Endocrine Disrupting Chemical (EDC) Activity and Health Effects of Identified Veterinary Growth Stimulants in Surface and Groundwater

Report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

INTRODUCTION

This was a multi-institutional study on a topic yet to be addressed in South Africa. The first part of the study served as a pilot where the aim was to identify the presence/absence of veterinary growth stimulants in the effluent of feedlots. This was done in consultation with Prof Heinrich Meyer (Germany), Prof Bruno Le Bisez (France), and Ms Ansie Burger (Consultant, WRC). A Geographic Information System (GIS) was used to plot the co-ordinates of the largest cattle feedlots from the 47 that are registered in South Africa. This information was then used by a geohydrologist to overlay the necessary geographic systems such as rivers, wetlands, underground water sources and soil types of the particular areas. With this information available together with a complete literature review, sample sites were identified. Both groundwater and surface water were considered as possible collecting points.

The main study consisted of six phases. Phase 1 included the collection and chemical analyses of samples, Phase 2 and 3 the bio-assay for endocrine disrupting activity in the water samples and identified veterinary compounds, respectively. Phase 4 was a reproductive toxicology study on rats using the identified mixtures of growth stimulants and Phase 5 was the aquatic toxicology studies. Phase 6 was added to the project and investigated the impacts of cattle feedlot activity and the use of growth stimulating hormones on aquatic ecosystems outside the feedlots. Independent samples were collected for Phase 6.

RATIONALE

In South Africa, at national level, the focus of health authorities is on acute conditions and infectious diseases. Most environmental risk research over the last 30 years has attempted to establish causal links between putative contaminants and a range of health effects. In all of these instances, the major problems were to achieve sufficient individual exposure and outcome data to provide definitive answers. It is, however, becoming increasingly obvious; both in developed and developing nations, that chronic conditions, especially those caused by exposure to environmental chemicals will constitute a greater threat to human health in the future. Humans, like other forms of life on earth, are dependent upon the capability both of local ecosystems and of the global ecosphere for maintaining health.

Reports of the endocrine disrupting potential of common environmental contaminants and the effects on reproductive health, e.g. increasing breast and reproductive tract cancers, reduced fertility, and in sexual development, are common in the literature. Endocrine Disrupting Chemicals (EDCs) are also linked to impaired intellectual and childhood development and psychological effects. Current research is reaching consensus on the observation that the focus on cancer as the main outcome of environmental health enquiry has severely limited the understanding of environmental health.

Instead, it is now clear that, other major effects specifically mediated through EDCs, are on the foetus, and that these effects may be trans-generational.

Health effects associated with EDC exposure are not limited to reproduction. Neurological effects have been suggested as a result of EDC exposure as the brain is one of the sensitive sites of action of steroids *in utero*. Immunotoxic responses may also occur when the immune system acts as a passive target of chemical insults, leading to altered immune function.

A number of hormones are involved in endocrine regulation of growth. In general, these hormones enhance body protein accumulation and metabolise fat stores resulting in increased growth rates. Efficiency of growth promotion ranges between 0% to 20% depending on the prerequisites like species, breed, gender, age, reproductive status, and body score or feeding of the animals. Estrogens and androgens mediate their activity via intracellular receptors – directly in muscular tissue as well as indirectly via stimulation of growth hormones from the pituitary and other growth factors from liver plus several other organs.

Humans are exposed to these chemicals through food and water, as these EDCs end up in our limited water resources. Indirect environmental exposure to veterinary growth stimulants might potentially pose a health risk. Very little is known on this topic in South Africa.

AIMS AND OBJECTIVES

Phase 1

1. To identify monitoring points and the presence/absence of veterinary drugs in the effluent of feedlots through a Geographic Information System (GIS) and information obtained of drug use in South Africa.
2. To identify growth stimulants used in South Africa, and identification of drugs to be tested through a proper literature study and consultation with international experts.
3. To collect samples at three different feedlots at a particular point and to identify growth stimulants used in South Africa through chemical target analyses.

Phase 2

To screen water sources close to selected feedlots in South Africa for estrogenic and androgenic activity, using bio-assays.

Phase 3

To determine the endocrine disrupting (estrogenic and androgenic) activity of growth stimulants, as identified in Phase 1, by using a battery of screening bio-assays.

Phase 4

To do a reproductive toxicology study, using the rat model, on the identified mixture of compounds, at environmentally relevant concentrations.

Phase 5

To perform a toxicological study on sharptooth catfish, *Clarias gariepinus* and *Oreochromis mossambicus* in an aquarium after exposure to sub-lethal concentrations of veterinary compounds originating from livestock production units, investigating the gonadosomatic index (GSI), histology of the testes and papilla (C. gariepinus only) and gametogenesis in both species and to determine blood hormone levels of 11-ketotestosterone and estradiol and plasma VTG.

Phase 6

To investigate the impacts of selected cattle feedlots on aquatic ecosystems at different sites. Samples were collected independently outside the cattle feedlots at different sites than for the other Phases.

PHASE 1: SAMPLING AND CHEMICAL ANALYSIS

The original project plan was designed to conduct a survey of ground and water samples of a large number of feedlots. During Phase 1 of the project it became apparent that each feedlot has a unique geographic design and virtually no similarities could be drawn between the different feedlots. In addition to this, the feedlots in South Africa are all privately owned and they were erected over a large number of years. The environmental legislation of South Africa was changed and the impact of "newer" feedlots on the environment was assessed with environmental impact (EIA) studies. The endocrine disruptive effects of synthetic and natural hormones on the environment are however not part of the current EIA requirements. As a result of these facts and minor geographic discrimination between the feedlots, it was concluded that each feedlot must be assessed as a unique unit and cannot be included as in a generic model.

During the study, water was collected from 44 sampling points in three different feedlots, over three different time periods. Samples collected ranged from boreholes within the feedlots, settling ponds, surface water and water from the closest river. The samples were analysed with an AB 4000 QTRAP LC-MS/MS system. The analytes covered in the study included both natural and synthetic hormones that can be expected to be present in and around feedlots in South Africa. Only a limited amount of metabolites were covered and no conjugated compounds were covered in the chemical analysis. The detection limits reached during the study was well within the international accepted limit of 10 ng/ℓ.

Low levels (below the detection limit of 10 ng/ℓ) of estrone, ethinylestradiol, diethylstilbestrol and testosterone were detected a few water samples. In 42% of the sites, concentrations of zilpaterol were detected. The concentrations found ranged from 69 ng/ℓ to the highest concentration of 23 703 ng/ℓ. The significance or impact of these concentrations found in the different water sources are however not clear since no other literature could be found that evaluated the possible estrogenic effects of zilpaterol.

Until such a time that more information is available on the possible endocrine disruptive effects of zilpaterol, no clear conclusions can be drawn towards the possible short and long term effects of this compound on the environment.

PHASE 2: EDC ACTIVITY OF SELECTED WATER SAMPLES IN AND AROUND SELECTED FEEDLOTS USING A BATTERY OF BIO-ASSAYS

In-vitro bio-assays have been widely used as screens to determine if specific compounds or environmental samples for example water or sediment have endocrine disrupting activity. In this study the recombinant yeast estrogen screen (YES) were used, as well as the T47D-KBluc reporter gene assay for estrogenic activity and the MDA-kb2 reporter gene assay or (anti-) androgenic activity in the water samples.

The results from the bio-assays indicated that compounds with estrogenic activity (0.02 ng/ℓ to 2.57 ng/ℓ in the T47D-KBluc assay) were present in water samples from the selected feedlots. This activity may not be solely attributed to the veterinary growth stimulants as other sources of estrogenic contamination may be present in the vicinity of the sampling point. Cytotoxicity was found in seven of the eleven samples tested and four samples were positive but not quantifiable. Four of the samples that were below the detection limit of the YES assay tested positive for estrogenic activity (0.04-2.57 ng/ℓ) in the T47D-KBluc assay. This could be explained by the fact that the yeast cells contain only the ERα, but the T47D-KBluc cells contain both the endogenous ERα and Erβ, making the T47D-KBluc assay more sensitive for estrogenic activity compared to the YES assay. The absence of androgenic activity measured in the MDA assay might be attributed to the complexity of the samples and also the fact that the pH was not adjusted (pH 3) prior to extraction. At present the US Environmental Protection Agency (EPA) is investigating an alternative assay for measuring androgenic activity in environmental samples. These results support the GWRC recommendations for the use of a battery of bio-assays to determine EDC activity in environmental samples.

PHASE 3: EDC ACTIVITY OF INDIVIDUAL VETERINARY GROWTH STIMULANTS USING A BATTERY OF BIO-ASSAYS

Individual growth stimulants selected from Phase 1 of the project were tested for EDC activity, using the same battery of bio-assays as in Phase 2 of the project. Diethylstilbestrol, α-zearalanol and estriol had the highest estrogenic activities.

β-Trenbolone, androstanolone, zilpaterol and epiandrosterone showed much weaker estrogenic activity (relative potency between 0.004% and 0.006%), but were still able to obtain full dose-response curves. Testosterone, methyltestosterone, androstenedione, progesterone and medroxyprogesterone were however not able to reach the maximum response obtainable by E_2 at the tested concentrations. The RP of testosterone is 0.001%; therefore a 100 000x higher concentration is necessary to reach the EC_{50} compared to E_2. The maximum estrogenic activity obtainable with testosterone is only 77% of the activity obtained with E_2.

In the T47D-KBluc reporter gene assay estrone had the highest estrogenic activity (RP = 49%), followed by diethylstilbestrol, estriol and α-zearalanol. Epiandrosterone, zilpaterol, androstanolone and testosterone had weak estrogenic activities with androstanolone and testosterone not being able to reach

maximum activity obtainable with E_2. Possible anti-estrogenic activities were detected in medroxyprogesterone acetate, methyltestosterone, nandrolone, progesterone and β-trenbolone.

In the MDA-kb2 reporter gene assay androstanolone, nandrolone, β-trenbolone, testosterone, methyltestosterone and medroxyprogesterone acetate had the highest androgenic activities with RPs above 29%. Androstenedione had a slightly weaker androgenic activity with a RP of 0.8%. E_2, epiandrosterone, estrone and progesterone were not able to reach maximum activity obtainable with dihydrotestosterone (DHT) at the tested concentrations and their RIE were all below 65%. Diethylstilbestrol, estriol, α-zearalanol and zilpaterol did not react in the MDA-kb2 assay and no anti-androgenic activity was detected in any of the samples.

PHASE 4: REPRODUCTIVE TOXICITY IN THE RAT MODEL

Based on the compounds detected in Phase 1 and the subsequent biological activity in Phase 3 of the study the following compounds were identified for use in this phase of the project, zilpaterol, diethylstilbestrol (DES), α-zearalanol, ß-trenbolone, and methyltestosterone. The dosing concentrations were decided according to the detection limit of the compounds in Phase 1 with an additional 20% added, in order to make the dosage environmentally relevant. It was decided to group the chemicals as an Estrogenic group (Group 2), an Androgenic group (Group 3) and a Combination of all the compounds in Group 4. Group 1 was the Control and Sprague-Dawley rats were used in the modified OECD 415 protocol for Reproductive Toxicity Studies.

No statistical differences between the control group and experimental groups for the mean body mass, total testicular mass, mean epididymal mass and mean liver mass were found. A statistically significant difference for the mean anogenital distance between the control group (40.900 mm) and group 3 (38.167 mm; $p = 0.0117$) were found.

The mean seminal vesicle mass for group 4 (0.6271 g) was lower ($p = 0.0074$) than the control group (0.7488 g). The mean prostate mass for group 4 (0.7789 g; $p = 0.0151$) was also statistically significantly lower than the control (0.9277 g). The total sperm count showed a statistically significant difference between the control group (57.036×10^6) and group 3 (41.083; $p = 0.0337$), with a lower sperm concentration in group 3.

A statistically significant difference for the T4, between the control group (64.395 nmol/ℓ) and group 2 (74.190 nmol/ℓ; $p = 0.0089$), control group and group 3 (74.464 nmol/ℓ; $p = 0.021$), with a higher T4 in group 3 and 4 compared to the control group, were found. No differences in the T3 for the control group and any experimental groups were found.

A statistically significant difference in the total lumen diameter of the seminiferous tubules was found between the control group and group 3 ($p = 0.0455$), and between the control group and group 4 ($p = 0.0289$). There was no difference between the control group and experimental groups for the seminiferous tubule diameter and epithelium thickness. Histological evaluation of the testes indicated that

all 14 stages of spermatogenesis were present. Some histological abnormalities, such as vacuolization, were observed and in group 3, two rats had immature germ cells present in the lumen.

The data from this study indicates that veterinary growth stimulants did have an influence on reproductive and thyroid parameters in maternally and directly exposed male rats. This is cause for concern as essentially these compound mixtures end up in the aquatic system.

PHASE 5: FISH TOXICITY STUDY

The objectives were to expose the partial lifecycle of two species, *C. gariepinus* and *O. mossambicus* to the selected concentrations of steroid hormones, estrogenic compounds and a mixture of these (similar to Phase 4). The endpoints included (1) mortality, hatching time and success of the lifecycle stages (2) macroscopic observations and (3) histological analyses of the dead life stages. Juveniles were investigated after three months' growth for gonadosomatic index (GSI), histology of the testes and papilla and gametogenesis in both species and to determine blood hormone levels of 11-ketotestosterone and estradiol and plasma VTG. The dose selection was similar as for the rat study (Phase 4).

The partial life cycle of *O. mossambicus* was exposed to an estrogenic, androgenic and an estrogenic-androgenic mixture. The estrogenic mixture included *trans*-diethylstilbestrol (DES)(0.24 µg/ℓ) and α-zearalanol (2.4 µg/ℓ) while the androgenic mixture consisted of β-trenbolone (12.0 µg/ℓ) and methyltestosterone (MT) (6.0 µg/ℓ). The estrogenic-androgenic mixture consisted of a mixture of the androgens and estrogens listed above. For preparation of stock solutions, chemicals were dissolved in 96% ethanol. Here was insufficient zilpatrol and therefore it was not included in the estrogenic mixture.

During all the exposures and as well as in the control/solvent control eggs/free swimming larvae/juveniles died on a daily basis and some of these deaths could be as result on natural causes. In every natural breeding cycle of these species there seems to be a variation in the speed of growth/development in the same and in different clutches as well as a number of non-survival juveniles/eggs. Apart from the toxicological factors most developing aquatic organisms have predisposing factors that can cause deformities and abnormal growth/development. These include environmental factors, hereditary material, trauma and infections. The only dead eggs were however observed during day one of the estrogenic exposure whereas the only other early deaths were observed in the mixture exposure.

The androgenic exposure group day 28 juveniles were bigger than the rest of the surviving fish at the same age from the different exposure groups. The androgenic exposure consisted of β-trenbolone (12.0 µg/ℓ) and MT (6.0 µg/ℓ) from which trenbolone is a steroid used by feedlots/veterinarians on livestock to increase muscle growth and appetite. Trenbolone has a high affinity for binding to the androgen receptor and once this drug is metabolized it stimulates the uptake of nitrogen in muscle and consequently an increase in protein synthesis, therefore an increase in fish size. Although MT is not as potent an androgen as the synthetic steroid methyldihydrotestosterone it also enhances muscle growth and masculine behaviour. During this study no signs of spinal deformities were observed in the androgenic exposure.

Usually when fish are exposed to androgens such as MT it biased a greater male population, but this was impossible to evaluate during this study as there were only 3 fish left (Control). In female fish however exposure to trenbolone has resulted in reduced plasma steroids and VTG protein levels and it caused masculinisation of secondary sex characteristics.

The anatomical abnormalities and deformities were mainly observed in the estrogenic (28%) and mixture (20%) exposures. The estrogenic mixture that included *trans*-diethylstilbestrol (DES) (0.24 µg/ℓ) and α-zearalanol (2.4 µg/ℓ) seemed to be quite potent to the developing fish. The most prominent finding in this exposed group was spine deformities and the slow growth rate of the different life stages.

The mortality rate of the juveniles from all the groups especially those of the estrogenic and mixture exposures could also have been as a result of feeding difficulty due to developmental abnormalities of the oesophagus leading to starvation. This could not been established using histology.

A few recommendations are proposed to future exposure studies in this regard:
Firstly, the routine methodology used in the exposure system can be refined to have optimum conditions for the exposed fish to grow in. This is necessary as most of the control fish also died before the markers such as histology, VTG and other reproductive endpoints could be measured. Secondly, to establish a certain result of the effects of these chemicals in future, it is necessary to expose clutches with a higher number of eggs to ensure more juveniles for comparable analyses. Also there would a higher number of 3 month old fish available for further analyses such as the presence of intersex, VTG and other reproductive endpoints. Thirdly more endpoints of the exposed juveniles should be measured before the preparation thereof, to have a better projected result that's not based on visual evaluation.

PHASE 6: IMPACT ON AQUATIC ECOSYSTEMS

With regards to the present ecological state (PES) of upstream and downstream sites the following conclusions can be made:

- The periodical increases in the conductivity and pH of downstream sites associated with feedlots are probably caused by effluent from the feedlots. The most marked changes are observed in the river associated with Feedlot A. These observed changes show increases during the high flow survey, indicating a mobilisation of nutrients and not a dilution. No other potential sources of effluent were observed in the area that could affect the water quality of the streams associated with the feedlots.
- Metal concentration, with the exception of Pb, does not indicate seasonal or site perseverance and is subsequently not the result of feedlot activity. Pb concentrations, however, are periodically higher downstream from feedlot activity than upstream. With the exception of Mn, all metal concentrations are at levels of concern and much higher than the TWQR for aquatic ecosystems.
- SASS5 scores do show some changes at downstream sites with a smaller diversity and lower sensitivity scores. This was especially true during the high flow assessment.

Macro-invertebrate Community Structure

With regards to the macro-invertebrate community structure and composition the following conclusions can be drawn:

- Macro-invertebrate community structures (diversity and abundances) do differ at upstream and downstream sites associated with feedlot activities.
- At the same time clear seasonal differences can also be observed.
- Further interpretation is needed to ascertain which macro-invertebrate community structures are prominent downstream and which water quality variables are driving the compositions.

Biomarkers

As far as the biomarkers, for growth hormone exposure, are concerned. The following can be concluded:

- There is a significant difference in the CEA of the group exposed to trenbolone acetate, compared to that of the Diethylstilbestrol exposure group. Trenbolone acetate exposed fish utilised more of their allocated energy suggesting a negative stress response.
- The CEA of the diethylstilbestrol exposure group increases over the time of the exposure, indicating increased lipid and protein production in these fish. Suggesting a typical oestrogen exposure response.
- The application of metabolomics as a functional biomarker was done with some success. A dose response was obtained after exposure to a single concentration of both growth stimulants at set intervals. The exposure to Diethylstilbesterol caused greater response both after 5 and 10 days of exposure. This was not the case with the Trenbolone acetate group, which reflected a slight sub-cellular response after 5 days of exposure. Furthermore the application of metabolomics aided in determining which metabolites are responsible for the sub-cellular response observed between exposed organisms. An increase in the exposure concentrations of diethylstilbesterol and trenbolone acetate would probably provide more marked effects.

CONCLUSION AND RECOMMENDATIONS

This is the first study in South Africa, looking from an EDC perspective on water, at the possible impact of growth stimulants used at cattle feedlots. The sample sites selected might not necessarily be representative of other feedlots as compounds use, the management of the feedlot, and the geographical landscape may differ between sites. The time and number of sample collection may also affect the outcome.

Several challenges were experienced with the preparation of collected samples and the technique development of analytical methods. As most of the identified growth stimulants used in South Africa was found to be below the detection limit in the collected samples, there is a clear need for refinement of methodology and analytical techniques. Zilpaterol tested positive in most of the water samples.

The combination of different bioassays for estrogenic activity in the selected feedlot samples seems to be successful and detecting such activity in most of the samples. The estrogenic activity could be quantified in most samples where there was no cytotoxicity.

No androgenic activity could be measured in the collected samples. This might be because of the half-life of the compounds. Metabolites should be tested in future studies.

The bioassays were also successfully applied to measure the estrogenic and androgenic activity and potency of the selected growth stimulants used in South Africa.

Unexpectedly, the identified mixture of relevant growth stimulant at environmental concentrations had effects on reproductive endpoints in laboratory rats. The animals in the androgenic mixture group and the total mixture of androgenic and estrogenic compounds were the most affected. Not only were body mass affected, but also ano-genital distance, sperm concentration, and the histological parameters of the testes.

