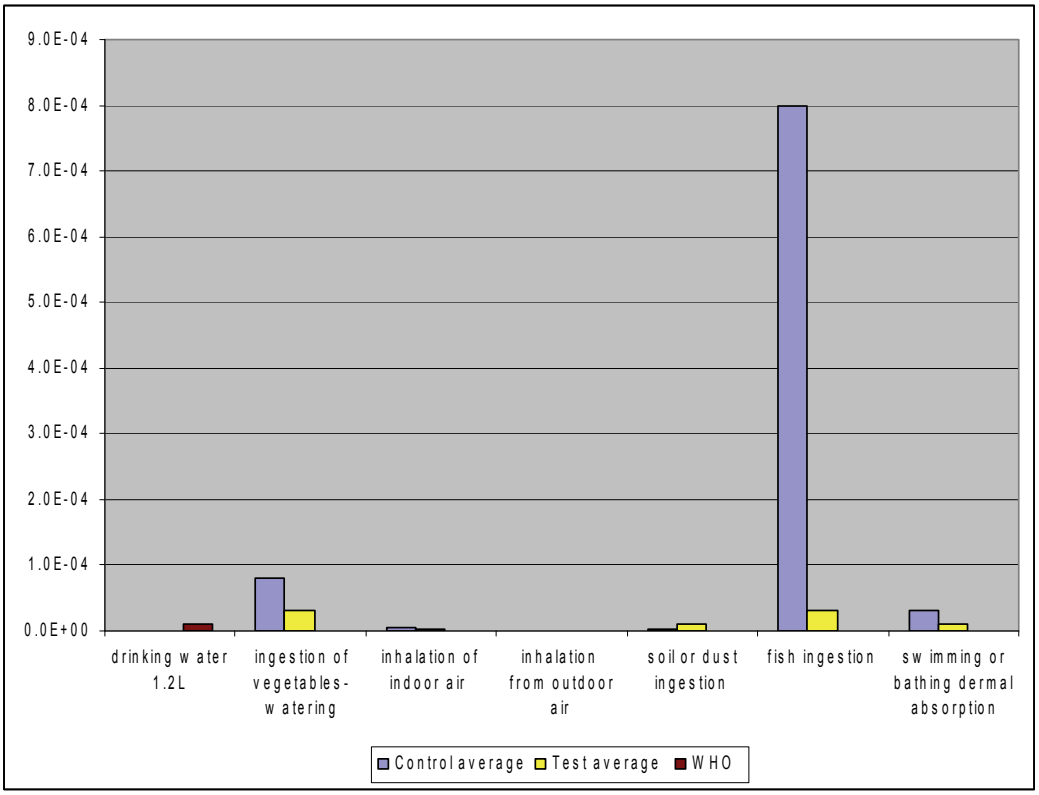

Figure 14.2: Cancer risks based on total average DDT concentrations.