In addition to the rat toxicology, effects were seen in life stages of the exposed fish species. The mortalities in the estrogenic mixture and the total mixture of compounds were earlier in the development after exposure. Furthermore the spinal deformities and delayed growth were also much higher in these groups than in the androgenic group and compared to the controls.

Although the site and sample collection for the study on the impact of growth stimulants on aquatic ecosystems were different than for the rest of the study, the results give an indication of ecological impact associated with exposure. Macro-invertebrate community structures (diversity and abundance) do differ from upstream and downstream sites associated with feedlot activities. Seasonal differences were also observed.

Evidence and observations from this study suggest a proper investigation of cattle feedlots regarding the use of EDCs and other veterinary compounds as there is reason to suspect an impact on surface and groundwater. Currently no environmental impact studies are done for registration of EDCs. It is suggested that monitoring should become part of the licencing process of these potentially harmful compounds.

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PHASE 1

Endocrine Disruptive Chemical (EDC) Activity and Health Effects of Identified Veterinary Compounds in Surface and Groundwater

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LIST OF ABBREVIATIONS

APCI	Atmospheric Pressure Chemical Ionisation
BSTFA	N,O-bis(trimethysilyl)trifluoro-acet-amide
DCM	Dichloromethane
DES	Diethylstilbestrol
DTE	Dithiothereitol
E1	Estrone
E2	17β-estradiol
E3	Estriol
EDC	Endocrine-Disrupting Compound
EE2	17α-ethinylestradiol
EI	Electron Impact
ESI	Electrospray Ionization
GC	Gas Chromatography
GC/MS/MS)	Gas Chromatography/Mass Spectrometry
GIS	Geographic Information System
HPLC	High-Performance Liquid Chromatography
LC/MS/MS)	Liquid Chromatography/Mass Spectrometry (tandem)
LOD	Limit of Detection
m/z	mass-to-charge ratio
MeOH	Methanol
MRL	Maximum Residue Levels
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MSTFA	N-Mehtyl-n-trimethylsilyl-trifuoracetamide
MTBE	Methyl Tert-Butyl Ether
ND	Not Detected
NI	Negative Ionization
RP-HPLC	Reversed Phase-High-Performance Liquid Chromatography
Rt	Retention time
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
TIC	Total Ion Chromatogram
TMIS	Trimethyliodosilane
TMS	Trimethychlorosilane

1.1 INTRODUCTION AND OBJECTIVES

The discovery, production and medicinal use of pharmaceuticals, synthetically derived or extracted from natural materials, are one of our society's greatest medical assets. Human and animal health has been improved substantially by the introduction of antibiotics, analgesics, and condition-specific formulations for heart disease, hypertension and other episodic and chronic health problems. An unsurprising but unintended consequence of the universal use of pharmaceuticals has been the introduction of these compounds or their metabolites into surface water and groundwater (Editorial, 2000; Matthiessen et al., 2006). Apart from human excretion of metabolised and unmetabolised drugs passing into the sewage or septic systems and leaching into surface and groundwater, direct discharge of excretions by livestock can be a potential source of contamination.

The cattle lot feeding industry in South Africa is an important sector of the domestic beef industries. More than 75% of all beef produced in South Africa originates from feed lots. It delivers all year round production of a product of consistent quality which is readily accepted by its customers, and is an important value adding component to both the beef and grains industries. Feedlots are important influences on regional economies as well as the economy generally. In general, the number and size of feedlots across South Africa is increasing. With this increasing intensity come the increasingly important duty to ensure minimum interference with the enjoyment of life and property outside the feedlot, and the duty to prevent adverse impacts on the environment.

If one considers the definition of a feedlot: namely a confined yard area with watering and feeding facilities where cattle are held and completely hand or mechanically fed for the purpose of production, one should also look at the impact of the feedlot on the surrounding environment. The main aim of this phase of the project is to determine the concentration of natural and synthetic hormones in water sources in and around feedlots.

1.2 IDENTIFICATION OF THE MARKERS TO BE TESTED DURING THE CHEMICAL ANALYTICAL PROCESS

In South Africa the use of both growth promoters and growth stimulants are legal and are used in combination with other feed additives to enhance the growth of cattle. Various compounds are registered for use such as REVALOR (Trenbolone), Compudose (Estradiol), Zilmax (Zilpaterol) and Zeraplix (Zeranol). These growth hormones are normally administered as an injection behind the ear (see Figure 1.1) of the cattle or in the case of Zilmax as an additive in the feed. However, the use of synthetic growth hormones, the concentration of natural hormones excreted by cattle in water must also be considered as a potential source of contamination of natural water.

Figure 1.1: Hormone application directly behind the ear of the cattle

Thus, to identify the hormones (both natural and synthetic) that should be included in the study, a variety of factors were considered. These include: metabolism of the hormones, excretion pathways, environmental stability, and the availability of an analytical methodology and the technical capabilities of laboratories in South Africa. To assist with this process, two international experts where consulted and Workshops were held with them, respectively.

Workshop 1: Prof Dr Bruno le Bizec (from France)

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The Workshop was held at the end of June 2007 with Prof Le Bizec to discuss the different analytical techniques that can be used to identify the different growth stimulants. The following list of compounds, as mentioned in the project proposal, was discussed:

Synthetic estrogens:

- Diethylstilbestrol (DES), ethynylestradiol

Androgenic steroids:

- Trenbolone acetate, nortestosterone, methyltestosterone,

Natural hormones:

- 17β-estradiol, testosterone, progesterone

Thyrostatics:

- Thio-methyl and propyl-uracil

Estrogenic action:

- Zeranol

B-agonists:

- Clenbuterol, salbutamol, mabutamol, zilpaterol

It was decided to exclude the following drugs due to the fact that they are either not registered and/or used in South Africa:

Total group of thyrostatic drugs
Ethynylestradiol (only used by humans)
Salbutamol
Mabutanol
Zilpaterol – is a receptor II drug (the importance in this study, compared to the reactivity in terms of an EDC activity of some drugs such as DES, was questioned).

After consultation with the veterinarian, Prof Heinrich Meyer and the management of feedlots, the list was finalised as summarised in Table 1.1.

Table 1.1: Potential drugs that was identified to be included in the study

Group name	Identification	Individual compounds	EDC activity
Stilbenes	Synthetic estrogen	Diethylstilbestrol (DES)	ESTROGEN
Natural hormones	Natural estrogen	Estradiol (E2), 17α and 17β Estrone (E1)	ESTROGEN
Testosterone	Natural hormone	17α Testosterone 17β Testosterone Epiandro-sterone Etiocholanolone (DHEA) 5α-androstane-3β, 17α-diol Androstaniol Stanolone Androstenedione	ANDROGENS
19 Nortestosteroon (Nandrolone)	Natural hormone or synthetic	17β Nortestosterone 17α Nortestosterone	ANDROGENS
Trenbolone Acetate	Synthetic hormone	17α and 17β Trenbolone 17β Tbketo	ANDROGENS
Resorcylic Acid Lactone	Synthetic hormone	Zeranol (α-Zearalanone) Taleranol (β-Zearalanol)	ESTROGEN
Beta agonist	Beta-2-receptor	Zilpaterol	UNKNOWN

1.3 GENERAL DESCRIPTION OF THE STEROIDS INCLUDED

Diethylstilbestrol was used in the past for growth-promoting purposes in livestock and as an anti-abortive in humans, but the FDA banned its use in 1971. In South Africa all related formulations of DES are banned and no remedies containing DES are available. However, it was included in the study to evaluate if it is still present from previous usage or as a result of fraudulent use. 17β-Estradiol can be used, alone or in combination with other natural or synthetic steroid

hormones (testosterone, progesterone, trenbolone acetate), to improve the rate of weight gain or feed efficiency in cattle. 17β-estradiol is primarily converted to estrone and further to estriol.

A variety of sulphate and glucuronide conjugates are also excreted. While estradiol is an endogenous hormone, animals may be treated with further supplements to promote growth. The only licensed way of application is subcutaneous injection of slow-release implant preparations into the rear side of the ear (See Figure 1.1). In some countries such as the United States of America, Canada, Argentina, Australia, New Zealand and South Africa, the use of hormonal growth promoters is allowed. The international regulatory bodies examined these natural compounds in the context of Maximum Residue Limit (MRL) determination. In July 1995, the Codex Alimentarius Commission stated that for all the natural hormones used as growth promoters, Maximum Residue Levels (MRLs) were unnecessary (Codex Alimentarius 1995). It states that "available data on the identity and concentration of residues of the veterinary drug in animal tissue indicate a wide margin of safety for the consumption of residues in food". The focus however of this study was not to determine residues, but to determine if these natural hormones could be detected in water sources due to the intensive production practices being used in feedlots.

The synthetic hormones used in birth control pills and post menopausal hormone replacement therapy (ethynylestradiol) is strictly not used in feedlots, but because it is excreted from the human body, it was included for academic purposes.

Testosterone secreted by the testes is the main androgen in males, along with its similarly active metabolite dihydrotestosterone (see Figure 1.2). These natural androgens are metabolized and excreted in urine as both free steroids and water-soluble conjugates.

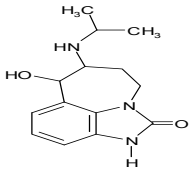
The major urinary metabolites are etiocholanolone and androsterone, both of which are physiologically weak or inactive. In addition to endogenous testosterone, testosterone or testosterone propionate may be used as a Hormonal Growth Promotant (HGP). Trenbolone is a synthetic androgenic steroid. It is used to promote growth and enhance the efficiency of feed utilization in beef cattle. Trenbolone is administered to feedlot cattle as trenbolone acetate, which is hydrolysed to form the potent androgen receptor agonist 17β-trenbolone.

Zeranol (α-zearalanol) is a non-steroidal estrogenic growth promoter, which increases live-weight gain in food animals. Zeranol is also a semi-synthetic product derived from the naturally occurring mycotoxin zearalenone and may occur naturally in urine and bile from sheep and cattle following metabolism of the mycotoxins zearalenone and α-zearalenol which can contaminate animal feedstuff (Kinani et al., 2008). Once administered, Zeranol is predominantly metabolised to α-zearalanone and taleranol (β-zearalanol).

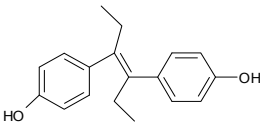
Zilpaterol is a β-adrenergic repartition agent demonstrated to enhance carcass leanness, improve growth rates, and decrease feed consumption in cattle and sheep (Van Hoof et al., 2005; Shelver et al., 2006).

Beta-agonists can bind to beta-2-receptors. Stimulation of these receptors results in relaxation of smooth muscles. Beta-agonists are therefore frequently used as bronchodilator for the treatment of pulmonary diseases in humans and animals. In addition, they also improve carcass composition as they decrease fat in favour of a higher percentage of muscle (repartitioners).

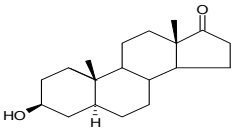
The chemical structures of the compounds of interest are summarized in Figure 1.2.



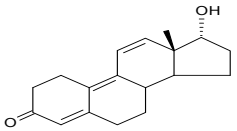
Zilpaterol



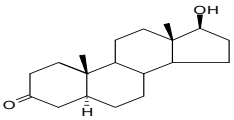
trans-Diethylstilbestrol



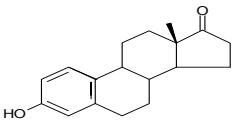
Epiandrosterone



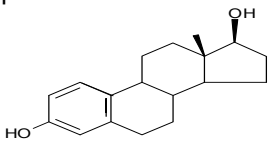
β Trenbolone



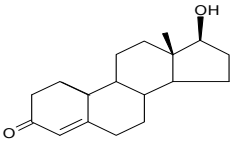
Androsstanolone (Stanolone)



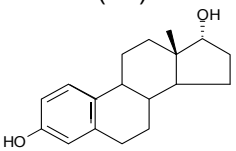
Estrone:(E1)



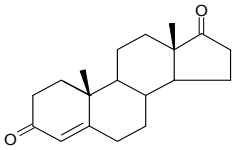
17β Estradiol (E2)



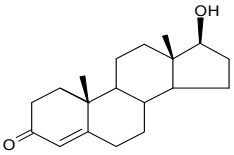
Nandrolone (19-nortestosterone)



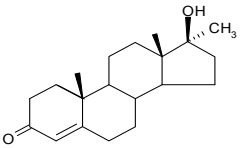
17α Estradiol



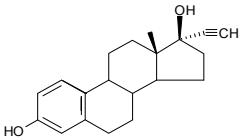
Androstenedione



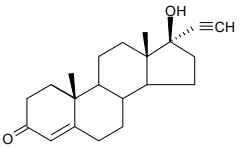
Testosterone



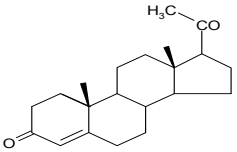
Methyltestosterone



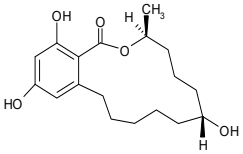
Ethynyl Estradiol



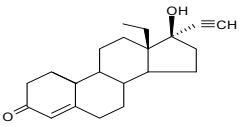
Ethisterone (ethynyltestosterone)



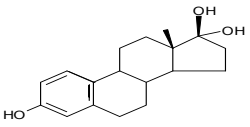
Progesterone



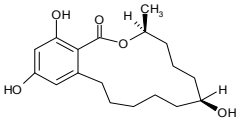
Zeranol



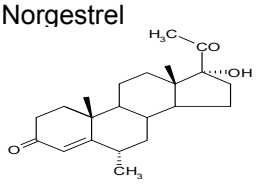
Norgestrel



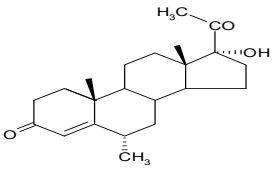
Estriol E3



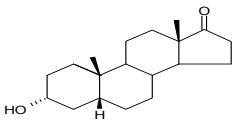
b-zearalenol (not taleranol)



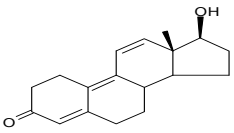
Medroxy Progesterone



Medroxyprogesterone acetate



Etiochloanolone



17α Trenbolone

Figure 1.2: Selection of compounds that were considered for the study together with their molecular structure.

1.4 ENVIRONMENTAL FATE OF STEROIDAL HORMONES

An updated summary of the fate of veterinary drugs is given in an article published by SJ Khan (Khan et al., 2008). The following is an overview of the section that describes the stability of the EDC's.

The stability of the hormones, once excreted, is largely dependent on the environmental conditions such as UV radiation, soil type and microbial contents of that particular site. The mobility of the drugs or drug metabolites in the soil and the geographical layout of the area will largely predict if the drugs can pose a threat to the groundwater or the rest of the environment such as aquatic or terrestrial organisms. Unfortunately not enough information is available at this stage that explains the mechanism and factors involved in the degradation of these hormones in the environment. The following is a summary of the most prominent aspects as mentioned in the article by Khan:

- Unconjugated steroidal hormones are chemically very stable, non-volatile, have low water solubility and are moderately hydrophobic (Hanselman et al., 2003; Layton et al., 2000).
- 17β-estradiol and estrone have high sorption affinities to soils (Casey et al., 2004; Colucci et al., 2002).
- Testosterone appears to behave differently, with lower soil sorption affinity only weakly correlating with soil particle size, organic matter and specific surface area (Casey et al., 2004).
- Degradation/transformation of 17β-estradiol occurs in the sorbed phase in soil and the process is rapid (Casey et al., 2003).
- Although it was found that testosterone degraded more readily than 17β-estradiol, it appeared to have a greater potential to migrate in the soil because it was not as strongly sorbed (Casey et al., 2004). These results are consistent with field observations where testosterone was shown to reach groundwater, while estrogen remained bound to the upper crust of the soil (Shore et al., 2003).
- Synthetic hormones, trenbolone and melengestrol acetate appear to behave similarly to testosterone, having a significant affinity to the organic fraction of soils, leading to a high retardation, but remaining nonetheless mobile in agricultural soils (Lange et al., 2002).
- It has been proposed that overland flow of steroidal hormones during rain events may be a much more significant means of transport to surface waters compared to seepage through soils (Kolodziej et al., 2004).
- Few studies have been undertaken to trace the fate of zeranol in the environment. However, interestingly, this compound has been observed at low ng ℓ^{1} concentrations in municipal sewage effluents and in a river receiving sewage discharges (Laganá et al., 2004).

While data is lacking, it has been suggested that steroidal hormones are biodegraded in the environment by many types of organisms (Hanselman et al., 2003). In soils, estradiol is converted, biotically or abiotically, to estrone, which is slowly further degraded or mineralised (Colucci et al., 2002; Shore et al., 2003).

1.5 SITE SELECTION OF SAMPLE

For this project to be supported by the feedlots in South Africa the Chairperson of the South African Feed Lot Society, Dr Dave Ford, was contacted and a meeting was set-up to discuss the co-operation of the Society with the team leaders of the project. During our discussion, it became obvious that the Feedlot Society is under a lot of strain from the general consumer in terms of current practices. He therefore made it clear that in order for us to obtain co-operation from them, emphasis must be placed on the confidentiality of the data generated during the study. The data generated must therefore not be published in the general media without considering the implications thereof to the feedlot industry in general. He also referred Ms Swemmer to Dr Johan van Niekerk, an environmental consultant, who is currently contracted by the Feedlot Society to compile a manual on Hazard Analysis and Critical Control Point (HACCP) practices in feedlots. Several meetings were held with Dr van Niekerk to establish the best practices that should be followed to ensure the co-operation of the feedlot owners. All the feedlots in South Africa are owned by private enterprises and co-operation for the collection of water samples can only happen with their full collaboration. It was subsequently decided that Dr van Niekerk will interact with the feedlots and represent the project team in order to maintain a good working relationship. From the discussions with Dr van Niekerk, it became apparent that, in general, feedlots could be classified according to the following classification:

1. Those that are registered but were established before 1998 and thus not under obligation to perform an EIA.
2. Those that are registered after 1998 and which are under obligation to perform an EIA study under the National Environmental Management Act 107 of 1998,
3. Those that are not even registered with the feedlot association.

It was subsequently decided that the research would be focused on the first two types of feedlots. It also became apparent that the levels of hormones (both natural and synthetic) accumulated in the environment do not form part of the requirements of the EIA study.

With this information at hand, Dr van Niekerk draw up a Questionnaire that will be applied at the feedlots once they are identified as being part of the study. This questionnaire is attached as Appendix A.

The GIS expert, Mr Louis Botha, from Groundwater Square, was contacted to set up the GIS information maps that are necessary to establish the groundwater geography as a possible risk assessment strategy. It was however discovered that from the 57 feedlots that are registered with the Feedlot Society of South Africa, less than halve were mapped on the GIS system. It was subsequently necessary to first obtain the GIS co-ordinates before mapping was possible. The feedlots were subsequently evaluated and a ranking process was applied. This data as well as the ranking criteria are available in Appendix B of the report.

After the evaluation of the information gathered, it became evident that although the feedlots were ranked in terms of a common risk model, it would be difficult to use a selection of feedlots as generic models. Each feedlot is defined by a particular design, geographic layout, management processes and a variety of other factors such as production years, soil type and feeding capacity that can either contribute positively or negatively to the potential risk the feedlot could pose on the environment.

It was decided to continue with the project on the basis that some information is needed as a baseline to evaluate whether feedlots does pose a threat to the surrounding water sources in terms of EDC activity. Three feedlots that were willing to co-operate and to assist with the sampling of the water were chosen to continue with the project. In essence the main aim was to sample water sources upstream and downstream from the adjacent river or water streams. In order to describe the location of the sampling points, the general design of the feedlots was studied.

1.6 FEEDLOT DESIGNS: (GUIDELINES FOR THE ESTABLISHMENT AND OPERATION OF CATTLE FEEDLOTS IN SOUTH AUSTRALIA, 2006)

The main by-products form cattle feedlots are the manure harvested from the surface of the pens and liquid effluent collected during rainfall runoff events. Good feedlot pad management requires a balance between environmental and animal health considerations and the economic cost of pen cleaning. One of the most cost effective ways to ensure that runoff water does not contaminate river water or that leaching through soil to groundwater is not prominent is to design the feedlot in such a way as to minimize these threats. In order to understand the description of the sampling points taken, it is necessary that the basic design of the feedlots is discussed briefly.

Most feedlots follow a rectangular layout with multiple feeding pens. They are easy to expand, but extensive earthwork is often required to ensure clean pens with good drainage. The basic rectangular feeding pen has a feed bunk along the top and a drainage channel at the bottom. They are arranged in a straight line to make feeding easy. This repeating rectangular block layout has two variations; one for a flat site and one for a sloping site (see Figure 1.3).

Both Feedlots sampled belong to the rectangular layout, with sloping sites that make use of the contour hills. The benefits of these design type they can:

- Have good feed handling, traffic and cattle flow.
- Have good provision of water and services.
- Are easy to expand.
- Requires pre-construction earthwork to establish grades and drainage.
- Takes advantage of natural topography.

- Usually excellent for drainage.
- Good feeding and waste handling.

(Another less common layout is a pie-shaped lot. The drainage, feeding and expansion possibilities are more limited).

The general design principles in terms of waste/drainage that are commonly followed are:

1. To maintain clean, dry pens a survey needs to be done of the site and the necessary earthworks done to obtain a uniform 2-4% pens slope.
2. Locate feeding pen runoff drains outside of the feeding pens. This makes the best use of pen area. Two options are to have combined cattle handling alley and drain or to have separate alleys and runoff drains.
3. Design the runoff drainage system not to interfere with feeding roadways.

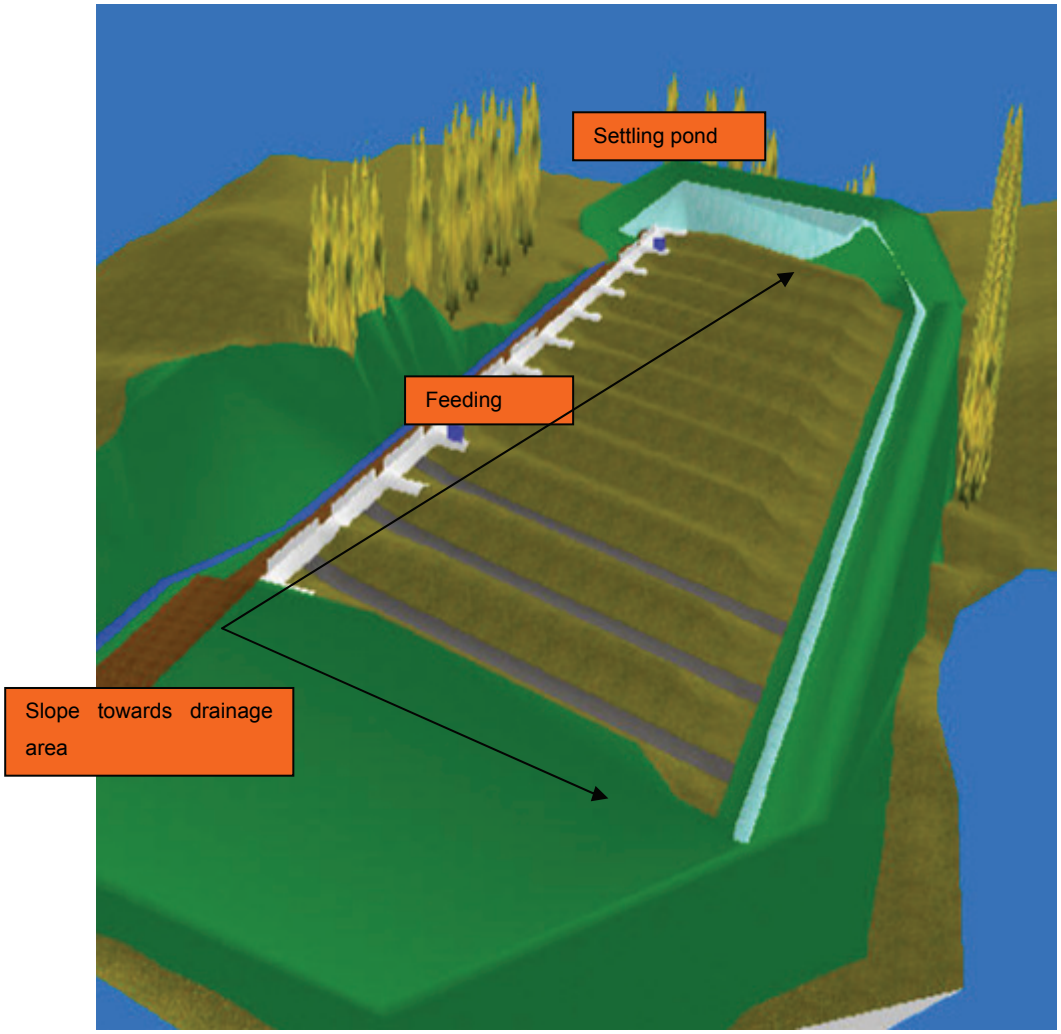


Figure 1.3: Typical lay-out of a sloped site, linear layout of a generic feedlot

Three different sites were selected to collect samples. The following Tables 3-5 provide a general background description of each feedlot sampled. None of the feedlots selected is situated next to a major river. This study therefore concentrates on the analysis of underground (borehole) water within feedlots and the environment adjacent to the feedlots. Humans and animals are dependent on this water.

Table 1.2: Description of Feedlot A

Background on the Feedlot	Details
Capacity	18 000
Vegetation strip available	Yes
Water sources	Only boreholes – approximately 150 m deep
ISO 1400 accreditation	In process
Divisions	None
Slope	2-5%

Table 1.3: Description of Feedlot B

Background on the Feedlot	Details
Capacity	18 000 head of cattle
Vegetation strip available	Yes – not fully functional – approximately 500 m deep
Water sources	Only boreholes
ISO 1400 accreditation	Yes
Divisions	East and a West block
Slope	2-5%

Table 1.4: Description of Feedlot C

Background on the Feedlot	Details
Capacity	21 000
Vegetation strip available	None
Water sources	Boreholes – depth varies around 7 m
ISO 1400 accreditation	Yes
Divisions	None
Slope	Between 5-10%

Table 1.5: General description of the sampling points that we sampled during the study

Sampling point	Feedlot	Sampling date	Site description
Sampling 1	A	27/02/2007	Groundwater from a bore hole 150 m below the surface – upstream from the feedlot
Sampling 2	A	27/02/2007	General drinking water available to the cattle
Sampling 3	A	27/02/2007	Runoff water from the feedlot gathered in a settling dam
Sampling 4	A	27/02/2007	Water management dam from the feedlot – inlet
Sampling 5	A	27/02/2007	Water management dam from the feedlot – central
Sampling 6	A	27/02/2007	General rain water from the road within the feedlot

Sampling point	Feedlot	Sampling date	Site description
Sampling 7	B	02/03/2007	Water puddle within the feedlot – next to road
Sampling 8	B	02/03/2007	Water from the feeding trough
Sampling 9	B	02/03/2007	Evaporation dam from the feedlot
Sampling 10	B	02/03/2007	Processing borehole – approximately 100 m depth
Sampling 11	B	02/03/2007	First settling dam
Sampling 12	B	02/03/2007	Overflow from the first dam
Sampling 13	B	02/03/2007	Borehole water – in feedlot
Sampling 14	B	02/03/2007	Borehole water – in feedlot
Sampling 15	B	02/03/2007	Reservoir in the feedlot
Sampling 16	B	02/03/2007	Settling dam
Sampling 17	B	02/03/2007	Borehole in the wetland
Sampling 18	B	02/03/2007	Inlet of general water dam
Sampling 19	B	02/03/2007	General water from the settling dam
Sampling 20	B	02/03/2007	Water downhill from the feedlot
Sampling 21	B	02/03/2007	Borehole used for cattle
Sampling 22	B	02/03/2007	Borehole – water used for hospital in feedlot
Sampling 23*	C	15/03/2007	Borehole 10 m beneath the feedlot
Sampling 24*	C	15/03/2007	Borehole available for human consumption
Sampling 25	C	15/03/2007	Water from the river – downstream from the feedlot
Sampling 26	C	15/03/2007	Borehole water available for human consumption
Sampling 27	C	15/03/2007	Borehole available for human consumption
Sampling 28	C	15/03/2007	Water from the third overflow of the settling pond
Sampling 29	C	15/03/2007	Primary settling pond
Sampling 30	C	15/03/2007	Sample uphill from feedlot
Sampling 31	C	15/03/2007	Rain water along the road
Sampling 32	C	15/03/2007	Rain water/filtration water
Sampling 33	C	6/02/2008	Borehole water – uphill

Sampling point	Feedlot	Sampling date	Site description
Sampling 34	C	6/02/2008	Borehole water – downhill
Sampling 35	C	6/02/2008	Irrigation water – downhill
Sampling 36	C	6/02/2008	River water – downstream
Sampling 37	C	6/02/2008	Borehole water – uphill
Sampling 38	C		Bottle broke during transportation
Sampling 39	B	29/01/2008	Bore hole – uphill
Sampling 40	B	29/01/2008	Bore hole – downhill from feedlot in wetland
Sampling 41	B	29/01/2008	Bore hole – downhill
Sampling 42	B	29/01/2008	Water from settling pond
Sampling 43	B	29/01/2008	Borehole at feedlot hospital
Sampling 44	B	29/01/2008	Reservoir water
Sampling 45	B	29/01/2008	2 km downstream from feedlot
Sampling 46	B	29/01/2008	4 km downstream from feedlot

*** Samples were lost during sample preparation**

1.7 SCOPE OF THE CHEMICAL ANALYSIS

During the initial planning of the project, samples would have been sent to a laboratory in South Africa that could analyse for the range of EDC compounds. The aim of the chemical analysis was to use one chromatographic method to determine the total range of EDCs. However no laboratory in South Africa was able to test for the range of EDCs in environmental samples. Logistically it was not viable to distribute the water samples between different laboratories, as each laboratory required a litre of water to reach the required detection limit of approximately 10 ng/ℓ. It was subsequently decided that a multi-residue method will be validated in the Residue Laboratory at the Onderstepoort Veterinary Institute (OVI) for the detection and identification of anabolic steroids in water samples. The original method plan was that gas chromatography coupled to mass spectrometry would be used for this purposes (Van Hoof et al., 2005; Layton et al., 2000). The outline of the method was provided by Bruno le Bizec (Le Bizec et al., 1993). However, the method provided was developed for urine and not for water, and the equipment described in the method was a tandem GC-MS. The laboratory at the OVI however only had a single quadropole GC-MS available. Notwithstanding these limitations, the method was altered and validation attempts were made to detect the following steroids: DES, 17α and 17β-estradiol, zeranol, taleranol, ethynyltestosterone, 17α- and 17β-testosterone, methyltestosterone, and nandrolone, 17β-trenbolone, medroxyprogesterone, epi-androsterone, 5α-androstane-3β, 17α-diol, ethynilestradiol estrone and estriol. However, some of the analytes such as trenbolone, estrone and progesterone, were poorly derivatised during the sample preparation and could not be detected on the GC-MS with good sensitivity. Subsequently, it was decided to alter the extraction method and to analyse these poorly recovered analytes on a tandem LC-MS system.

1.8 SAMPLING PROTOCOL

The sampling protocol was discussed with Prof Le Bizec and was also used in a previous study (Soto et al., 2004). Consideration must be given to sample handling and preparation to ensure that samples are representative of the environment being assessed. For aqueous samples, analysis is often undertaken immediately following sampling or, if that is not feasible, they are chemically preserved. Samples taken were immediately, after collection, preserved by adding 0.02% Sodium azide to prevent microbial degradation.

Surface samples were collected as grab samples from various streams in the vicinity of the feedlot. All samples were collected in 1 ℓ shot bottles pre-rinsed with methanol and deionised water. Clear bottles were wrapped in foil to protect the content from light. The samples were stored in a refrigerator at 4°C until analysis commenced. The pH of the water samples was checked prior to analyses and pH values obtained were between 5 and 7 for all samples apart from water collected directly from or in the settling ponds. Hands were protected with nitrile gloves during sampling. To avoid clogging of the SPE cartridges, all environmental samples were filtered through glass micro fiber filters prior to analyses. Samples with a high percentage of suspended matter were centrifuged at 4000 rpm for 10 minutes to further avoid clogging the filter paper. No pH adjustments were made during the extraction procedure.

1.9 MATERIALS AND EQUIPMENT

1.9.1 Reagents and standards

During the analysis, unless specified, all the reagents were of analytical grade quality. Only ultra pure or distilled water was used. The enzymatic hydrolysis was accomplished with *Helix Pomatia* juice (β-glucuronidase-aryl sulfatase, Merck – 4114) Methyl Tert-Butyl Ether (MTBE) was used as the eluant for the SPE step.

The anabolic steroids were provided by Steraloids, Dr Ehrenstorfer, National Horse Racing Authority of South Africa and Sigma Aldrich. All the primary solutions were prepared in ethanol, at 1 μg/μℓ$^{-1}$ and 100 ng/μℓ$^{-1}$ and stored frozen in the dark (≤ -16°C). Working solutions were prepared by dilution of the primary solution in ethanol and stored the same way.

The analytical standard, Zilpaterol was only commercially available from Dr Ehrenstorfer, lot number 80806, with a purity stated on the certificate of analysis of 56%. A purity adjustment to 100% was calculated during the optimisation of the instruments.

Derivatisation reagents:-

N-Methyl-N-trimethylsilyl-trifluoroacetamide: MSTFA (Fluka 9479),
Trimethyliodosilane: TMIS (Fluka 58118),
Dithiothreitol: DTE (Aldrich 15,046-0),
Preparation of the silylation reagent MSTFA/TMIS/DTE 1000:5:5 (v/v/w). Add in an anhydrous way 2000 (± 20) μℓ of MSTFA, 10 (± 2) μℓ of TMIS and 10 (± 2) mg of DTE.

1.9.2 GC-MS equipment

An Agilent GC-MS system consisting of 7890A gas chromatograph (7890A) and mass spectrometer (7890A) was used. Data processing was accomplished with MSD Chemstation software version E.01.00.237. Chromatographic separation was achieved with a capillary column, splitless injection and a programmable temperature oven up to 300°C. The transfer line was set at 280°C. An automatic injector (Hewlett Packard – 7673) was used. A Non polar phase capillary column, with low bleeding DB 1701 with dimensions: Length = 30 m, Internal diameter = 0,25 mm and Film thickness = 0,25 μμm was used. Detection in SIM mode was used for improved sensitivity.

1.9.3 LC-MS/MS equipment

An Applied Biosystems API 4000 QTRAP LC/MS/MS system equipped with a Shimatzu Prominence autosampler and binary LC pump was used. Ionisation was achieved by Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) using a Turbo V ionization source. All compounds were monitored with two Multiple Reaction Monitoring (MRM) transitions per compound.

1.9.4 Extraction procedure

The process used to quantify estrogenic hormones involved a series of steps to concentrate the hormones, separate them from interfering compounds, and for GC analysis, convert them into derivatives that were more easily detected.

A schematic representation of the analytical technique is provided in flow diagram and is described below. The method comprises the following steps:

1. Pre-preparation of the samples.
2. Enzymatic hydrolysis of conjugates.
3. Solid Phase Extraction (SPE) on Oasis HLB cartridges (supplied by Waters).
4. Splitting of the eluant for GCMS and LC-MS/MS analysis.
5. Derivatisation of GC-MS fraction.
6. Detection and identification by GC-MS and LC-MS/MS.

1.9.4.1 Pre-preparation of the samples

As a precautionary measurement, all samples were filtered through a glass fibre pre-filter. This step of the method was previously validated to ensure that during the analyses of real environmental samples, the pre-filtering of the sample would not influence the recovery of the hormones. Samples with high percentage of suspended matter were centrifuge prior to filtration.

1.9.4.2 Enzymatic hydrolysis

Helix Pomatia juice (β-glucuronidase-aryl sulfatase) was used as the enzyme to enhance the deconjugation of the hormones from the matrix. The success of this step in environmental samples cannot be guaranteed, but from the publications of Le Bizec, this step seems to be important during the analyses of urine samples (Le Bizec et al., 1993). It was expected that a large number of water samples taken, will contain a significant percentage of urine and deconjugated of the steroids was therefore desirable.

1.9.4.3 Solid Phase Extraction (SPE)

Solid Phase Extraction (SPE) was evaluated as the method for choice for cleanup and enrichment of the sample. The Oasis HLB cartridge was recommended by Dr Le Bizec during the workshop and subsequent investigation confirmed the usefulness of the Oasis® HLB: (Hydrophilic-Lipophilic Balance Sorbent reversed-phase sorbent). The protocol describe by the manufacturer were followed whereby 1 ℓ of water was passed through the cartridge and the cartridge were subsequently eluted with methyl tert-butyl ether. Further investigations on the HLB cartridge indicated that it is essential to use glass tubes during the extraction procedure in order to avoid unnecessary side reaction during the derivatisation step.

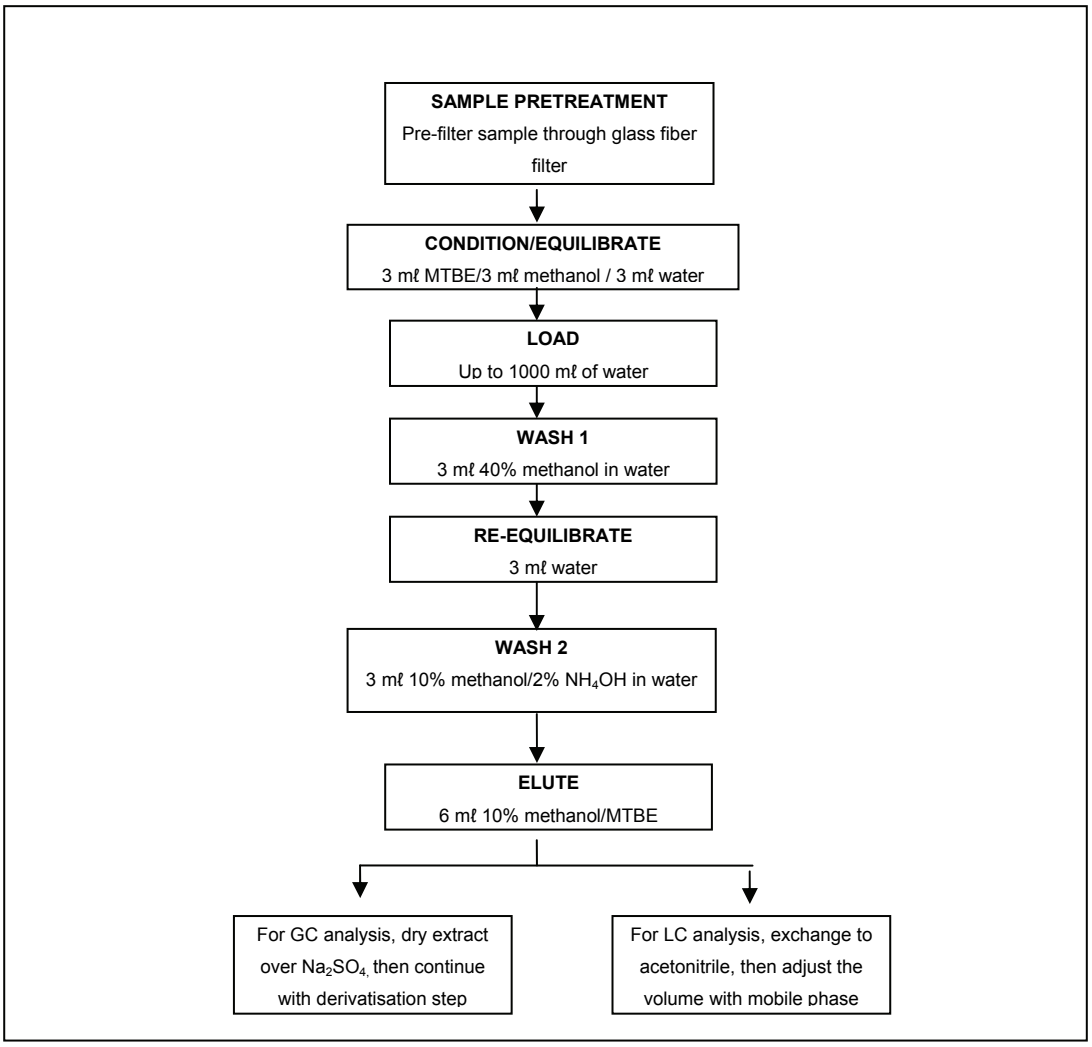


Figure 1.4: Flow diagram of the sample preparation steps

1.9.5 GC-MS analysis

The determination of the hormones in the environmental samples was based on solid phase extraction, silylation and detection by GC/MS. Derivatisation was carried out in the case of the thermo labile, polar, and low volatile compounds, such as estrogens, to avoid thermal decomposition and to improve the chromatographic separation and the sensitivity of the analysis. The derivation step targets the hydroxyl functional groups present in the molecular structure of the steroids.