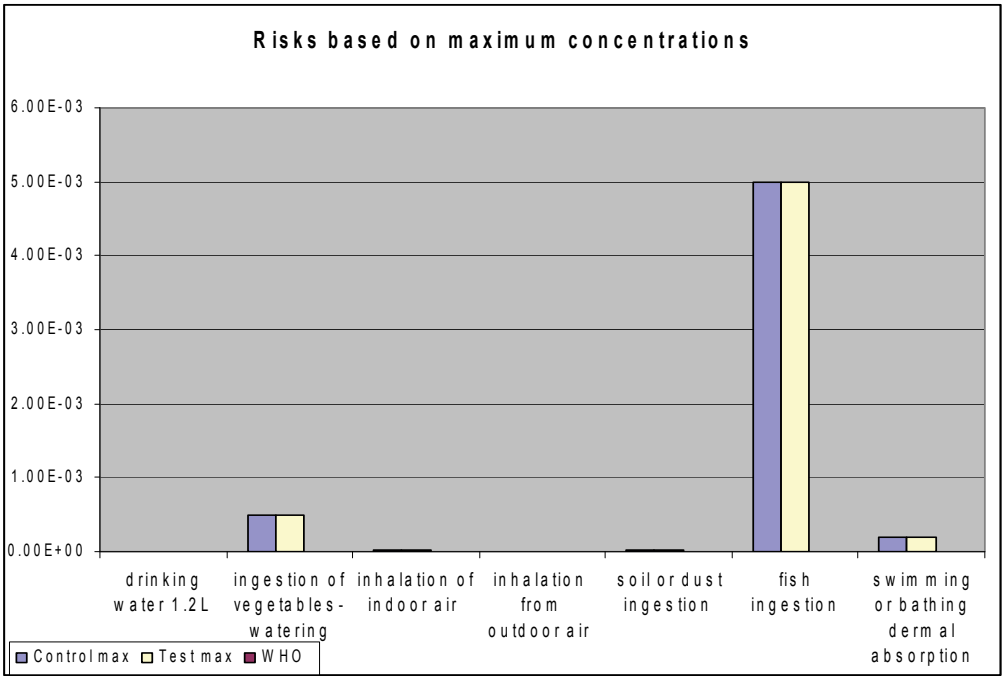

Figure 14.3: Risks based on total maximum DDT concentrations.

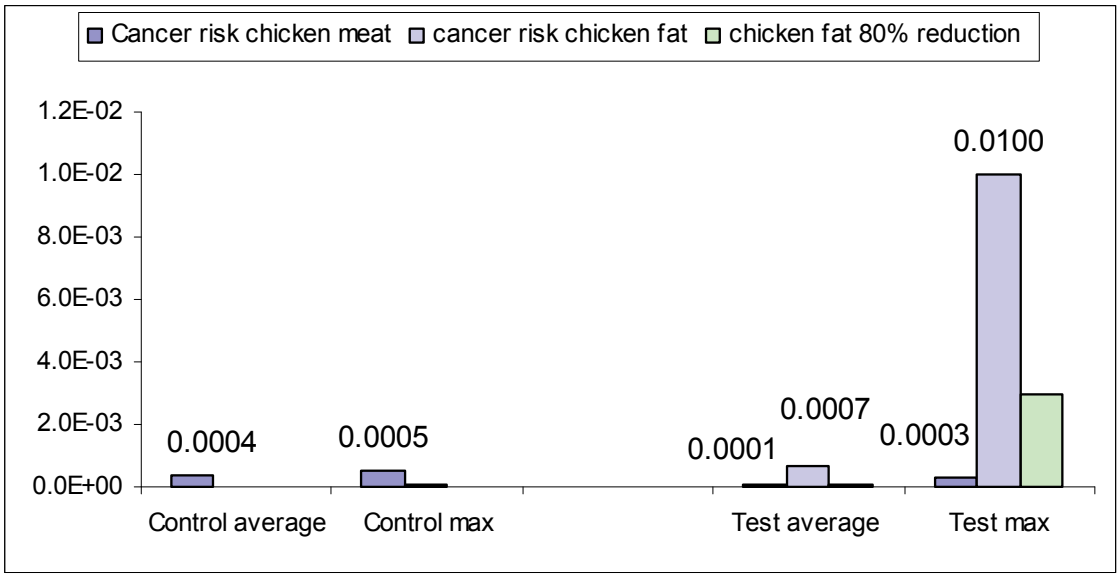

Figure 14.4: Risks based on total maximum DDT concentrations in chicken in test and control villages.

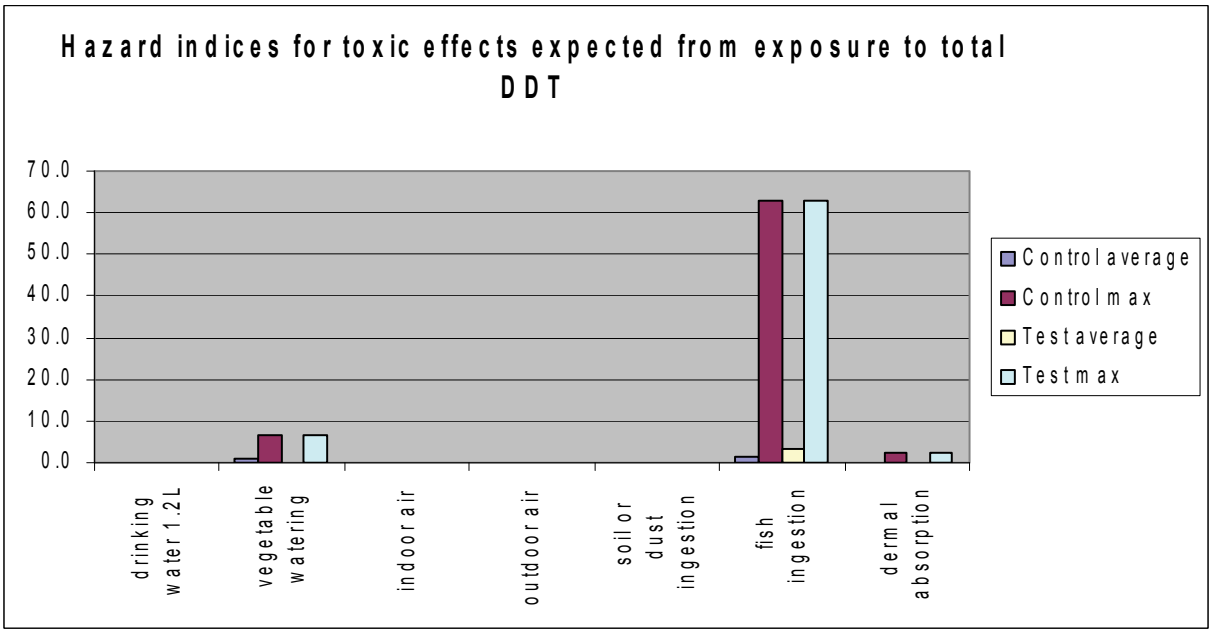

Figure 14.5: Hazard indices for toxic effects of total DDT concentrations.

(A hazard index over 1.0 is considered not to be safe for a life time exposure)

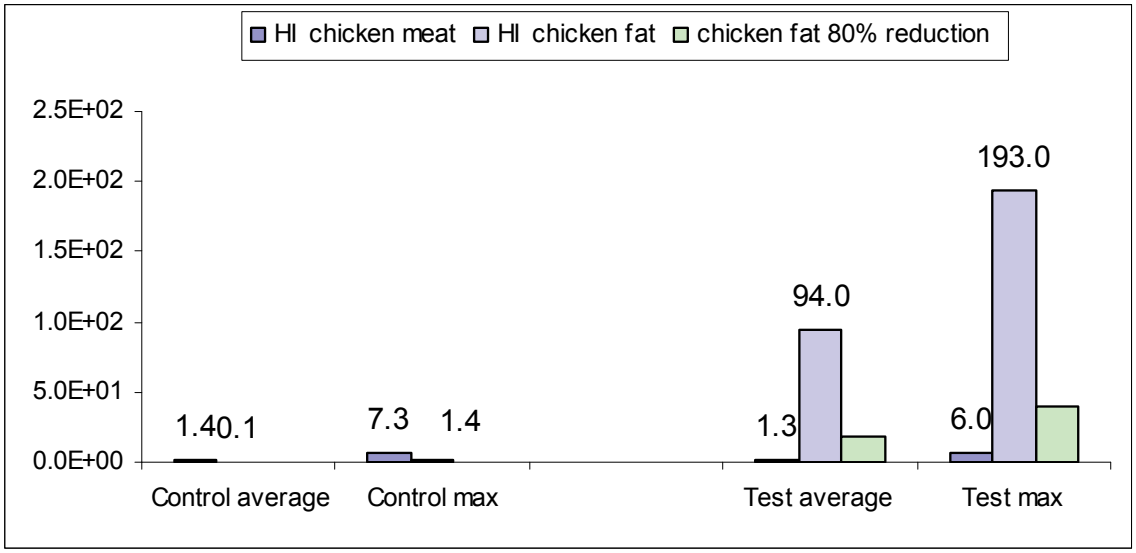

Figure 14.6: Hazard indices for toxic effects of total DDT concentrations in chicken in a test and control village.

In general, the health risk assessment indicates that the potential for serious health effects exists if people are exposed to the water and food from the test areas as well as part of the control areas.

People will be exposed to an unacceptable risk to develop cancer, ranging from 7 in 10,000 in the test village, to 4 in 10,000 in the control village, as a result of possible exposure to chicken. Additional effects in the form of toxic effects can be anticipated if they make use of the dam water. Ingestion of chicken and /or fish results in the highest risk of developing cancer and toxic effects. Carcinogenic risk from direct ingestion of drinking water is in the "acceptable" risk level according to the WHO definition of acceptable risk of 1 in 100,000 (Tables 14.4 & 5). The same was found as a result of exposure to DDT through dust and soil.