1.9.5.1 Derivatisation

Silylation was used as the preferred choice for the derivatisation and subsequent stabilisation of the selected steroids during the chromatographic step. The reagents available are known to replace the active hydrogen from acids, alcohols, thiols, amines and ketones with a trimethylsilyl group. The derivatisation reagents recommended by Dr Le Bizec, MSTFA/TMIS/DTE (Le Bezic et al., 1993), was developed and evaluated by his group specifically for the derivatision of the bulk of the group of hormones investigated (Hanselman et al., 2003).

1.9.5.2 Chromatographic detection

The detection of the hormones was accomplished either via a GC-MS system both in SCAN and Single Ion Monitoring (SIM) mode. The GC-MS injection was accomplished via splitless injection at a temperature of 250°C and at an injection pressure of 250 kPa. A volume of 1 µℓ was injected onto a DB 1701 analytical column.

The following table provides a summary of the hormones that were considered in the study on the GC-MS, the retention times and the ions that were used for identification and quantitation purposes.

Table 1.6: GC-MS analysis of selective steroids in SIM mode

Analyte	Retention time	Base peak	Other ions
Zilpaterol	13.3	308	291,218,405,309
Epiandrosterone	16.068	347	362,272,155,129
Androstanolone	16.419	129	272,347,257,149
Nandrolone	17.013	129	256,346,331,
Testosterone	17.626	129	360,270,226,
Zeranol	17.798	433	307,335,523,
B-Trenbolone	18.253	342	211,209,237,181
B-Zearalenol	19.015	536	446,305,333,431
Ethisterone	19.042	369	317,125,
Androsenedione	19.946	286	124,244,148,107
Norgestrel	19.946	355	140,303,209,370

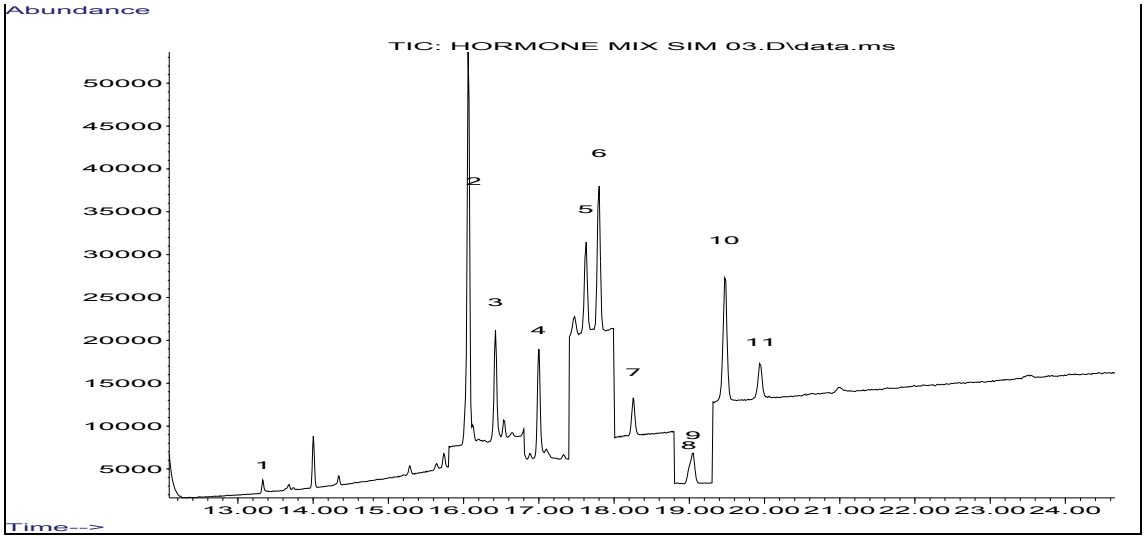


Figure 1.5: Chromatographic separation on a GC-MS of the steroids listed in Table 1.6

1.9.6 LC-MS/MS analysis

An Applied Biosystems 4000 QTrap LC-MS/MS system equipped with a Shimadzu Prominence autosampler and binary LC pump was used. Ionization was achieved by Electrospray Ionization (ESI) using a Turbo V ionization source. All compounds were monitored by two Multiple Reaction Monitoring (MRM) transitions per compound. A dwell time of 50 ms was used for each compound. The source parameters were as follow: Temperature 450ºC, Gas 1 = 45, Gas 2 = 30. Optimal mass spectrometer settings are illustrated in Table 1.8.

Chromatographic separation was achieved on a reversed phase Synergi Fusion (150 x 2.0 mm, 4 um), C18 column. An injection volume of 20 µℓ was used. The mobile phase conditions and elution profile are shown in Table 1.9. The total analysis time was 25 minutes per sample. To improve ionization efficiency both positive and negative ionization was utilized in Table 1.7. For negative ionization a solution containing aqueous ammonia, 10% (v/v), were infused with the aid of a Harvard syringe pump (10 µℓ / min) into the chromatographic effluent prior to mass spectrometric detection. The mobile phase consisted of water containing formic acid, 0.1% (v/v), and acetonitrile containing formic acid, 0.1% (v/v). The formic acid was omitted for analysis in the negative ionization mode due to ion suppression. A typical MRM elution profile of the steroids is illustrated in Figures 1.6-1.9. The quantification limits of the respective steroids are listed in Table 1.11.

Table 1.7: A summary of the LC-MS/MS ionisation mode, either positive or negative for each steroid as well as the two MRM transitions used during the analysis.

No:	Steroid	MRM 1	MRM 2	Ionization mode
1	Estrone (E1)	269 - 145	269 - 143	Negative
2	Estriol (E3)	287 - 171	287 - 145	Negative
3	17β Estradiol (E2) and (17α)	271 - 14	271 - 183	Negative
4	Ethinyl Estradiol (EE2)	295 - 145	295 - 159	Negative
5	Zearalanone	319 - 160	319 275	Negative
6	*trans*-Diethylstilbestrol	267 - 222	267 - 237	Negative
7	Zeranol (α-zearalanol)	321 - 277	321 - 303	Negative
8	Progesterone	315 - 297	315 - 279	Positive
9	Methyltestosterone	303 - 285	303 - 267	Positive
10	Trenbolone	271 - 199	271 - 253	Positive
11	Nandrolone (19-nortestosterone)	275 - 109	275 - 257	Positive
12	Zilpaterol	262 - 244	262 - 185	Positive
13	Medroxyprogesterone acetate	387 -123	387 - 327	Positive
14	Testosterone	289 - 109	289 - 97	Positive
15	Androstenedione	287 - 97	287 - 109	Positive
16	Epiandrosterone	291- 273	291 - 255	Positive
17	Androsstanolone	291 - 255	291 - 273	Positive
18	Ethisterone (ethynyltestosterone)	313 - 109	313 - 97	Positive
19	Norgestrel (IS)	313 -245	313 - 157	Positive

In order to avoid false positives, two MRM transitions as set out in Table 1.7 were monitored, provided that the relative abundance of the two selected precursor ion-product ion transitions were within 20% of the ion rations obtained for the standard. Only then was a positive identified. All quantification was done by matrix assisted calibration curves.

Table 1.8: Optimal mass spectrometric settings for each steroid as determined on the LC-MS/MS

Compound	MRM Transition (*m/z*)	Declustering Potential (V)	Collision Energy (CE)	Collision Exit Potential (V)
α-Ethynyl estradiol	295 - 145	- 95	- 52	- 9
	295 - 159	- 95	- 48	- 9
Estriol	287 - 171	- 115	- 48	- 9
	287 - 145	- 115	- 54	- 9
Estrone	269 - 145	- 90	- 48	- 11
	269 - 143	- 90	- 74	- 7
Estradiol	271 - 145	- 105	- 54	- 9
	271 - 183	- 75	- 56	- 11
α-Zearalanol	321 - 277	- 95	- 30	- 17
	321 - 303	- 95	- 30	- 17
β-Zearalenol	319 - 160	- 85	- 40	- 9
	319 - 130	- 85	- 50	- 9
Diethylstilbestrol	267 - 222	- 85	- 44	- 11
	262 - 237	- 80	- 38	- 4
Progesterone	315 - 297	+ 91	+ 23	+ 12
	315 - 279	+ 76	+ 25	+ 12
Trenbolone	271 - 199	+ 91	+ 33	+ 10
	271 - 253	+ 91	+ 89	+ 4
Androstanolone	291 - 255	+ 86	+ 23	+ 12
	291 - 273	+ 86	+ 21	+12
Testosterone	289 - 109	+ 76	+ 31	+ 4
	289 - 97	+ 76	+ 35	+ 4
Norgestrel	313 - 245	+ 76	+ 27	+ 10
	313 - 157	+ 76	+ 33	+ 6
Zilpaterol	262 - 157	+ 56	+ 51	+ 8
	262 - 185	+ 56	+ 51	+ 8
Androstanolone	291 - 273	+ 81	+ 13	+ 12
	291 255	+ 81	+ 21	+ 12
Androsterone	309 - 274	+ 66	+ 17	+ 12
	309 - 255	+ 66	+ 27	+ 5
Ethisterone	313 109	+ 66	+ 35	+ 4
	313 - 97	+ 66	+ 37	+ 4
Nandrolone	275 - 109	+ 81	+ 39	+ 4
	275 - 239	+ 81	+ 25	+ 12
Androstenedione	287 - 97	+ 81	+ 33	+ 4
	287 - 109	+ 81	+ 35	+ 4

Compound	MRM Transition (*m/z*)	Declustering Potential (V)	Collision Energy (CE)	Collision Exit Potential (V)
Medroxyprogesterone	387 - 123	+ 76	+ 43	+ 6
	387 - 327	+ 71	+ 21	+ 8
Methyltestosterone	303 - 97	+ 76	+ 59	+ 4
	303 - 109	+ 91	+ 41	+ 6

Table 1.9: Mobile phase conditions as used during the analysis of the steroids

Time (min)	Phase A (%)	Phase B (%)	Flow Rate (mℓ / min)
0.01	90	10	0.200
2.00	90	10	0.200
8.00	40	60	0.200
15.00	10	90	0.200
18.00	10	90	0.200
19.00	90	10	0.200
25.00	90	10	0.200

Table 1.10: Quantification limits (LOQ) of the steroids analysed

Compound	LOQ (ng/ℓ)	Signal To Noise at LOQ
α-Ethynyl estradiol	10	17
Estriol	10	35
Estrone	10	163
β-Estradiol	10	22
α-Zearalanol	10	280
β-Zearalenol	10	33
Diethylstilbestrol	10	11
Progesterone	10	33
Trenbolone	10	217
Androstanolone	10	105
Testosterone	10	445
Norgestrel	10	673
Zilpaterol	50	11
Androsterone	10	45
Ethisterone	10	426
Nandrolone	10	268
Androstenedione	10	12
Medroxyprogesterone	10	715
Methyltestosterone	10	672

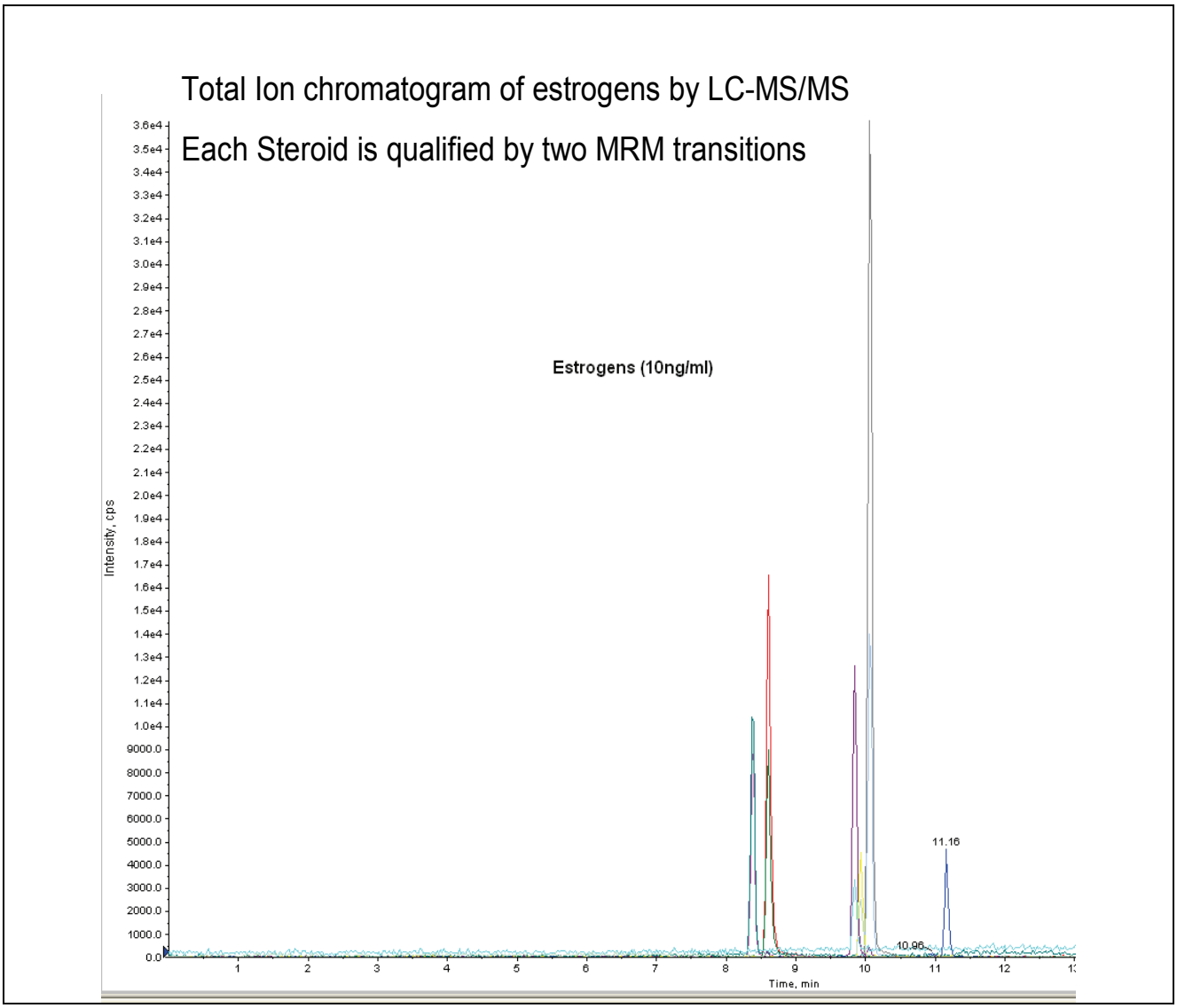


Figure 1.6: Total Ion Chromatogram of estrogens by LC-MS/MS.

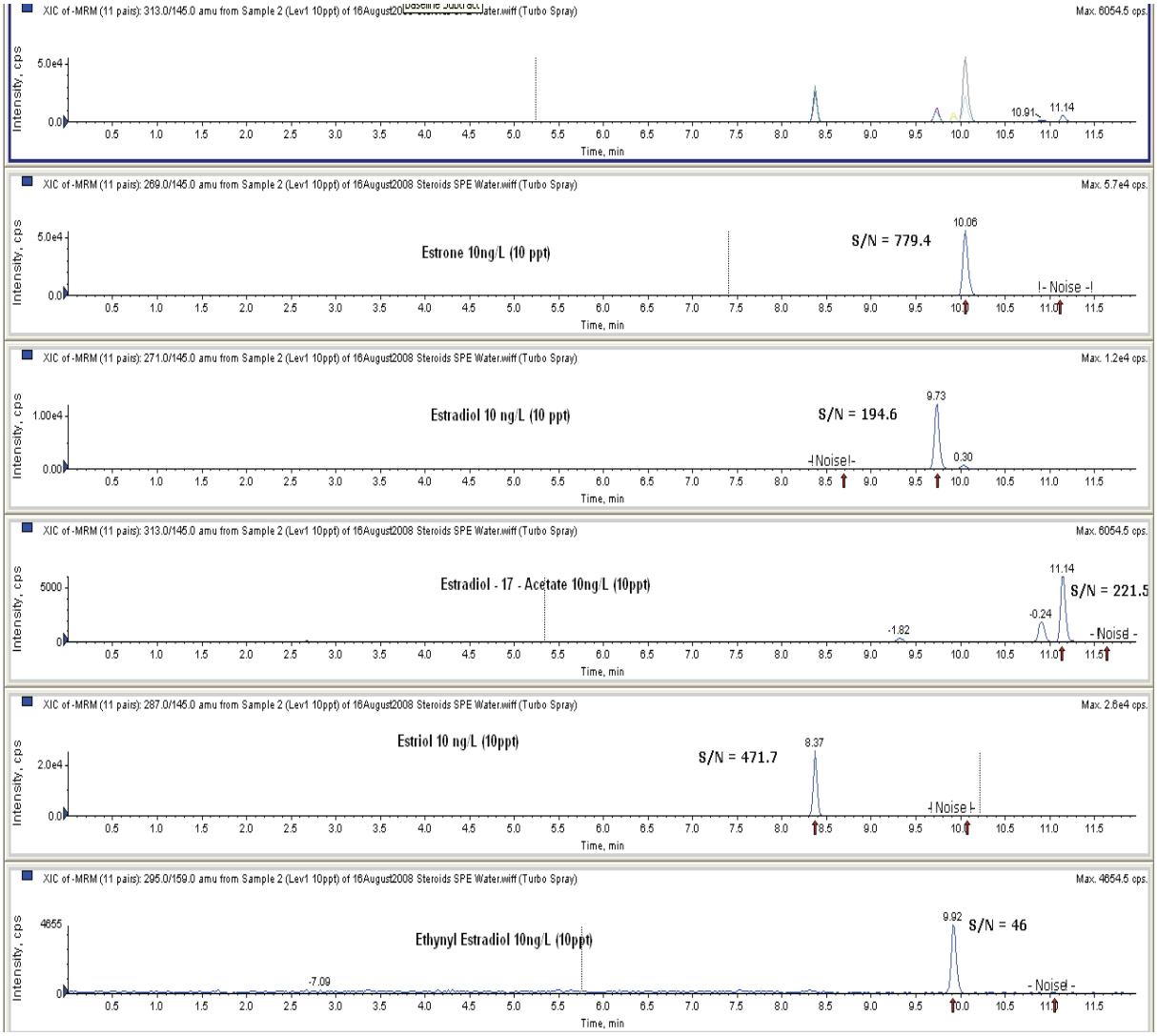


Figure 1.7: Extracted Ion Chromatogram of the different estrogens determined during the study

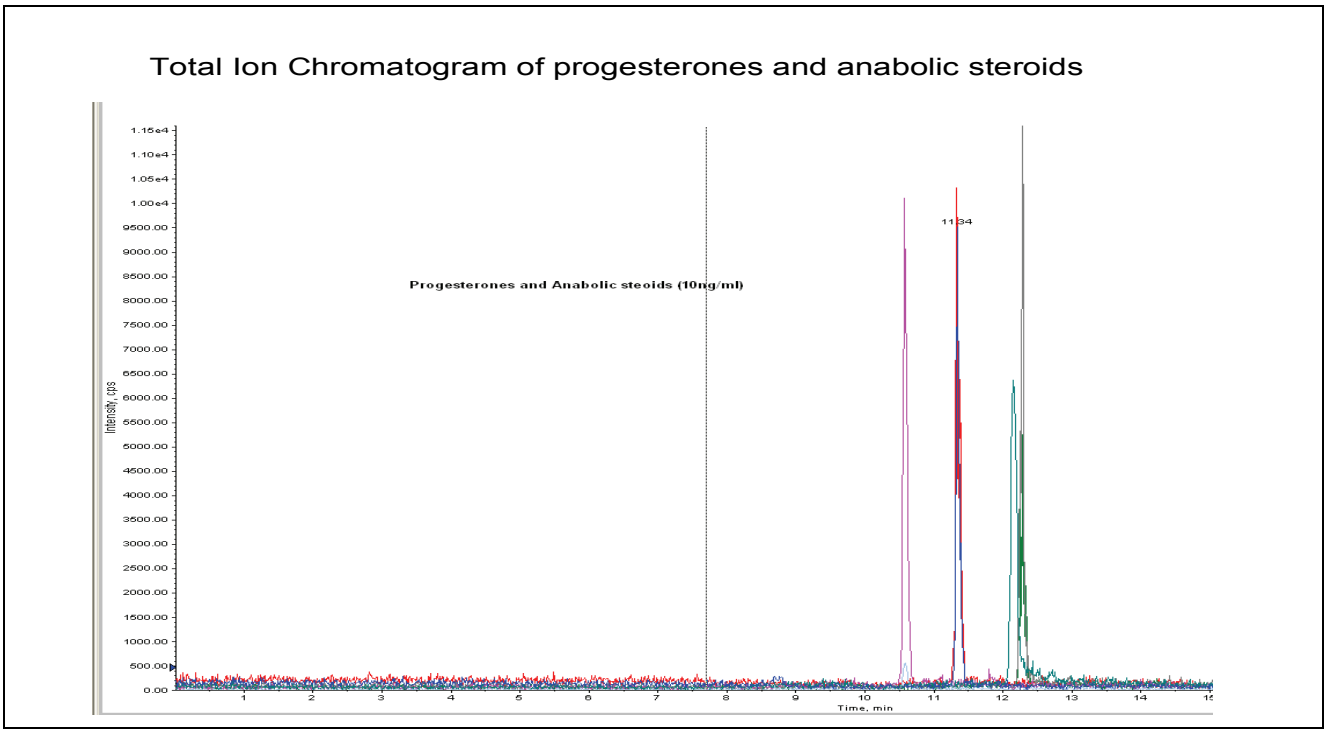


Figure 1.8: Total Ion Chromatogram of progesterone and anabolic steroids by LC-MS/MS.