Results of uncertainty analysis using probabilistic risk assessment with @Risk Monte Carlo simulations are provided in Figures 14.7-10. This provides an indication of the results that might be expected with a 1 to 2 order of magnitude range of expected risks.

Dermal exposure to the water (assuming one is exposed on a daily basis for 12 minutes) results in risks in the range of 3 in 100,000 to 2 in 100,000, as a result of absorption of the chemicals in the water. DDT was present in concentrations individually resulting in an unacceptably high health risk. Any possible synergistic effects of other chemicals in combination with DDT could not be taken into account in the health risk assessment. The

risks calculated showed test and control dams to have similar DDT concentrations and therefore potential health risks.

Using dam water from both test and control areas for vegetable watering may expose people to a higher risk of developing cancer than the recommended 1 in 100,000 level as a result of ingestion of these vegetables (Table 14.4 & 5). DDT concentrations in vegetables in the control village were below the detection limit of 10 µg/kg. Hypothetical values were therefore used. A value of half the detection limit (5 µg/kg) was used as the average value and 9 µg/kg as a maximum value to represent a value just below the detection limit. Risks calculated using these hypothetical values resulted in very low carcinogenic risks as well as low hazard indices, indicating that there would be little reason to anticipate risks as a result of consumption of vegetables in the control village. The risk in the test village would also be low based on actual DDT concentrations in vegetables. This may be an underestimation of risk as the predicted concentrations based on trans- medium equations indicate a higher risk. This may be due to inefficient extraction methods and laboratory analytical methods. Another possible explanation may be that not as much water is used as predicted for the cross-media transfer model and the vegetables are therefore not exposed to as much DDT.

Chicken meat and fat were found to contain unacceptably high levels of total DDT, resulting in a predicted carcinogenic risk of 7 in 10,000 based on average fat concentrations in the test village. DDT concentrations in the chicken fat tested in the control village were within a range resulting in 2 in a million risk of developing cancer based on hypothetical assumptions described. No toxic effects are anticipated as a result of the DDT levels in the chicken fat found in the control village.

Literature has indicated that cooking reduces DDT in food by between 15-80% (Mes et al., 1992; Khanna et al., 1997; Wilson et al., 1998). A hypothetical value of maximum removal was used to determine whether this would allow for a reduction in DDT to acceptable levels. This unfortunately did not occur, with risks remaining at 1 in 10,000 for average concentrations and 3 in 10,000 for the maximum concentration in the test village. The risks in the control village were far lower with a cancer risk of only 2 in 1,000,000 for average DDT concentrations assuming an 80% reduction of DDT levels in the chicken fat (Table 14.4).

14.8 Uncertainty

All risk estimates involve some degree of uncertainty. Uncertainty in the health risk assessment 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. Uncertainty analyses were performed using Monte Carlo simulation making use of @Risk (Palisade) and summarised in Figures 14.7-10. These uncertainty results are not shown in detail in this document. Different distributions were used to represent the data. Larger uncertainty was seen for the contaminant concentrations in environmental media than for the predicted risks.

This health risk assessment is based on hypothetical exposure scenarios. Therefore, uncertainty in the results will be present. Uncertainty in the dose-response (carcinogenic and toxicological) data also exists. 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 the waters if used for domestic purposes and for vegetable watering.

These risks predicted are an under-representation of health risks as they only portray contact via water and ingestion of fish. Other chemicals are present and therefore the true health risk will in fact be greater than what is presented in this report.

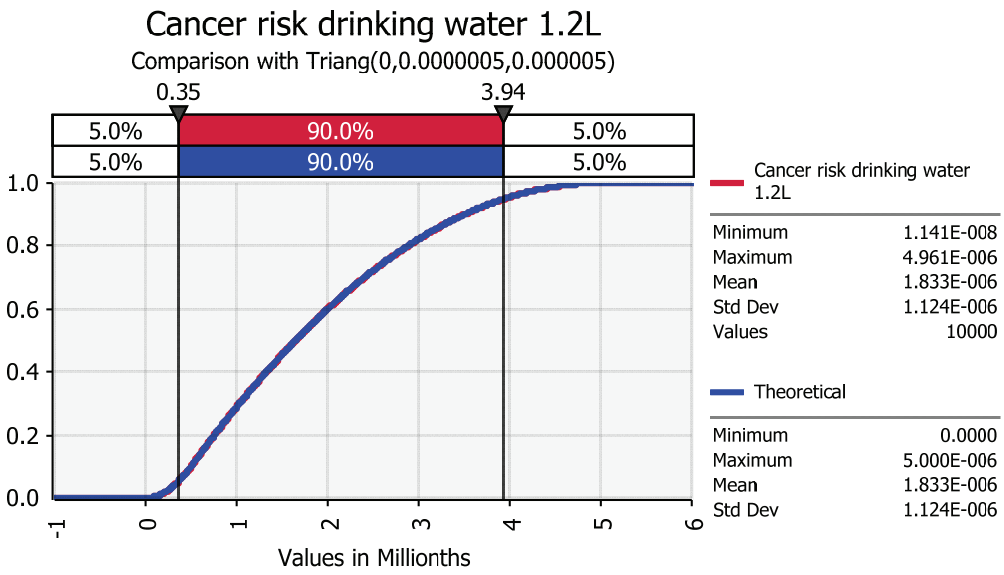

Figure 14.7: Uncertainty analysis for the risk of developing cancer as a result of exposure to drinking water – 10,000 iterations using Monte Carlo Analysis.

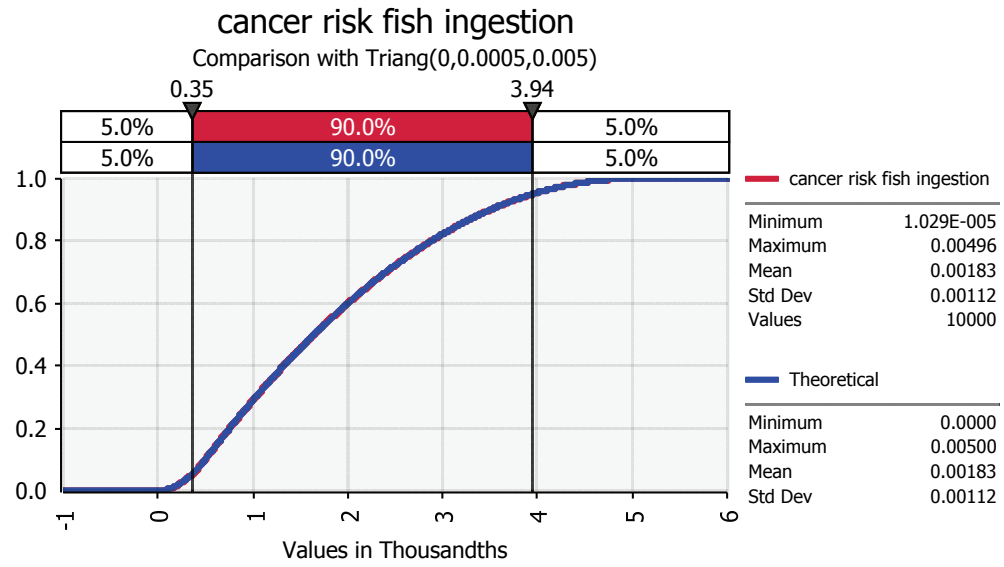

Figure 14.8: Uncertainty analysis for the risk of developing cancer as a result of ingestion of fish – 10,000 iterations using Monte Carlo Analysis.