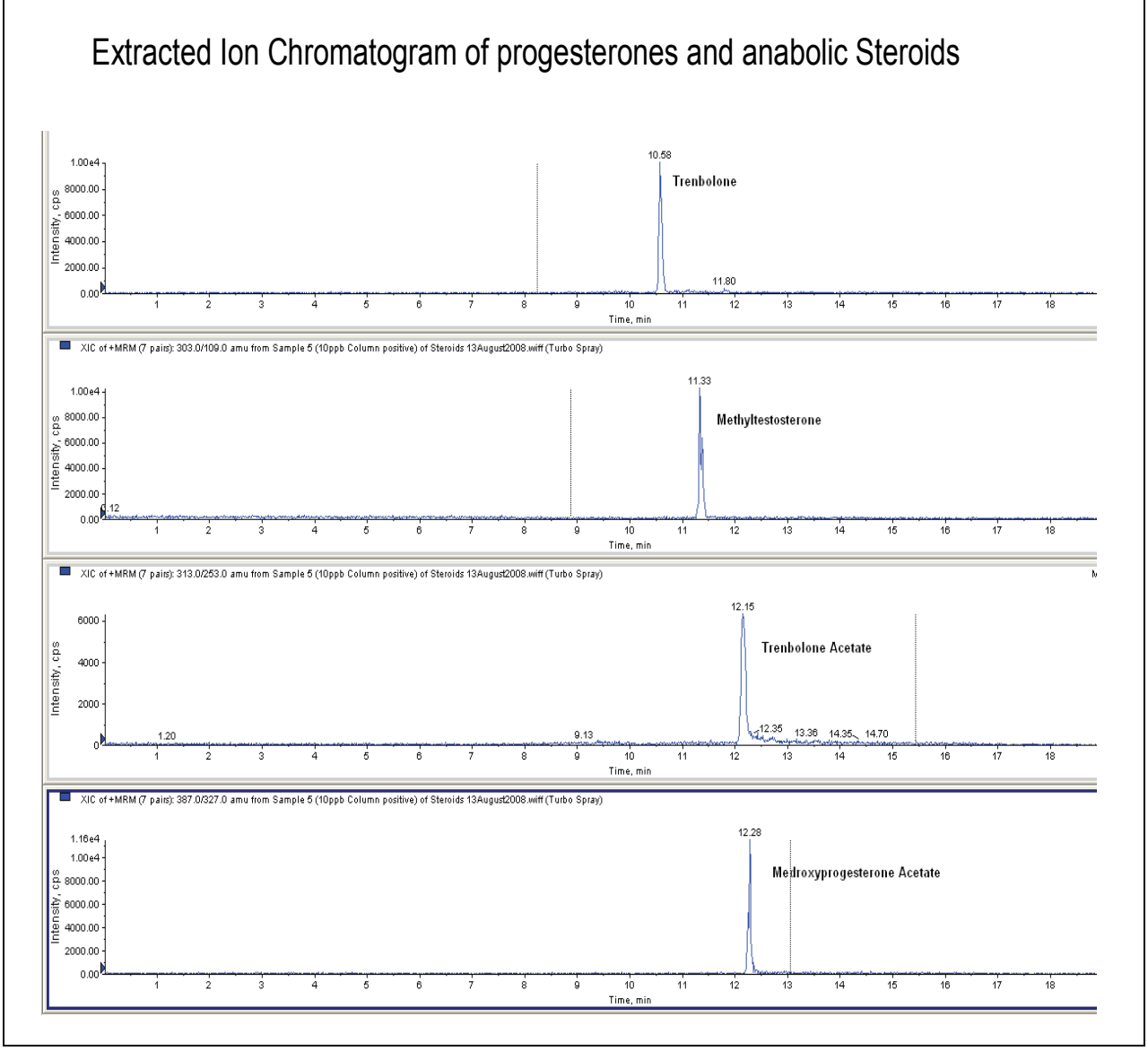


Figure 1.9: Extracted Ion Chromatogram of the progesterone and anabolic steroids determined during the study

Typical calibration curves obtained for the analysis of steroids

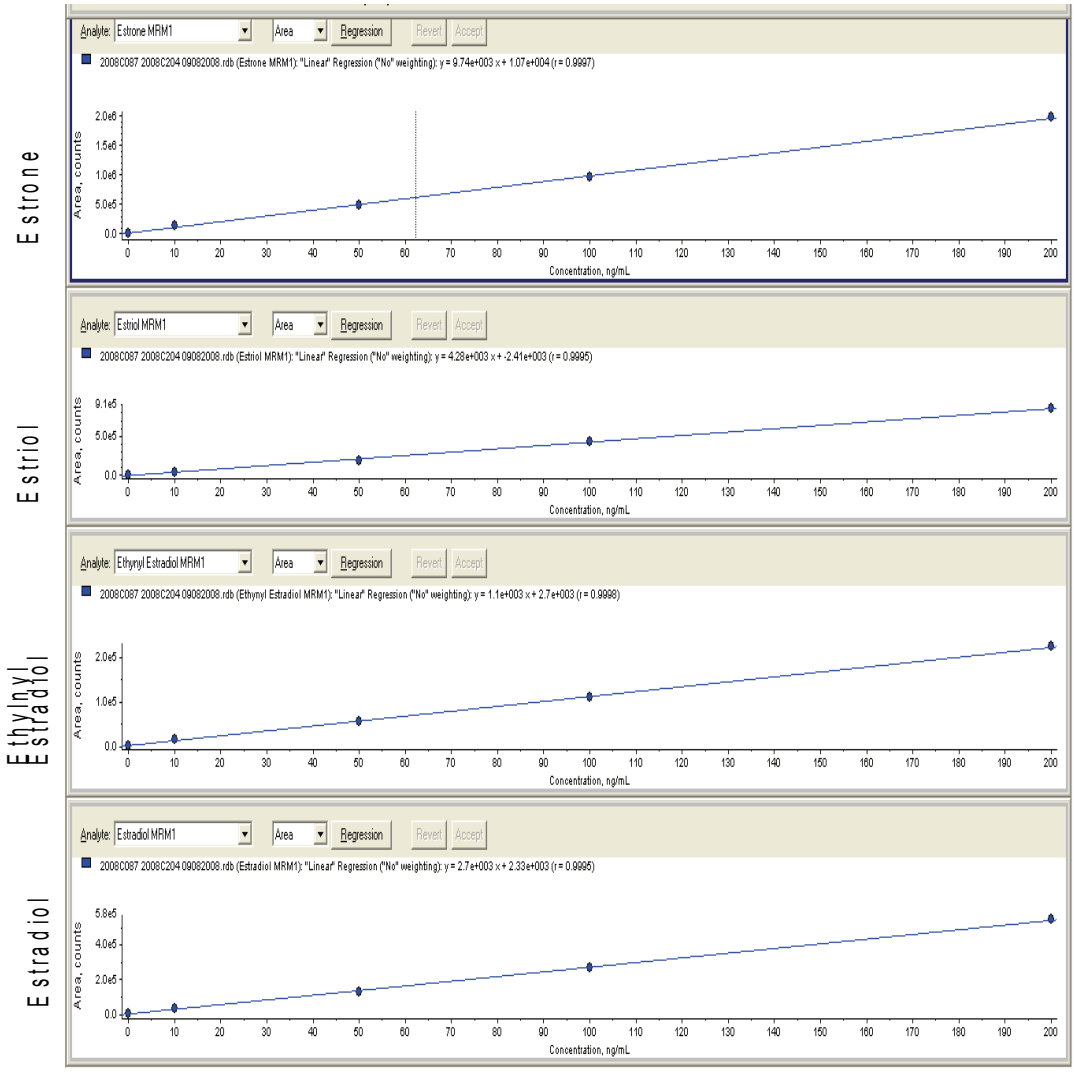


Figure 1.10: Typical matrix assisted calibration curves obtained during the study

1.10 RESULTS

Table 1.12 lists the concentrations of the steroids included in the study that was detected at the different sampling points with the tandem LC/MS. The sampling point listed in the first column of the table refers to the general description of the sampling point in Table 1.5. If a column values is indicated as ND – not detected, no detectable concentration of the steroids was detected for that particular sample. If a concentration below the detection limit of 10 ng/ℓ was detected, the table indicated a detection limit of <10 ng/ℓ. The pH of most of the samples was measured and is listed in the second column of the table.

Table 1.11: Recovery values for the range of analytes in both positive and negative mode.

Positive ESI	Low Concentration 15 ng/ℓ	High Concentration 400 ng/ℓ	Average Recovery %
Methyltestosterone	116	92	**104**
Progesterone	132	74	**103**
Trenbolone	116	102	**109**
Medroxyprogesterone	87	80	**84**
Androstanolone	76	84	**80**
Testosterone	119	88	**104**
Ethisterone	128	86	**107**
Nandrolone	108	94	**101**
Norgestrel	135	86	**111**
Androstenedione	102	127	**115**
Androsterone	70	91	**81**
Methyltestosterone	116	82	**99**
Zilpaterol	Below DT	67	**67**
Negative ESI	Low Concentration 15 ng/ℓ	High Concentration 400 ng/ℓ	Average Recovery %
Estrone (E1)	85	152	**119**
Estradiol (E2)	41	155	**98**
Estriol (E3)	95	133	**114**
Ethynylestradiol (EE2)	140	139	**140**
Zearalanol	60	140	**100**
Beta Zearalenol	69	153	**111**

Table 1.12: Concentration of the individual analytes determined per site

Site Point	pH	E1 ng/ℓ	E2 ng/ℓ	E3 ng/ℓ	EE2 ng/ℓ	ZER ng/ℓ	DES ng/ℓ	ZER2 ng/ℓ	PRO ng/ℓ	MTES ng/ℓ	TREN ng/ℓ	NAN ng/ℓ	ZIL ng/ℓ	MPRO ng/ℓ	TES ng/ℓ	ANDO ng/ℓ	EANDO ng/ℓ	ANDROS ng/ℓ	ETES ng/ℓ
FEEDLOT A																			
1	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	69	ND	ND	ND	ND	ND	ND
2	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	132	ND	ND	273	ND	ND	ND
3	8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3373	ND	ND	ND	ND	ND	ND
4	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	409	ND	ND	ND	ND	ND	ND
6	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	291	ND	ND	ND	ND	ND	ND
7	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	220	ND	ND	ND	ND	ND	ND
FEEDLOT B																			
8	6	< 10	ND	ND	< 10	ND	ND	ND	ND	ND	ND	ND	252	ND	ND	ND	ND	ND	ND
9	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	190	ND	ND	ND	ND	ND	ND
10	6	< 10	ND	ND	< 10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
11 (42)	8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	8260	ND	ND	ND	ND	ND	ND
12	9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	23703	ND	ND	ND	ND	ND	ND
13 (39)	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
15	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16	8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1537	ND	ND	ND	ND	ND	ND
17 (40)	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	< 10	ND	ND	ND	ND
18	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	185	ND	ND	ND	ND	ND	ND
19	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	338	ND	ND	ND	ND	ND	ND
20	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	220	ND	ND	ND	ND	ND	ND
21	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	< 10	ND	ND	ND	ND
22 (43)	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	< 10	ND	ND	ND	ND
FEEDLOT C																			
25 (36)	6	ND	ND	ND	ND	ND	< 10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
26 (34)	6	ND	ND	ND	ND	ND	< 10	ND	ND	ND	ND	ND	ND	ND	< 10	ND	ND	ND	ND
27(33)	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
28	8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3328	ND	ND	ND	ND	ND	ND
29	10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	12616	ND	ND	ND	ND	ND	ND
30(37)	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
31	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	70	ND	ND	ND	ND	ND	ND
32	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	< 10	ND	ND	ND	ND	ND	ND

FEEDLOT C																		
Site Point	pH	E1 ng/ℓ	E2 ng/ℓ	E3 ng/ℓ	EE2 ng/ℓ	ZER ng/ℓ	DES ng/ℓ	ZER2 ng/ℓ	PRO ng/ℓ	MTES ng/ℓ	TREN ng/ℓ	NAN ng/ℓ	ZIL ng/ℓ	MPRO ng/ℓ	TES ng/ℓl	ANDO ng/ℓ	EANDO ng/ℓ	ANDROS ng/ℓ	ETES ng/ℓ
33(27)	-	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
34 (26)	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	71	ND	ND	ND	ND	ND	ND
35 (24)	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	427	ND	ND	ND	ND	ND	ND
36 (25)	7	ND	ND	ND	ND	< 10	ND	ND	ND	ND	ND	ND	82	ND	< 10	ND	ND	ND	ND
37 (30)	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	82.5	ND	ND	ND	ND	ND	ND
FEEDLOT B																			
39 (13)	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
40 (17)	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	735	ND	ND	ND	ND	ND	ND
41	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	86	ND	ND	ND	ND	ND	ND
42 (11)	8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1873	ND	ND	ND	ND	ND	ND
42 B	7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2408	ND	ND	ND	ND	ND	ND
43 (22)	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
45	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
46	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
47	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

E1=Estrone, E2=17β estradiol, E3=Estriol, EE2=17α ethinylestradiol, ZER=α - Zearalanol, DES=Diethylstilbestrol, ZER2=Zeranol, PRO=Progesterone, MTES=methyltestosterone, TREN=trenbolone, NAN=Nandrolone, ZIL=Zilpaterol, PRO=Medroxyprogesterone, TES=testosterone, ANDO=Androstenedione, EANDO=Epiandrosterone, ANDROS=Androsstanolone, ETES=Ethisterone ** Samples 23 and 24 was lost in sample prep

1.11 DISCUSSION

1.11.1 Sampling

In the present study, samples were taken as grab samples from the various points. Due to the relative low concentration of EDC's in environmental samples, further studies should be undertaken with a passive sampler, similar to the Polar Organic Chemical Integrative Sampler (POCIS) described in the literature (Matthiessen et al., 2006). Briefly the POCIS consists of solvent-washed solid-phase adsorption medium (trade name OASIS) which is able to trap hydrophilic molecules including steroids. The sorption medium is sandwiched between two disc-shaped semi-permeable plastic membranes held in place by two metal compression rings which are in turn mounted inside a protective perforated stainless steel cylinder. The benefit of these passive systems is that low concentrations of EDCs can be trapped over a longer period of time – from a few days to a few months.

1.11.2 Choice of methodology

Enzyme-Linked Immunosorbent Assay (ELISA) and Radioimmunoassay (RIA) have been used for the determination of endogenous estrogens in aquatic environment, due to its relatively simple protocol and high sensitivity (Huang et al., 2001; Isobe et al., 2003). However, the testing kits available are limited and are generally only available for the endogenous estrogens such as 17β estradiol, estrone and ethynylestradiol. An ELISA test kit method for trenbolone is also available. However, these kits are generally designed to detect single compounds or a group of compounds belonging to a specific chemical family. If a wide range of compounds are needed, typically for a survey, the total range of kits is relatively expensive. Due to the range of analytes (17 in total) in this project, the ELISA and RIA techniques were not a cost effective option. Chromatographic techniques were an alternative for the chemical detection.

The introduction of tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) has largely improved the performance of the detection techniques by reducing the detection and quantification limits and enhancing analyte identification (Van Poucke et al., 2002). However, this instrumentation is not readily available in South Africa for research or commercial purposes. The instrument only became available for method development in May 2008 and subsequently water analysis commenced after three months.

1.11.3 Enzymatic deconjugation

Enzymatic hydrolysis is normally used to deconjugate hormones that are metabolised in biological systems. It was however published that this step is generally successful for the deconjugation of the glucuronide conjugate, but less successful with the sulphate conjugate (Huang et al., 2001). It is therefore necessary to keep in mind that the enzymatic hydrolysis may underestimate the concentration of the conjugated hormones, especially the sulphate conjugates.

In future a method that can determine both the active and the conjugated form should therefore improve the quantitative analysis of the compounds. Such methods have been published at least for steroid estrogens and their conjugates (Isobe et al., 2003).

1.11.4 Solid Phase Extraction (SPE) cleanup

Various SPE cartridges have been used in the literature to extract the hormones and steroids from environmental samples. A polymer cartridge, Oasis HLB, is mentioned in various publications as the method of choice (Isobe et al., 2003; De Alda et al., 2001). This cartridge has both a lipophilic and hydrophilic function as a sorbent and was suitable for the extraction of the wide variety of compounds in the study. It is however necessary that the packing material is packed in glass syringes rather than the normal plastic syringes to avoid contamination during the extraction step.

1.11.5 GC-MS technique

Some analytical methods were also published for endogenous b-E2 and E1 and exogenous EE2 using GC-EI-MS and GC-NCI-MS following derivatisation of the hydroxyl functional groups present in the molecular structure of the molecule (Diaz-Cruz et al., 2003). For the derivatisation of the hydroxyl functional groups present in the molecular structure of steroids, diverse derivatisation agents, such as N-methyl-N-(*tert*-butyldimethylsilyltrifluoracetamide (MTBSTFA), BSTFA and pentafluoropropionic acid anhydride (PFPA), have been used *tert*-Butyldimethylsilyl (tert-BDMS) and pentafluoropropionyl (PFP) derivatives are formed more quickly and are less sensitive to hydrolysis than many silyl derivatives, e.g. trimethylsilyl (TMS) derivatives. However, when using these reagents, partial derivatisation, and in many cases derivatisation in only the hydroxyl group of the unsaturated ring occurs, which in turn, results in less improvement in the sensitivity. Water decomposes both TMS reagents and derivatives. The derivatisation step must therefore be performed under complete anhydrous conditions otherwise the reagent is hydrolysed. If care is not taken, the derivatisation is not complete and the chromatographic separations seem to be poor and irreproducible. Working under anhydrous conditions is not always easy from a practical point of view and special precautionary measurements must be introduced. Because of the reactivity of the derivatisation reagent, the use of all plastic material was avoided during the sample cleanup process.

In the chromatographic step, taleranol, the metabolite of zeranol could not be distinguished from the natural metabolite of zearalenol. It is possible that the reduction on the molecule either happen during the injection/derivatisation step of the method, or the analytical standard provided was not provided with the desired purity.