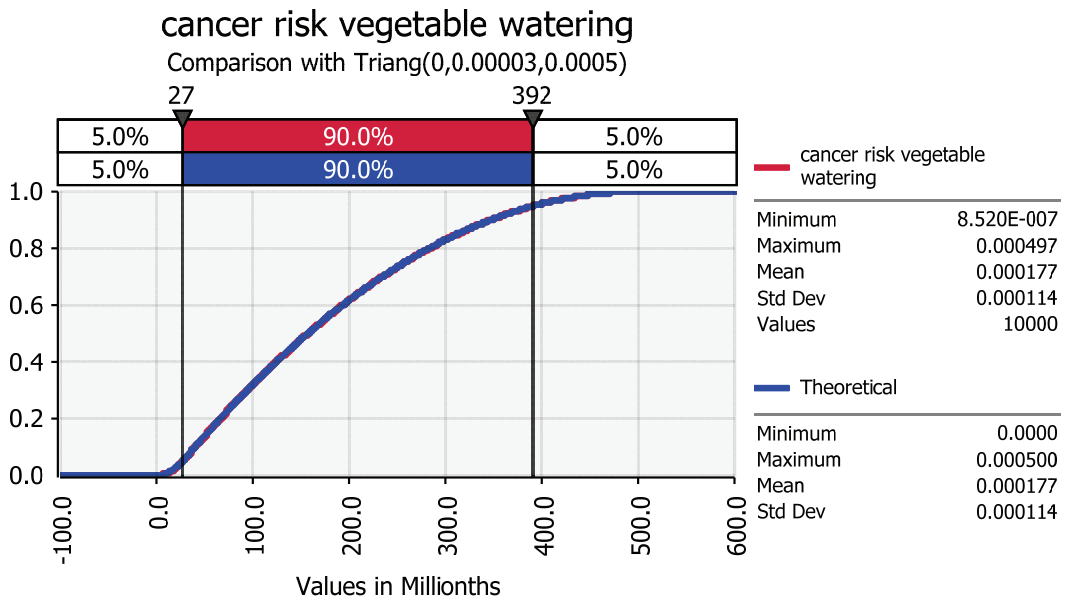

Figure 14.9: Uncertainty analysis for the risk of developing cancer as a result of ingestion of vegetables – 10,000 iterations using Monte Carlo Analysis.

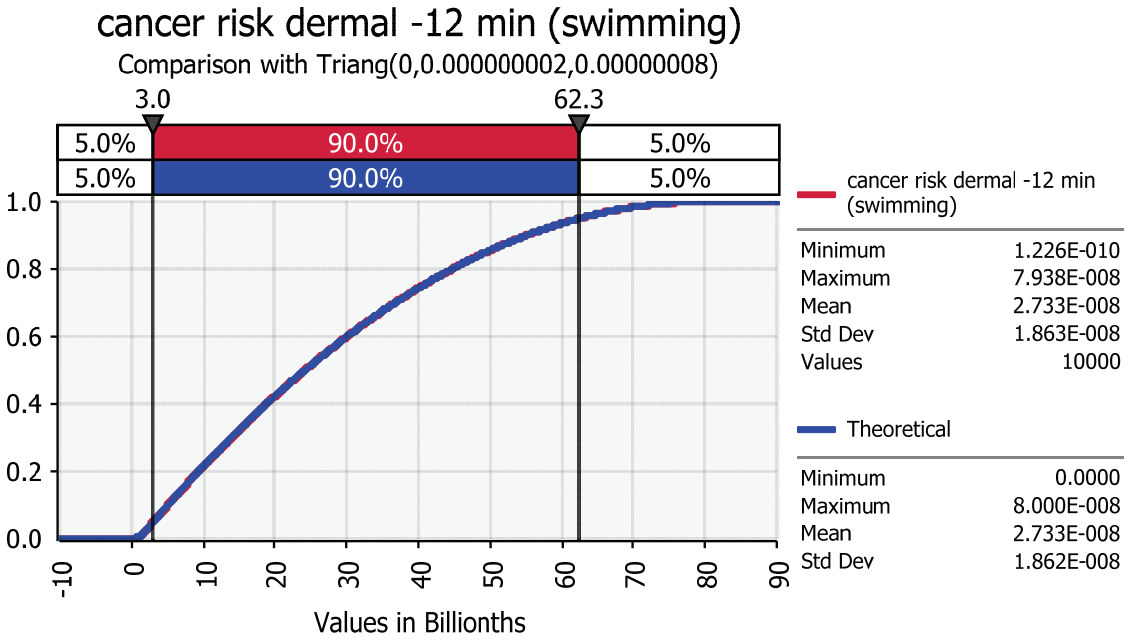

Figure 14.10: Uncertainty analysis for the risk of developing cancer as a result of dermal absorption – 10,000 iterations using Monte Carlo Analysis.

14.9 Qualitative risk assessment

Endocrine disrupting effects are of serious concern with little data available to conduct a quantitative risk assessment. It is possible to make use of qualitative data however.

A qualitative health risk assessment was conducted making use of the available data collected from the study area. Endocrine disrupting effects were observed at the cellular level with the T47D-KBluc assay detecting estrogenicity in 33% of samples, i.e., 3 of 9 samples analysed. The aquatic animals also displayed endocrine disrupting effects in catfish, with increased intersex fish, increased apoptosis, reduced sperm motility, reduced sperm counts, and macroscopic differences observed.

In addition, a cross sectional study looked at DDT and DDE levels in blood and semen samples of males living in DDT sprayed villages compared to non-sprayed villages. The investigation looked at human semen quality associated with DDT exposure in the same study area and showed that semen quality was impaired (Aneck-Hahn et al., 2007). Mean lipid adjusted *p,p*'-DDE values were found to be **215 µg/g**. In previous studies **potent anti-androgenic effects** were reported (Kelce et al., 1995). These effects were observed at **63.6 µg/g** lipid adjust DDE concentrations. One can therefore anticipate the observed semen impairment where DDE concentrations were found to be between 2- 3 times higher and assume that other anti-androgenic effects can be anticipated.

Based on the anti-androgenic effects of DDE and observed ecological effects there is a 'likely probability' that endocrine effects can be expected in a human population. One must also consider that the probability of endocrine disruption is an **additional one** 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 food as media of exposure. People are also exposed to chemicals via air and additional food and therefore the true health risk can in fact be far greater than what is presented in this report.

14.10 DDT and malaria control

Malaria is endemic in the study area and it is reported as one of the global top killers. The reduction in malaria incidence and case fatality rates has been reported to have resulted from indoor DDT spraying and led to the continued use of DDT with indoor spraying to

control malaria. The numbers report a drop from 150 per 100,000 without spraying to 50 per 100,000 with DDT spraying (or a reduction of 100 per 100,000 in case fatalities. However to look at these numbers in depth one should examine the incidence and case fatalities of local data in areas where indoor spraying was stopped with indoor spraying of DDT.

When DDT was not in use, the numbers of reported deaths per annum ranged from 100-425. This reduced to 10-30 when DDT indoor spraying was re-introduced. In over 30 years we have <60 average deaths per year over the whole country, or in the 32 years from 1971 to 2002 a total of 1715 deaths have been reported.

Table 14.6: Malaria incidence in South Africa.

YEAR	CASES	DEATHS	YEAR	CASES	DEATHS
1971	364	5	1987	10 374	10
1972	1 792	23	1988	9 317	48
1973	331	12	1989	7 055	30
1974	1 623	16	1990	6 822	34
1975	1 821	4	1991	4 693	19
1976	1 747	6	1992	2 872	14
1977	3 513	1	1993	13 285	45
1978	7 103	36	1994	10 289	12
1979	2 022	12	1995	5 991	12
1980	3 109	10	1996	11 047	42
1981	2 343	9	1997	22 052	104
1982	2 184	13	1998	26 445	198
1983	2 130	13	1999	51 535	402
1984	4 642	19	2000	61 934	423
1985	11 358	32	2001	25 337	80
1986	7 491	20	2002	3 454	11

14.11 Summary

In summary the predicted risks for both carcinogenic and toxic risks are high, with chicken and fish ingestion causing the highest risks, followed by vegetable ingestion, (where DDT levels result from DDT in water used to irrigate the vegetables). Dermal exposure also results in high risks. The lowest risk is from direct exposure to water through ingestion and highlights

the importance of examining all possible exposure routes when conducting a risk assessment.

Endocrine disrupting effects were also observed and can be anticipated in the exposed population with effects observed in both the human and animal population and at the cellular level.

CHAPTER 15
INTEGRATED, STANDARDISED SOUTH AFRICAN RELEVANT TOOLKIT MANUAL OF TESTS FOR WIDER APPLICATION IN OTHER SPRAYING AREAS

Bornman MS and De Jager C

15.1 Bioassays

Substantial effort has been put in recent years into the development of biological methods to assess the estrogenicity of water including drinking water, ground water, surface water and wastewater. Methods that rely on biological activity are finding increasing utility as screening tools, because the chemical nature of the endocrine disrupting sample may be unknown and difficult to identify, and the biological method may be the best (or only) indicator of biological activity.

The bio-assays for estrogenic activity applied in this study (the recombinant yeast estrogen screen (YES) and T47D-KBluc reporter gene assay) are included in the report for WRC project 1816: *Guidelines for EDC management in water resources: Volume 3: Bio assays and chemical analysis for EDCs.* This study aims to develop a standard operation manual, to be used and applied in other areas in South Africa. The outcome of that study will therefore also address the requirements of a currently DDT-sprayed area and recommendations will not be made here.