Apart from these problems, GC-MS methods, in general cannot determine any conjugated compound such as the estradiol-3-sulfate and estradiol-3-glucuronide in the environment. Although it is suspected that these conjugates transform to the unconjugated format in the aquatic environment, little information on the behaviour and distribution of these conjugates are known (Isobe et al., 2003).

As was also found in this study, the chemical determination of EDC's in the environment is not an easy task; firstly, because of the complexity of the environmental matrices, and secondly, because of the usually low concentrations of the target compounds.

Despite the sensitivity of the GC-MS technique, accurate quantification is difficult because of high concentration factors required. More recently the tandem GC-MS technique is applied that is less susceptible to interferences by natural organic matter (Khan et al., 2008; Diaz-Cruz et al., 2003). This equipment was however, not available in South Africa during this study.

1.11.6 LC-MS/MS technique

The introduction of tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) improved the performance of the technique by reducing the detection limits and enhancing analyte identification (Diaz-Cruz et al., 2003). However, initially the instrumentation was not readily available in South Africa for commercial purposes. The instrument only became available in the FDA Laboratories for method development in May 2008 and subsequently water analysis commenced after three months.

An important consideration with the technique is the potential problems that may arise from matrix interference, especially when dealing with complex matrices, such as environmental samples. Matrix effects can be observed with any analytical tool because of insufficient co-extractive removal during the sample preparation stage. Ionisation suppression is a known phenomenon in LC-MS/MS by ESI, which occurs in the ionisation chamber, modifying the ionisation yield. To combat this problem, the laboratory used thorough extraction/clean up procedures for the removal of interfering contaminants, used an eluent additive to increase ionisation in the ionisation chamber and used matrix assisted calibration curves. The detection limits as specified in Table 1.10 is all below 10 ng/ℓ. This limit is well within the detection limits that are quoted in the literature (Stavrakakis et al., 2008; Noppe et al., 2005). Some of the theoretical detection limits could be lowered according to the S/N ration given in the table, however, the method was only validated with matrix assisted calibration curves up to the specified Limit of Quantification (LOQ).

1.12 RESULTS

The results of the analysis are set out in Table 1.11. The sampling points, as indicated in the first column of the table, correspond with the site description as explained in Table 1.5. Sampling points that can be compared as “duplicates” are indicated in brackets in column 1 of the table.

It is however, important to note that these sampling points were sampled at different time points (refer to the dates indicated in Table 1.5), and cannot be regarded as duplicate sampling points due to the changes in climatic conditions. The amount of cattle that were fed during that particular period within the feedlot also should have an effect on the particular sampling points sampled. Duplicate sampling points, should therefore not be interpreted as real duplicates because of all the variables involved between the sampling dates.

All pH values measured are indicated in column one of the table. All values were between 6 and 8 apart from water taken direct in or from the settling ponds.

Both these sites are water from sources that is expected to contain large amounts of urine and inorganic materials and thus the pH can be expected to be outside the normal range. The pH of the samples was not adjusted during the extraction procedure.

The method described by Waters in their application notes, do not indicate that samples must be adjusted when low level estrogens are determined. A high pH may lead to breakthrough in the extraction step. The consequence is that the values reported may be slightly underestimated

The detection limits of the method are set out in Table 1.10. From the S/N level indicated in the table, some of the compounds could be detected well below the value of 10 ng/ℓ. However, since the target DL of the method was set as 10 ng/ℓ during validation, no value below that limit could be quantified with an acceptable uncertainty value. Low levels of some of the analytes could however be detected and are indicated in the table. Apart from the detection of Zilpaterol, only 1.6% of the total amount of sampling points per analyte was positive for small concentrations of EDC's.

In 42% of the sites, concentrations of zilpaterol were detected. The values ranged from 69 ng/ℓ to the highest value of 23 703 ng/ℓ sampled. The sample originates from water underneath the surface and predominantly water holes within the feedlot. Water from sites 26 and 27 that are used for human consumption, contained no traces of zilpaterol. Four of the sites sampled, 25, 36, 46 and 47 were taken from river water. Three of the sites contained no detectable zilpaterol concentration, but site 36 contained a low level of zilpaterol, 82 ng/ℓ, during one of the time points when samples were collected. However, the use of a passive sampler as explained earlier, should be used in future to evaluate the importance of the concentrations measured in river water during such a study. Detectable concentrations of zilpaterol were found in most of the settling ponds. Water from the settling ponds is normally water directly gathered from the feedlot and is expected to contain the highest level of EDCs. The high levels found, between 3373 ng/ℓ and 23703 ng/ℓ could therefore be expected from those sampling points. From the values detected in the settling ponds, it seems as if zilpaterol is quite stable under the current environmental conditions. No literature up to date could be found on the environmental stability of Zilpaterol.

Settling ponds, for example, site 9, contained values of zilpaterol despite the argument that the water in this pond was gathered over a period of 3 to 36 months. On the permeability of zilpaterol through soil, the results were inconclusive. Sampling of water sources at site 17 and 40 would have given a clear indication of the permeability of zilpaterol. However, on one occasion, the values were below the detection limit and on the second occasion, a value of 735 ng/ℓ was detected.

From the results obtained and the above mentioned discussion, it can be concluded that relative high levels of zilpaterol could be found in and around water sources in the vicinity of the feedlots. The significance of the concentrations found is however, not clear since no other publications could be found that evaluated the possible estrogenic effects of zilpaterol.

Until such a time that more information is available on the endocrine descriptive effect of zilpaterol, the fate and behavior of this compound and other hormones in the aquatic environment, no risk assessment is possible.

1.13 CONCLUSIONS

The original project plan was designed to conduct a survey of ground and water samples of a large number of feedlots. During Phase 1 of the project it became apparent that each feedlot has a unique geographic design and virtually no similarities could be drawn between the different feedlots. In additional to this, the feedlots in South Africa are all privately owned and they were erected over a large number of years. During this period of time the environmental legislation of South Africa was changed and the impact of “newer” feedlots on the environment were assessed with environmental impact (EIA) studies. The endocrine disruptive effects of synthetic and natural hormones on the environment are however not part of the current EIA requirements. As a result of these facts and minor geographic discrimination between the feedlots, it was concluded that each feedlot must be assessed as a unique unit and cannot be included as in a generic model.

During the study, water was collected from 44 sampling points in three different feedlots, over three different time periods. Samples collected ranged from boreholes within the feedlots, settling ponds, surface water and water from the closest river. The samples were analysed with an AB 4000 QTRAP LC-MS/MS system. The analytes covered in the study included both natural and synthetic hormones that can be expected to be present in and around feedlots in South Africa. Only a limited amount of metabolites were covered and no conjugated compounds were covered in the chemical analysis. The detection limits reached during the study was well within the international accepted range of 10 ng/ℓ.

Low levels (below the detection limit of 10 ng/ℓ) of estrone, ethinylestradiol, diethylstilbestrol en testosterone were detected in a few water samples. In 42% of the sites, concentrations of zilpaterol were detected.

The concentrations found ranged from 69 ng/ℓ to the highest concentration of 23 703 ng/ℓ. The significance or impact of these concentrations found in the different water sources are however not clear since no other literature could be found that evaluated the possible estrogenic effects of zilpaterol. Until such a time that more information is available on the possible endocrine disruptive effects of zilpaterol, no clear conclusions can be drawn towards the possible short and long term effects of this compound on the environment.

1.14 RECOMMENDATIONS

The following recommendations could be drawn from Phase one of the project:

- All feedlots should be assessed on an individual basis towards the possibility that the excretion of natural and synthetic hormones could have an effect on the environment.
  It is also recommended that any new EIA within the South African environmental legislation should include a thorough study towards the fate of synthetic and natural hormones in the vicinity of a feedlot.
- Since the analytical methodology became available to include conjugated hormones in the analytical protocol, the detection of these substances should form part of any future study.
- If the sampling of water and stream water is included in future studies, water should be collect with the aid of passive samplers rather than grab samples as was used in this study. The use of passive samplers will reflect a more accurate concentration level that might be present. A better understanding of the possible long term leaching of these EDC into the environment would be possible with low level exposure data.
- The environmental fate and behaviour of Zilpaterol should be studied to ensure that a proper risk assessment can be conducted in and around feedlots.

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PHASE 2

EDC Activity of Selected Water Samples in and Around Selected Feedlots Using a Battery of Bio-Assays

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LIST OF ABBREVIATIONS

Abs	*Absorbance*
AR	*Androgen Receptor*
DHT	*Dihydrotestosterone*
DHTEq	*Dihydrotestosterone Equivalents*
E_2	*17β-estradiol*
EC_{50}	*Half Maximal Effective Concentration*
EDCs	*Endocrine Disrupting Compounds*
EEq	*Estradiol Equivalents*
EPA	*Environmental Protection Agency (United States)*
ER	*Human Estrogen Receptor*
ERα	*Human Estrogen Receptor Alpha*
ERβ	*Human Estrogen Receptor Beta*
ERE	*Estrogen-Responsive Elements*
FBS	*Fetal Bovine Serum*
GR	*Glucocorticoid Receptor*
MTT	*3-(4, 5-Dimethylthiazol-2-y1)2, 5-Diphenyl Retrazolium Bromide*
OHF	*Hydroxyflutamide*
RIE	*Relative Induction Efficiency*
RP	*Relative Potency*
SPE	*Solid Phase Extraction*
YES	*Recombinant Yeast Estrogen Screen*

2.1 INTRODUCTION AND OBJECTIVES

In-vitro bio-assays have been widely used as screens to determine if specific chemicals or environmental samples such as water or sediment 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).

2.1.1 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 JP 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).

2.1.2 The T47D-KBluc reporter gene assay

The United States (US) Environmental Protection Agency (EPA) developed an estrogen-dependent stable cell line. The T47D human breast cancer cells, which contain both endogenous ERα and ERβ, 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 ERβ (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 dimmer binds to the ERE on the reporter gene construct and activates the luciferase reporter gene. The presence of the luciferase enzyme can then be assayed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The amount of light produced is relative to the degree of estrogenic activity of the test chemical. When testing chemicals using the T47D-KBluc cells, an estrogen is defined as a chemical that induced dose dependent luciferase activity, which could be specifically inhibited by the 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 and can be conducted in 96 well plates and consistent results are produced.

2.1.3 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 and 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.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 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.

2.2.2 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).

2.2.3 Sample processing

2.2.3.1 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).

2.2.3.1.1 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 10x concentrated yeast stock and incubated at 28°C in a rotating water bath at 150-155 upm until turbid (Aneck-Hahn, 2003; Aneck-Hahn et al., 2005; 2008; Bornman et al., 2007).

2.2.3.1.2 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 (2.724 µg/ℓ to 1.3 ng/ℓ) which was extended to a concentration of 1.19 x 10^{-15} M (3.24 x 10^{-13} g/ℓ). The plates were sealed with parafilm (Cat. No. P7793, Sigma) and placed in a naturally ventilated incubator (Heraeus, B290) at 32°C for 3 to 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 from yellow to light orange. 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 17β-estradiol standard curve was fitted (sigmoidal function, variable slope) using Graphpad Prism (version 4), which calculated the minimum, maximum, slope, EC_{50} 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.

2.2.3.2 The T47D-KBluc reporter gene assay

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

2.2.3.2.1 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).

2.2.3.2.2 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 LUMIstar OPTIMA luminometer and quantified as relative light units. Each well received 25 µℓ reaction 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 17β-estradiol standard curve was fitted (sigmoidal function, variable slope) using Graphpad Prism (version 4), 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.

2.2.3.3 The MDA-kb2 reporter gene assay

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

2.2.3.3.1 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).

2.2.3.3.2 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 24h 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 LUMIstar OPTIMA luminometer and quantified as relative light units. Each well received 25 µℓ reaction 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 4), 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.

2.3 RESULTS

2.3.1 YES assay

The results of the feedlot water samples using the YES assay are tabulated in Table 2.1.

Table 2.1: Estrogenic activity in water samples from selected feedlots using the YES assay

Sample	Estrogenic activity		Cytotoxicity
	Result	EEq (ng/ℓ)	
1	<dl		Detected in 0.25x concentrated sample and higher concentrations
3	<dl		Detected in 6x concentrated sample and higher concentrations
4	Positive	0.38 ± 0.15	Detected in 25x concentrated sample and higher concentrations
8	n/q		Detected in highest concentrated sample (50x)
13	<dl		Detected in highest concentrated sample (50x)
14	n/q		Detected in 3x concentrated sample and higher concentrations
20	n/q		Detected in 1.5x concentrated sample and higher concentrations
39	<dl		-
41	n/q		-
45	<dl		-
46	<dl		-

<dl: Below detection limit of the assay plate

n/q: EEq not quantifiable, for less than 3 point above the dl were obtained

- : No cytotoxicity observed

An EEq value could only be calculated for sample 4 (Water management dam from feedlot – inloop, 0.38 ± 0.15 ng/ℓ). Samples 8 (Water from feeding cradles), 14 (Borehole water – used in feedlot), 20 (Water downstream from feedlot) and 41 (Borehole – downstream) had points above the detection limit of the assay, but not enough points to be able to calculate an EEq value. Cytotoxicity was observed in most of the samples and could be responsible for masking of estrogenic activity if such activity was present in the samples.

2.3.2 The T47D-KBluc reporter gene assay

The results of the feedlot water samples using the T47D-KBluc assay are tabulated in Table 2.2.

Table 2.2: Estrogenic and anti-estrogenic activity in water samples from selected feedlots using the T47D-KBluc reporter gene assay

Sample	Estrogenic activity		Anti-estrogenic activity/Cytotoxicity
	Result	EEq (ng/ℓ)	
1	<dl		Detected in 0.1x concentrated sample and higher concentrations
3	Positive	2.57 ± 0.39	-
4	Positive	0.32 ± 0.04	Detected in unconcentrated sample and higher concentrations
8	Positive	0.02 ± 0.004	-
13	Positive	0.13 ± 0.03	-
14	Positive	0.14 ± 0.02	-
20	Positive	0.94 ± 0.67	Detected in 3x concentrated sample and higher concentrations
39	<dl		-
41	Positive	0.47 ± 0.01	-
45	Positive	0.25 ± 0.14	-
46	Positive	0.04 ± 0.007	-

<dl: Below detection limit of the assay plate

- : No cytotoxicity observed

Nine of the 11 feedlot water samples tested positive for estrogenic activity with EEq values ranging from 0.02 ng/ℓ to 2.57 ng/ℓ. However, only sample 3 (Runoff water from feedlot gathered in settling dam), exceeded the Predicted-No-Effect-Concentration (1 ng/ℓ) for 17β-estradiol in water. When different sample concentrations of sample 1 (Groundwater – upstream from feedlot), 4 (Water management dam from feedlot – inloop) and 20 (Water downstream from feedlot) were co-incubated with 100 pM (27 ng/ℓ) 17β-estradiol (positive control), the 17β-estradiol activity was reduced at higher concentrations of the sample extracts, indicating possible anti-estrogenic activity or cytotoxicity of the sample. In order to distinguish between an anti-estrogenic or cytotoxic reaction, a test for cytotoxicity (e.g. MTT (3-(4, 5-dimethylthiazol-2-y1) 2, 5-diphenyl tetrazolium bromide test) colorimetric test) would be required. Sample 4 and sample 20 tested positive for estrogenic activity, but also reduced 17β-estradiol activity at higher concentrations. The EEq values for these two samples are therefore probably underestimated due to the concurrent presence of a cytotoxic or anti-estrogenic substance in the sample mixture.

2.3.3 The MDA-kb2 reporter gene assay

No androgenic activity was observed in any of the feedlot water samples, as none of the samples were able to obtain a two-fold induction relative to the vehicle control. When different sample concentrations of sample 1 and sample 41 were co-incubated with 1 nM (290 ng/ℓ) DHT (positive control), the DHT activity was reduced at higher concentrations of the sample extracts, indicating possible anti-androgenic activity or cytotoxicity of the sample. In order to distinguish between an anti-androgenic or cytotoxic reaction, a test for cytotoxicity (e.g. MTT test) would be required.

2.4 DISCUSSION AND CONCLUSIONS

The results from the bio-assays indicate that compounds with estrogenic activity are present in water samples from the selected feedlots. This activity may not be solely attributed to the veterinary growth stimulants as other sources of estrogenic contamination may be present in the vicinity of the sampling point.

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 in the YES assay 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. This is confirmed by the estrogenic activity measured for five sample sites (3, 8, 13, 14 and 20) in the T47D-KBluc assay.

Some of the samples that were below the detection limit of the YES assay tested positive for estrogenic activity in the T47D-KBluc assay (Table 4.1). This could be explained by the fact that the yeast cells contain only the ERα, but the T47D-KBluc cells contain both the endogenous ERα and ERβ, making the T47D-KBluc assay more sensitive for estrogenic activity compared to the YES assay.

Although only sample 3 exceeded the Predicted-No-Effect-Concentration (1 ng/ℓ) for 17β-estradiol in water it must be noted that EEq values could be under estimations 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 water and sediment samples consist of a complex mixture of chemicals with possible (anti)-androgenic and (anti)-estrogenic activity, as well as other chemicals not measured, that could affect the outcome of the assay. It should therefore be noted that EEqs are only rough estimates, as the complexity of the sample, pH, extraction procedure and the nature of the assay (i.e. a biological system) might all have an influence on the results and may possibly lead to an under-estimation of the results or even to false negatives.

The absence of androgenic activity measured in the MDA assay might also be attributed to the complexity of the samples and the fact that the pH was not adjusted (pH 3) prior to extraction. At present the EPA is having difficulty in obtaining reliable responses in the MDA assay with environmental samples and an alternative assay for environmental samples is being considered. Therefore, this assay might not be the ideal choice when screening environmental samples (personal communication Dr V Wilson, 20-23 October 2008).

The results strengthen the argument for the use of a battery of screening bio-assays which can complement each other and give a more comprehensive assessment on the estrogenic activity in environmental samples.

Table 2.3: Summary of the bio-assay results in Phase 2

	Estrogenic activity YES		***Estrogenic activity K-Bluc***	
Samples	***Result***	***Cytotoxicity***	***Result***	***Cytotoxicity***
1	*<dl*	✓	*<dl*	✓
3	*<dl*	✓	*Positive*	-
4	*Positive*	✓	*Positive*	✓
8	*n/q*	✓	*Positive*	-
13*	*<dl*	✓	*Positive*	-
14	*n/q*	✓	*Positive*	-
20	*n/q*	✓	*Positive*	✓
39*	*<dl*	-	*<dl*	-
41	*n/q*	-	*Positive*	-
45	*<dl*	-	*Positive*	-
46	*<dl*	-	*Positive*	-

<dl: Below the detection limit of assay; n/q: positive but not quantifiable; ✓: cytotoxicity present;

* Same sample site different sampling period

2.5 RECOMMENDATIONS

The EPA is currently in the process of developing a new assay for detecting androgenic activity in environmental samples. The assay uses 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 that the cell line be included in future projects.

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PHASE 3

EDC Activity of Individual Veterinary Growth Stimulants Using a Battery of Bio-Assays

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LIST OF ABBREVIATIONS

Abs	*Absorbance*
AR	*Androgen Receptor*
DHT	*Dihydrotestosterone*
DHTEq	*Dihydrotestosterone Equivalents*
E_2	*17β-estradiol*
EC_{50}	*Half Maximal Effective Concentration*
EDCs	*Endocrine Disrupting Compounds*
EEq	*Estradiol Equivalents*
EPA	*Environmental Protection Agency (United States)*
ER	*Human Estrogen Receptor*
ERα	*Human Estrogen Receptor Alpha*
ERβ	*Human Estrogen Receptor Beta*
ERE	*Estrogen-Responsive Elements*
FBS	*Fetal Bovine Serum*
GR	*Glucocorticoid Receptor*
OHF	*Hydroxyflutamide*
RIE	*Relative Induction Efficiency*
RLU	*Relative Light Units*
RP	*Relative Potency*
SPE	*Solid Phase Extraction*
YES	*Recombinant Yeast Estrogen Screen*

3.1 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).

3.1.1 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 JP 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).

3.1.2 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 ERβ, 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 ERβ (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 dimmer binds to the ERE on the reporter gene construct and activates the luciferase reporter gene. The presence of the luciferase enzyme can then be assayed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The amount of light produced is relative to the degree of estrogenic activity of the test chemical. When testing chemicals using the T47D-KBluc cells, an estrogen is defined as a chemical that induced dose dependent luciferase activity, which could be specifically inhibited by the 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 and can be conducted in 96 well plates and consistent results are produced.