15.2 Monitoring human exposure

Human milk and blood have been used as markers of exposure of humans to certain pollutants. Both these human sample media can show comparable temporal trends in a particular population because they integrate environmental and dietary exposures. Milk also provides relevant information on transfer to infants and potential health effects.

Human milk is none-invasive and can be readily obtained in quantity from lactating women whose infants are considered a vulnerable population group. The levels of contaminants in maternal blood correlates well with the levels in human milk. Therefore, human milk and maternal blood can be used for monitoring on an equal basis. Maternal blood however, provides the exposure information for foetal circulation. Maternal blood pollutant levels are also likely the general driving force for human milk concentrations of POPs, but milk is the most sensitive exposure medium for the infant in terms of postnatal development.

p,p'-DDT levels in plasma represents current exposure to DDT and are usually higher following spraying. On the other hand, *p,p'*-DDE is the stable metabolite of *p,p'*-DDT and reflects chronic or historical exposure. Target chemical analysis with low detection limits of blood samples will provide useful information when specific health outcomes are being studied.

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APPENDIX:

Appendix 1

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TO WHOM IT MAY CONCERN

Illegal fishing and irresponsible/uncontrolled farming practices within the Luvuvhu River Catchment Management Area (CMA).

A fish reproductive health study was done on *Clarias gariepinus* and *Oreochromis mossambicus* from 2004 to 2008 at three sites within the Luvuvhu River CMA. This study by the University of Pretoria is funded by the WRC and entitled: "Environmental exposure and health risk assessment in an area where ongoing DDT spraying occurs".

During this four year period, the use of illegal gill and throw nets were consistently observed at various sites within the CMA. As aquatic scientists we are aware and subsequently extremely concerned about the impact of nets on the endemic fish populations. Very low fish numbers are evident in this CMA area – in one specific dam five nets were left for about 12 hours in a bay without any fish being caught.

We want to bring the following to your attention:

1) Illegal use of gill and cast nets

Throughout the sampling period from 2004 to 2008, cast nets and gill nets were a common feature along the banks of the Luvuvhu River. These sites included Hasana, upstream and downstream from the Xikundu weir, Mhinga as well as the Nandoni and Albasini dams. On enquiry, none of these

fishermen had the necessary permits for fishing. Unarguably, the use of these nets will lead to over-fishing of fingerlings and juveniles. Although some fish may be caught for subsistence purposes, most of these fishermen sell their catch. One individual (employed by the Department of Water Affairs and Forestry) informed us that he catches up to 160 fish per day and offered to sell specimens to us. These fishermen do not seem to realize the impact their actions will have on the numbers and diversity of the fish population. Cast nets were specifically prevalent at the inflow to the Nandoni Dam where the nets are used from the new bridge that leads to Dididi.

2) Xikundu weir fish ladder

Fish ladders are specifically constructed to allow fish to migrate upstream over man-made barriers within river systems. However, at the Xikundu weir, it was observed how the local children and fishermen gained access to the pools within the ladder on a daily basis. They use home-made cages and fish traps to empty the pools of the migrating fish. Thus, at the moment, the fish ladder only acts as a death trap for migrating fish and will potentially have a severe, negative impact on the fish numbers upstream from the weir (where over-fishing also occurs).

3) Feeding points

Fishermen walk around the Albasini Dam creating feeding points to lure fish only to net them in the shallow areas.

4) Crops on the river banks

Everywhere along the Luvuvhu River banks crops have been planted down to the water level. This is specifically problematic at the Xikundu weir where steep slopes create run-off of fertile soil into the water with rainfall. During the heavy rains in November 2007 and in January 2008, tons of soil was observed being washed into the Limpopo River. Not only does the soil erosion leave the farmers without land to grow crops, but create unfavourable surviving conditions to aquatic life.

5) Nature conservation and management

We regret to inform you, but nowhere was any staff member of Nature Conservation observed during the study period, neither at the dams nor along the Luvuvhu River. While we acknowledge that your staff has a vast area to cover, we are also sure that unless urgent measures are taken to restore management, not only fish diversity, but also numbers will be affected irreversibly.

Kind regards

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