3.1.3 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 and 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.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Sample collection and storage

Stock solutions (100 ppm) of the compounds were made up in ethanol by ARC Veterinary Residue Laboratory, at Onderstepoort and the FDA Laboratory, in Pretoria. These were collected and stored at -20°C until assayed.

3.2.2 Sample processing

3.2.2.1 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).

3.2.2.1.1 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 10x concentrated yeast stock and incubated at 28°C in a rotating water bath at 150-155 upm until turbid (Aneck-Hahn, 2003; Aneck-Hahn et al., 2005; 2008; Bornman et al., 2007).

3.2.2.1.2 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 (2.724 µg/ℓ to 1.3 ng/ℓ) which was extended to a concentration of 1.19 x 10^{-15} M (3.24 x 10^{-13} g/ℓ). The plates were sealed with parafilm (Cat. No. P7793, Sigma) and placed in a naturally ventilated incubator (Heraeus, B290) at 32°C for 3 to 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 17β-estradiol standard curve and veterinary growth stimulant curves were fitted (sigmoidal function, variable slope) using Graphpad Prism (version 4), which calculated the minimum, maximum, slope, EC_{50} 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.

3.2.2.2 The T47D-KBluc reporter gene assay

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

3.2.2.2.1 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).

3.2.2.2.2 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 LUMIstar OPTIMA luminometer and quantified as relative light units (RLU). Each well received 25 µℓ reaction 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 17β-estradiol and veterinary growth stimulant curves were fitted (sigmoidal function, variable slope) using Graphpad Prism (version 4), which calculated the minimum, maximum, slope, EC_{50} value and 95% confidence limits.

3.2.2.3 The MDA-kb2 reporter gene assay

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

3.2.2.3.1 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).

3.2.2.3.2 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 24h 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 LUMIstar OPTIMA luminometer and quantified as Relative Light Units (RLU).

Each well received 25 µℓ reaction 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 curves for DHT and selected veterinary growth stimulants were fitted (sigmoidal function with variable slope) using Graphpad Prism (version 4), which then calculated the minimum, maximum, slope, EC_{50} value and 95% confidence limits.

3.2.3 Relative Potency (RP) and Relative Induction Efficiency (RIE) calculations

RP and RIE values were calculated for veterinary growth stimulants that had estrogenic or androgenic activity.

RP were calculated with the following formula:

RP = EC_{50} of positive control/EC_{50} of test chemical * 100

RP gives an indication of the potency of a test chemical compared to the positive control (positive control = 100%), e.g. for a RP of 10 means that the test chemical is 10% as potent as the positive control and that a 10 times greater concentration would be needed to obtain the EC_{50} compared to the positive control.

RIE were calculated with the following formula:

RIE = max abs or RLU of test chemical/max abs or RLU of positive control * 100

RIE gives an indication of the maximum estrogenic or androgenic activity that could be obtained with a test chemical as a percentage of the positive control, e.g. for a RIE of 50 means that the maximum estrogenic or androgenic response of a test chemical is only 50% of the response that could be obtained with the positive control and will never reach the maximum activity that could be obtained by the positive control, not even by increasing the concentration of the test chemical.

3.3 RESULTS

3.3.1 YES assay

The reaction of the individual growth stimulants using the YES assay are graphically represented in Figure 3.1 and their EC_{50} values, RP and RIE values are tabulated in Table 3.1.

Table 3.1: EC_{50}-, RP- and RIE-values of selected veterinary growth stimulants using the YES assay

Sample	Estrogenic activity		
	EC_{50} (ng/ℓ)	RP (%)	RIE (%)
Androstanolone	348 267 ± 15 235	0.005	99
Androstenedione	6 819 333 ± 520 440*	0.0003*	59*
Diethylstilbestrol	247 ± 6.8	7	102
Epiandrosterone	398 033 ± 199 447	0.005	96
17β-estradiol	18.4 ± 8.4	100	100
Estriol	9 846 ± 1 498	0.2	97
Estrone	<dl		
Medroxyprogesterone acetate	14 380 000 ± 2 111 705	0.0001	51*
Methyltestosterone	5 935 333 ± 1 373 587	0.0003	59
Nandrolone	456 433 ± 30 908	0.004	98
Progesterone	4 453 000 ± 648 391*	0.0004*	68*
Testosterone	1 673 666 ± 57 500	0.001	77
β-Trenbolone	308 500 ± 12 010	0.006	99
α-Zearalanol	2 188 ± 288	0.8	96
Zilpaterol	445 867 ± 11 927	0.004	97

<dl: Below detection limit of the assay plate

* A full dose-response curve could not be obtained at the maximum concentration, therefore values are underestimated

Diethylstilbestrol, α-zearalanol and estriol had the highest estrogenic activities. β-trenbolone, androstanolone, zilpaterol and epiandrosterone showed much weaker estrogenic activity (RP between 0.004% and 0.006%), but were still able to obtain full dose-response curves.

Testosterone, methyltestosterone, androstenedione, progesterone and medroxyprogesterone were however not able to reach the maximum response obtainable by E_2 at the tested concentrations.

The RP of testosterone is 0.001%, therefore a 100 000x higher concentration is necessary to reach the EC_{50} compared to E_2. The maximum estrogenic activity obtainable with testosterone is only 77% of the activity obtained with E_2. Estrone did not react in the YES assay.

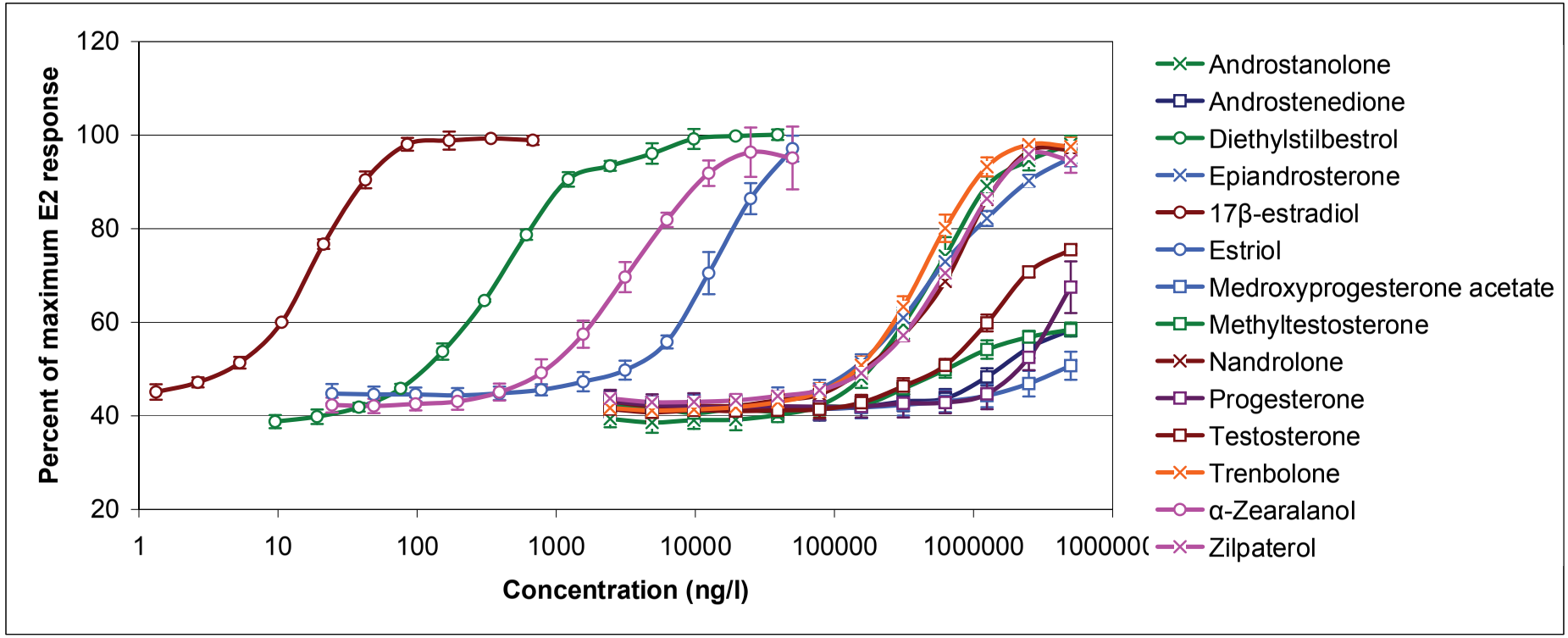

Figure 3.1: Estrogenic activity of selected veterinary growth stimulants using the YES assay

3.3.2 The T47D-KBluc reporter gene assay

The reaction of the individual veterinary growth stimulants using the T47D-KBluc assay are graphically represented in Figure 3.2 and their EC_{50} values, RP and RIE values are tabulated in Table 3.2.

Table 3.2: EC_{50}-, RP- and RIE-values of selected veterinary growth stimulants using the T47D-KBluc assay

Sample	Estrogenic activity			Anti-estrogenic activity
	EC_{50} (ng/ℓ)	RP (%)	RIE (%)	
Androstanolone	9301 ± 398	0.005	77	Not detected
Androstenedione	<dl			Not detected
Diethylstilbestrol	4.2 ± 0.4	10	109	Not detected
Epiandrosterone	5923 ± 902	0.007	118	Not detected
17β-estradiol	0.4 ± 0.05	100	100	Not detected
Estriol	9.8 ± 1.2	4	115	Not detected
Estrone	0.9 ± 0.1	49	103	Not detected
Medroxyprogesterone acetate	<dl			Detected
Methyltestosterone	<dl			Detected
Nandrolone	<dl			Detected
Progesterone	<dl			Detected
Testosterone	12948 ± 959	0.003	57	Not detected
β-Trenbolone	<dl			Detected
α-Zearalanol	11.2 ± 2.7	4	83	Not detected
Zilpaterol	5233 ± 286	0.008	89	Not detected

<dl: Below detection limit of the assay plate

Estrone had the highest estrogenic activity (RP = 49%), followed by diethylstilbestrol, estriol and α-zearalanol. Epiandrosterone, zilpaterol, androstanolone and testosterone had weak estrogenic activities with androstanolone and testosterone not being able to reach maximum activity obtainable with E_2. Possible anti-estrogenic activities were detected in medroxyprogesterone acetate, methyltestosterone, nandrolone, progesterone and β-trenbolone.

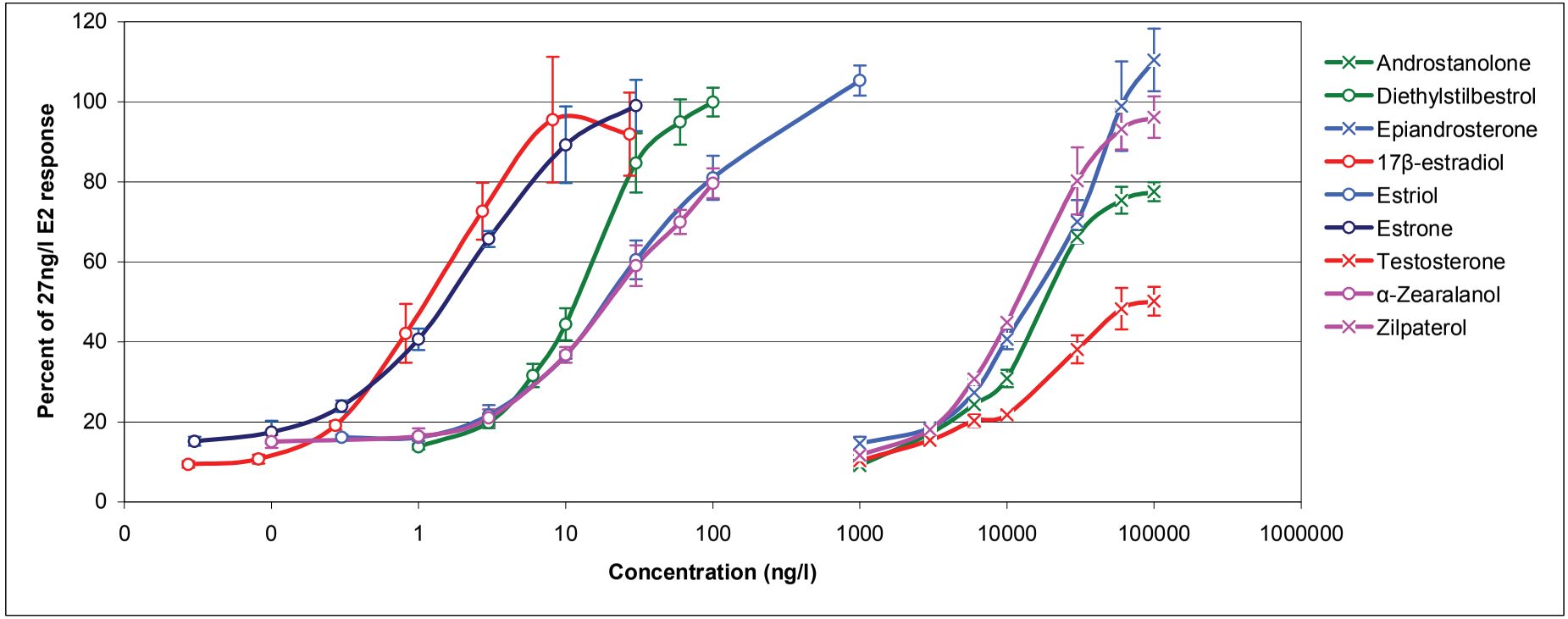

Figure 3.2: Estrogenic activity of selected veterinary growth stimulants using the T47D-KBluc assay

3.3.3 The MDA-kb2 reporter gene assay

The reaction of the individual veterinary growth stimulants using the MDA-kb2 assay are graphically represented in Figure 3.3 and their EC_{50} values, RP and RIE values are tabulated in Table 3.3.

Table 3.3: EC_{50}-, RP- and RIE-values of selected veterinary growth stimulants using the MDA-kb2 assay

Sample	Androgenic activity			Anti-androgenic activity
	EC_{50} (ng/ℓ)	RP (%)	RIE (%)	
Androstanolone (DHT)	21.5 ± 1.0	52	96	Not detected
Androstenedione	1360 ± 306	0.8	106	Not detected
DHT positive control	11.1 ± 3.9	100	100	Not detected
Diethylstilbestrol	<dl			Not detected
Epiandrosterone	23570 ± 13383	0.05	62	Not detected
17β-estradiol	26228 ± 15417	0.04	62	Not detected
Estriol	<dl			Not detected
Estrone	51048 ± 11613	0.02	45	Not detected
Medroxyprogesterone acetate	37.7 ± 2.8	29	157	Not detected
Methyltestosterone	38.9 ± 3.5	29	112	Not detected
Nandrolone	26.2 ± 1.8	42	107	Not detected
Progesterone	115845 ± 21623	0.01	37	Not detected
Testosterone	43.6 ± 1.1	25	98	Not detected
β-Trenbolone	27.1 ± 10.2	41	108	Not detected
α-Zearalanol	<dl			Not detected
Zilpaterol	<dl			Not detected

<dl: Below detection limit of the assay plate

Androstanolone, nandrolone, β-trenbolone, testosterone, methyltestosterone and medroxyprogesterone acetate had the highest androgenic activities with RPs above 29%. Androstenedione had a slightly weaker androgenic activity with a RP of 0.8%.

E_2, epiandrosterone, estrone and progesterone were not able to reach maximum activity obtainable with DHT at the tested concentrations and their RIE were all below 65%.

Diethylstilbestrol, estriol, α-zearalanol and zilpaterol did not react in the MDA-kb2 assay and no anti-androgenic activity was detected in any of the samples.

It is not clear why the veterinary test compound androstanolone (DHT) had a RP of only 52% compared to the DHT that was used as the positive control. Differences in purity and manufacturers of the chemicals could explain the difference in androgenic activity.

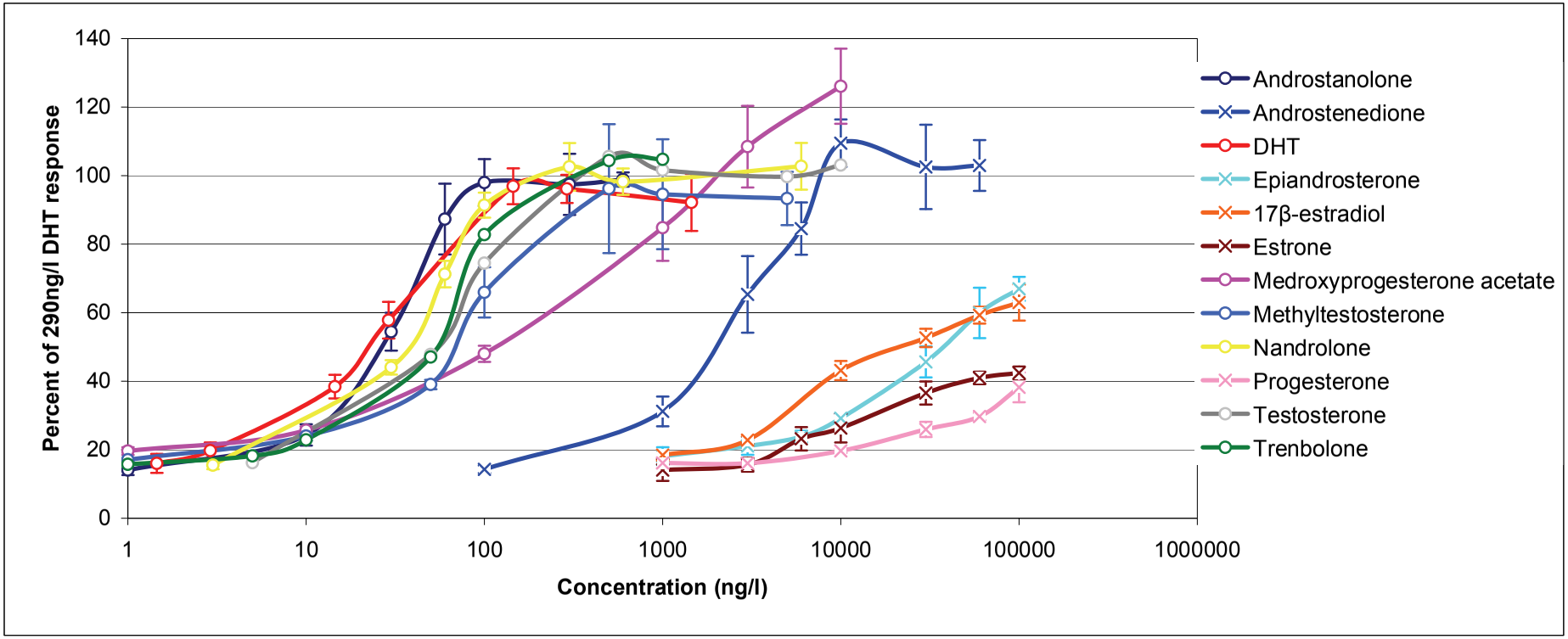

Figure 3.3: Androgenic and anti-androgenic activity of selected veterinary growth stimulants using the MDA-kb2 assay

3.4 DISCUSSION AND CONCLUSIONS

Test compounds compared well between the YES assay and the T47D-KBluc assay. Although the EC_{50} values for each compound differed greatly between the YES and T47D-KBluc assay, the RP values were close to each other for all of the chemicals, except for estriol and α-zearalanol. The yeast cells only contain ERα, whereas the T47D-KBluc cells contain both ERα and Erβ. Estriol has a greater binding affinity for ERβ than for ERα (Zhu et al., 2006), which could explain why the RP in the T47D-KBluc assay (RP = 4%) was higher than in the YES assay (RP = 0.2%). α-Zearalanol (zeranol) binds to both the ERα and Erβ (Takemura et al., 2007), which could explain why the RP is higher in the T47D-KBluc assay (RP = 4%) as this cell line has both receptor ERα and Erβ, compared to the YES assay (RP = 0.8%) which only has ERα.

Androstenedione, medroxyprogesterone acetate, methyltestosterone, nandrolone, progesterone and β-trenbolone tested positive for estrogenic activity in the YES assay, but were under the detection limit of the T47D-KBluc assay. Possible reasons are that samples can be tested at higher concentrations in the YES assay due to the assay protocol and secondly, samples are incubated for three to five days in the YES assay compared to one day in the T47D-KBluc assay, thereby missing estrogenic activity of slower reacting chemicals in the latter assay.

Estrone gave a good estrogenic response in the T47D-KBluc assay, but was below the detection limit of the YES assay. Zhu et al. demonstrated that estrone preferentially binds to ERα (Zhu et al., 2006), so it is not clear why estrone did not react in the YES assay containing the ERα.

The results strengthen the argument for the use of a battery of screening bio-assays, which can complement each other and give a more comprehensive assessment on the estrogenic and androgenic activity of veterinary growth stimulants.

3.5 RECOMMENDATIONS

It is recommended that a second screening assay for androgenic activity be included in future studies for a more comprehensive assessment of androgenic activity.

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PHASE 4

Interactive Effects of a Relevant Environmental Mixture of Veterinary Growth Stimulants on Fertility Parameters in Male Rats

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LIST OF ABBREVIATIONS

AGD	*Ano-Genital Distance*
EDC	*Endocrine Disrupting Chemical*
DDT	*Dichloro-Diphenyl Trichloro-Ethane*
DM	*Deltamethrin*
DES	*Diethylstilbestrol*
FSH	*Follicle-Stimulating Hormone*
LH	*Lutenizing Hormone*
OECD	*Organization for Economic Cooperation and Development*
p-NP	*p-Nonylphenol*
SA	*South Africa*
TBG	*Thyroid-Hormone-Binding Globulin*
TSH	*Thyrotropin*
T3	*Triiodothyronine*
T4	*Thyroxine*

4.1 INTRODUCTION AND OBJECTIVES

The hypothesis that xenobiotic chemicals used daily in commerce or natural chemicals released into the environment had the potential to disrupt the endocrine system of humans and animals was formulated as far back as the early 1990s (Guillette, 2006). This hypothesis has become known as the endocrine disrupting contaminant hypothesis (Colborn and Clement, 1992). Early work on endocrine disruption has been associated with wildlife, with observations describing estrogenic, anti-estrogenic, androgenic, anti-androgenic and anti-thyroid actions (Guillette, 2006).

It has been suggested that the apparent decline in human male reproductive health might be caused by an excess of estrogenic compounds which are often in daily use in industry, agriculture and in the home (Sharpe, 1993; Jensen et al., 1995). The effect of xenoestrogens is not limited to infertility, but is also increasingly associated with testicular and prostatic carcinoma, hypospadia, a high incidence of epididymal cysts and cryptorchidism. Many chemical compounds used as insecticides, pesticides, herbicides, etc. possess endocrine disrupting effects (Toppari et al., 1996).

The aquatic environment is a potential source of exposure, as studies have revealed that surface waters worldwide are contaminated with endocrine disrupting chemicals (EDCs) released from sewage treatment plants (Soto et al., 2004; Guillette, 2006) and paper pulp mills (Guillette, 2006). Another possible source of contamination are studies that focused on natural hormones released from animal waste used to fertilise agricultural fields (Orlando et al., 2004) and feedlot effluents (Soto et al., 2004). Veterinary drugs, such as growth stimulants and antibiotics are used extensively in agriculture, but few studies have been done on their presence in the aquatic environment near farms. In the USA anabolic agents are used in the cattle industry to increase growth. Primarily, the androgens, testosterone and trenbolone acetate; estrogens 17β-estradiol and zeranol and the progestogens, progesterone and melengestrol acetate are used (Lange et al., 2001; Soto et al., 2004). The fate of the excreted anabolic agents is unknown, but measurable amounts released from the farm animals reach the environment (Lange et al., 2002; Soto et al., 2004).

In South Africa (SA) there are different groups of environmental EDCs that contaminate water sources and that have shown some effects on health (Bornman et al., 2007). There is limited knowledge on the growth stimulants, used at cattle feedlots in SA, and if they are part of the mixture of EDCs that humans are exposed to. Numerous studies have documented the difficulty of establishing the link between exposure and effects in human populations (Guillette, 2006). Swan et al. (2005) found that the ano-genital distance, an end point in rodent developmental toxicology studies, decreased significantly with increased environmental exposure to phthalates, in prenatally exposed rodents (Guillette, 2006). Reproductive health effects in general are associated with EDC exposure. Therefore many endpoints in rodents may be used to predict adverse outcomes in humans (Guillette, 2006).

4.2 AIM

The objective of this project was to determine the possible effects of a relevant mixture of growth stimulants at environmental levels as determined in Phase 1, on male reproductive potential in exposed rats.

4.2.1 Materials and methods

4.2.1.1 Test System and Experimental Design

The study was performed after approval by the Ethics Committee of the University of Pretoria (**Project no: H031-07**). The Organization for Economic Cooperation and Development (OECD) 415 protocol for Toxicity Studies was modified to accommodate one control and three experimental groups (OECD, 1983). The design allowed for the study of the endocrine disrupting effects of maternally exposed rats (P1) to known EDCs on the fertility and reproductive parameters in lifetime exposed F1 males.

Twenty, seven day pregnant, female (P1) Sprague-Dawley rats were obtained from the University of KwaZulu-Natal – Biomedical Resource Unit. The rats were randomly divided into a control group (Group 1) and three experimental groups (Groups 2, 3 and 4) each containing five rats respectively (Figure 4.1). All animals were housed in standard polycarbonated cages in rooms with constant temperatures (22 ± 2ºC), constant humidity (55 ± 10%) and 12 hour day/night cycles. Rats were maintained on a stock pellet diet (Epol ®: Epol mice cubes, lot nr. 30101, Pta, SA) and had free access to food and tap water.

Seven day pregnant P1 females were orally gavaged for a three week period and during lactation till weaning of the F1 male pups (a subsequent three week period). After weaning twelve male offspring (F1) from each experimental and control group were subsequently dosed till 11 weeks of age. The individual animals were kept in separate cages and were directly exposed to the substances by oral gavage for the 11-week period at which time the F1 males were terminated for sample collection. All the males in the F1 generation were mated with the females (F1) of the same age (from the same groups) before termination in order to determine litter size outcome.

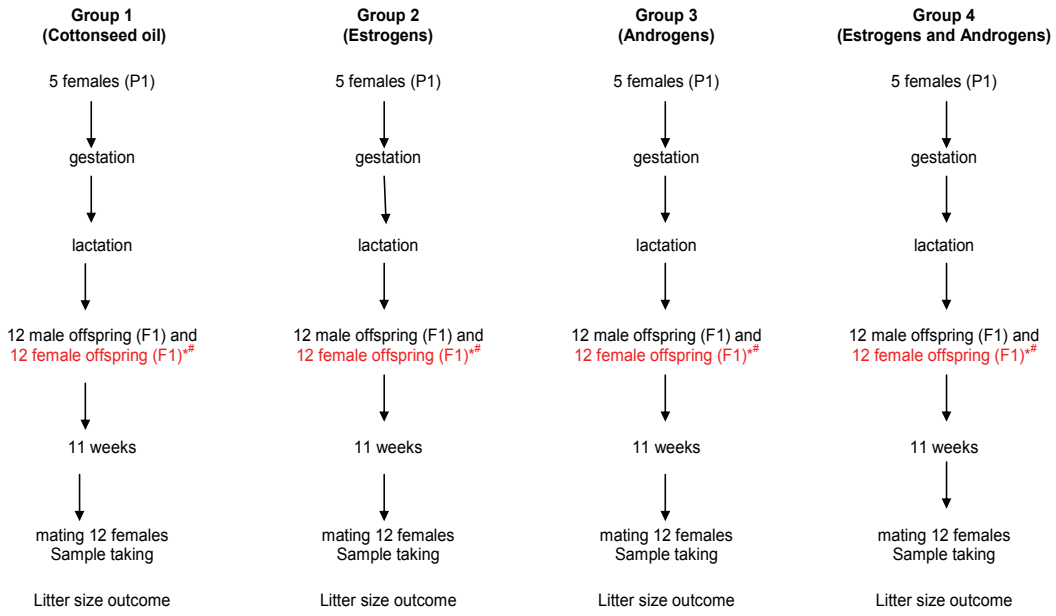

Figure 4.1: A schematic overview of the experimental design

4.2.1.2 Oral dosing

Based on the compounds detected in Phase 1 and the subsequent biological activity in Phase 3 of the study the following compounds were identified for use in this phase of the project, zilpaterol, diethylstilbestrol (DES), α-zearalanol, β-trenbalone, and methyltestosterone. The dosing concentrations were decided according the detection limit of the compounds in Phase 1 with an additional 20% added, in order to make the dosage environmentally relevant. It was decided to group the chemicals as an estrogenic group (Group 2), and androgenic group (Group 3) and a combination of all the compounds in Group 4 (Table 4.1).

The (P1) females were divided into 4 groups of 5 animals each. The control group was dosed with the vehicle only (cottonseed oil: Cat no 053K0077 Sigma-Aldrich, Steinheim, Germany). The following three groups were dosed with different mixtures of growth stimulants (as identified from Phase 1) till weaning (Table 4.1). Group 2 was dosed with a β-agonist, zilpaterol (Supplied to FDA laboratories by the feedlot) and two known estrogens, diethylstilbestrol (DES) (Sigma, 99%) and α-Zearalanol (Sigma, 97%). Group 3 was dosed with two known androgens β-trenbolone (Dr Ehrenstorfer GmbH, 95%) and methyltestosterone (Dr Ehrenstorfer GmbH), and Group 4 was dosed with a combination of growth stimulants used in Groups 2 and 3. All chemical mixtures were administrated intra-gastrically and the dose rate for all groups, including the control group, was 1 mℓ/kg adjusted according to body mass of each animal.

Table 4.1: The selected veterinary compound mixtures allocated to the three experimental groups at environmentally relevant concentrations

Group 1 (Control)	***Group 2 (Estrogens)***	***Group 3 (Androgens)***	***Group 4 (Estrogen and Androgen)***
Cottonseed oil only	*0.12 µg/kg Zilpaterol* *0.24 µg/kg Diethylstilbestrol (DES)* *2.4 µg/kg α-Zearalanol*	*12 µg/kg β-Trenbalone* *6 µg/kg Methyltestosterone*	*0.12 µg/kg Zilpaterol* *0.24 µg/kg DES* *2.4 µg/kg α-Zearalanol* *12 µg/kg β-Trenbalone* *6 µg/kg Methyltestosterone*

4.2.1.3 Observations and procedures

Throughout the experiment, the animals were observed daily for clinical health, behavioral changes, signs of toxicity and mortality. They were weighed and dosed at the same time every day to exclude any external variables.

The weights of the F1 males were recorded before anaesthesia. The males were anaesthetised with Isofor (provided by UPBRC). Cardiac puncture was used for blood collection. The blood collection, together with an overdose of Isofor vapour was used for termination. The blood was spun down and the serum used for TSH (thyrotropin), T3 (triiodothyronine) and T4 (thyroxine) estimation. After termination the ano-genital distance was measured, the testes, epididymides, seminal vesicles, prostate and the liver were weighed and the left testis fixed for histological evaluation.

4.2.1.3.1 Ano-genital distance

The ano-genital distance (AGD) was assessed by measuring the length of the perineum from the base of the genital tubercle to the centre of the anus when the skin was naturally extended, without stretching using a ruler and recorded in millimetres.

4.2.1.3.2 Testes, epididymides, seminal vesicles

The left and right testes and epididymides were removed, separated, cleaned and weighed individually. Both left and right seminal vesicles were removed and weighed. The mean testicular, epididymal and seminal vesicle mass were calculated. The left testis was placed in Bouins solution and was used for histology, with special reference to spermatogenesis (De Jager, 1999).

4.2.1.3.3 Cauda epididymal sperm count

An epididymal sperm count was done. The left cauda epididymis was separated from the caput-corpus and placed in 2 mℓ of phosphate buffered saline (PBS) medium (Cat no. P4417, Sigma) in a Petri dish. The cauda epididymis was cut up into very small pieces to free the sperm. The PBS with sperm was transferred to a Falcon tube. The Neubauer method (WHO, 1999) was used to determine the sperm concentration, expressed as million / mℓ.

4.2.1.3.4 Testicular histology and staging

Histological evaluations of the testis were done with special reference to spermatogenesis. Stages of spermatogenesis were determined. Bouins solution was used as fixative for the samples. Bouins was prepared by mixing saturated Picric acid (Cat no. 295544M) with 125 mℓ Formaldehyde (Merck (PTY) LTD, Cat nr. SAAR2436020LP) and 25 mℓ of Acetic acid (Merck (PTY) LTD, Cat no. AB00063.2.5.) Testes were fixed for three days in Bouins fixative. The testicular samples were then washed with running tap water, 30% ethanol, 50% ethanol and then 70% ethanol to remove the fixative (Carson, 1992). Fixed cross-sections of the testes were embedded in paraffin wax and the testicular tissue was dehydrated in a graded series of ethanol. Thin sections, 3 µm, were cut on a microtome and stained with a modified periodic acid-Schiff's reaction (PAS) and counterstained with hematoxylin. Staging of spermatogenesis was done using an Olympus BX 41 microscope and Altra 20 Olympus camera with 10x, 20x and 100x objectives. A computer software program on spermatogenesis, STAGES 2.1 (Vangaurd Media Inc., Illinois, UDA), was used for the staging process together with a histological atlas by Russell (1990). For each of the 47 F1 male rats, thirty randomly selected seminiferous tubules were staged to identify and classify the 14 stages of spermatogenesis. The tubular diameter, seminiferous epithelium and lumen diameter for all thirty tubules were measured horizontally and vertically. The mean values of the horizontal and vertical measurements for each parameter were used for the statistical analyses of the mentioned variables.

4.2.1.3.5 Thyroid function

Blood was collected from the heart and allowed to clot. It was then spun down at 3000 rpm for 15 minutes to collected serum that was then sent for TSH, T3 and T4 (Thyroid functions) estimations. Tests were done at the Faculty of Veterinary Science, Department of Production Animal studies, Reproduction Section. The following kits were used: Coat-A-Count Canine TSH IRMA (PIIK9T-5, 2006-12-29; Cat no IK 9T1), Coat-A-Count Total T3 (PITKT3-5, 2006-12-29; Cat no TKT31) and Coat-A-Count Canine T4 (PITKC4-5, 2006-12-29, Cat no TKC41). Coat-A-Count Canine TSH IRMA is an immunoradiometric assay designed to quantitative measurements of canine stimulating hormone (canine thyrotropin, cTSH) in serum. Coat-A-Count Total T3 is a solid-phase radioimmunoassay designed for the qualitative measurement of total circulating triiodothyronine (T3) in serum or plasma. Coat-A-Count Canine T4 is a solid-phase radioimmunoassay designed for the qualitative measurement of total thyroxine (T4) in canine serum.

4.2.1.4 Statistical Analysis

For between group comparisons of all variables one-way ANOVA (Analysis of Variance) was performed using ranks followed by pair-wise comparisons with Fisher's LSD (least significant difference method). Pair-wise comparisons between the control group and the treatment groups were performed at the Bonferroni adjusted level of significance (0.05/4 = 0.012) with the Wilcoxon Rank Sum test. For male F1 data the Kruskal-Wallis All-Pair-wise Comparisons Test was also performed but appears to be more stringent for between-group comparisons.

Therefore the ANOVA results on ranked data should be reported, followed by the Wilcoxon Rank Sum test for pair-wise comparisons with control group. Only significant findings and findings > 0.012 and < 0.05 were reported.

4.3 RESULTS

4.3.1 Daily observations

Throughout the experiment no behavioural changes or signs of toxicity were observed in any of the groups (P1, F1 males and F2 offspring). No birth defects or abnormalities were noted in F2 offspring.

4.3.2 P1 Females

According to the Fisher's LSD test there were no significant differences in the litter sizes between the groups. Using the same test there were also no significant differences in the male to female pup ratio between the groups.

4.3.3 F1 Males

The results for the F1 males are summarised in Table 4.2.

Table 4.2: A summary of the reproductive parameter results of the F1 males of the different treatment groups, using the Wilcoxon rank sum test.

Variables	*Group 1 Mean*	*Group 2 Mean*	*1:2 p-value*	Group 3 *Mean*	*1:3 p-value*	*Group 4 Mean*	*1:4 p-value*
Body mass (g)	*374.76*	*374.00*	*ns*	*360.64*	*ns*	*358.04*	*ns*
Ano-genital distance (mm)	*40.900*	*38.830*	*0.0670*	*38.170*	***0.0117***	*39.420*	*0.0865*
Seminal vesicle mass (g)	*0.7488*	*0.7916*	*ns*	*0.6862*	*ns*	*0.6271*	***0.0074***
Epididymal mass (g)	*0.5616*	*0.5687*	*ns*	*0.5830*	*ns*	*0.5964*	*ns*
Testicular mass (g)	*1.7807*	*1.8253*	*ns*	*1.7904*	*ns*	*1.8623*	*ns*
Seminiferous tubule diameter (µm)	*306.83*	*312.57*	*ns*	*316.83*	*ns*	*314.78*	*ns*
Seminiferous epithelium thickness (µm)	*100.19*	*101.70*	*ns*	*103.68*	*ns*	*102.56*	*ns*
Lumen diameter (µm)	*114.04*	*120.60*	*ns*	*121.69*	***0.0455***	*121.56*	***0.0289***
Total sperm count (x10^6	*57.036*	*46.300*	*ns*	*41.083*	***0.0337***	*47.033*	*n/s*
Prostate (g)	*0.9277*	*0.8778*	*ns*	*0.8600*	*ns*	*0.7789*	***0.0151***
Liver (g)	*18.132*	*17.364*	*ns*	*16.074*	*ns*	*16.362*	*ns*
T3 nmol/ℓ	*1.2127*	*1.2717*	*ns*	*1.2367*	*ns*	*1.2958*	*ns*
T4 nmol/ℓ	*64.395*	*74.190*	***0.0089***	*74.464*	***0.0210***	*64.793*	*ns*

Group 1: Control

Group 2: Zilpateral (0.12 µg/kg), DES (0.24 µg/kg), α-Zearalanol (2.4 µg/kg)

Group 3: β-Trenbalone (12 µg/kg), Methyltestosterone (6 µg/kg)

Group 4: Zilpateral, DES, α-Zearalanol; 3, β-Trenbalone, Methyltestosterone

Statistically significant: $p < 0.05$

The Kruswal-Wallis All Pair-wise Comparison and Fisher's LSD test showed no statistical differences between the control group and experimental groups for the mean body mass, total testicular mass, mean epididymal mass and mean liver mass. The Wilcoxon rank sum test indicated a statistically significant difference for the mean AGD between the control group (40.900 mm) and group 3 (38.167 mm; $p = 0.0117$), with a shorter AGD for group 3 (Table 4.2).

The Wilcoxon rank sum test indicated a statistically significant difference between the control group and group 4 ($p = 0.0074$).

The mean seminal vesicle mass for group 4 (0.6271 g) was lower than the control group (0.7488 g). The mean prostate mass for group 4 (0.7789 g; $p = 0.0151$) was statistically significantly lower than the control (0.9277 g). The total sperm count showed a statistically significant difference between the control group (57.036) and group 3 (41.083; $p = 0.0337$), with a lower total sperm count in group 3. TSH data was not analysed due to concentrations being too low (probably below the detection limit of the method) in rats. The Wilcoxon rank sum test indicated a statistically significant difference for the T4, between the control group (64.395 nmol/ℓ) and group 2 (74.190 nmol/ℓ; $p = 0.0089$), control group and group 3 (74.464 nmol/ℓ; $p = 0.021$), with a higher T4 in group 3 and 4 compared to the control group, as indicated in Table 4.2. The Fisher's LSD test indicated no differences in the T3 for the control group any experimental groups.

Using the Wilcoxon rank sum test a statistically significant difference in the total lumen diameter was found between the control group and group 3 ($p = 0.0455$), and between the control group and group 4 ($p = 0.0289$). Therefore, a larger lumen diameter in group 3 and 4 compared to the control group, as indicated in Table 4.2. There was no difference between the control group and experimental groups for the seminiferous tubule diameter and epithelium thickness. Histological evaluation of the testes indicated that all 14 stages of spermatogenesis were present. No abnormalities such as vacuolization were observed, although in group 3, two rats had immature germ cells present in the lumen (Figure 4.2 C).

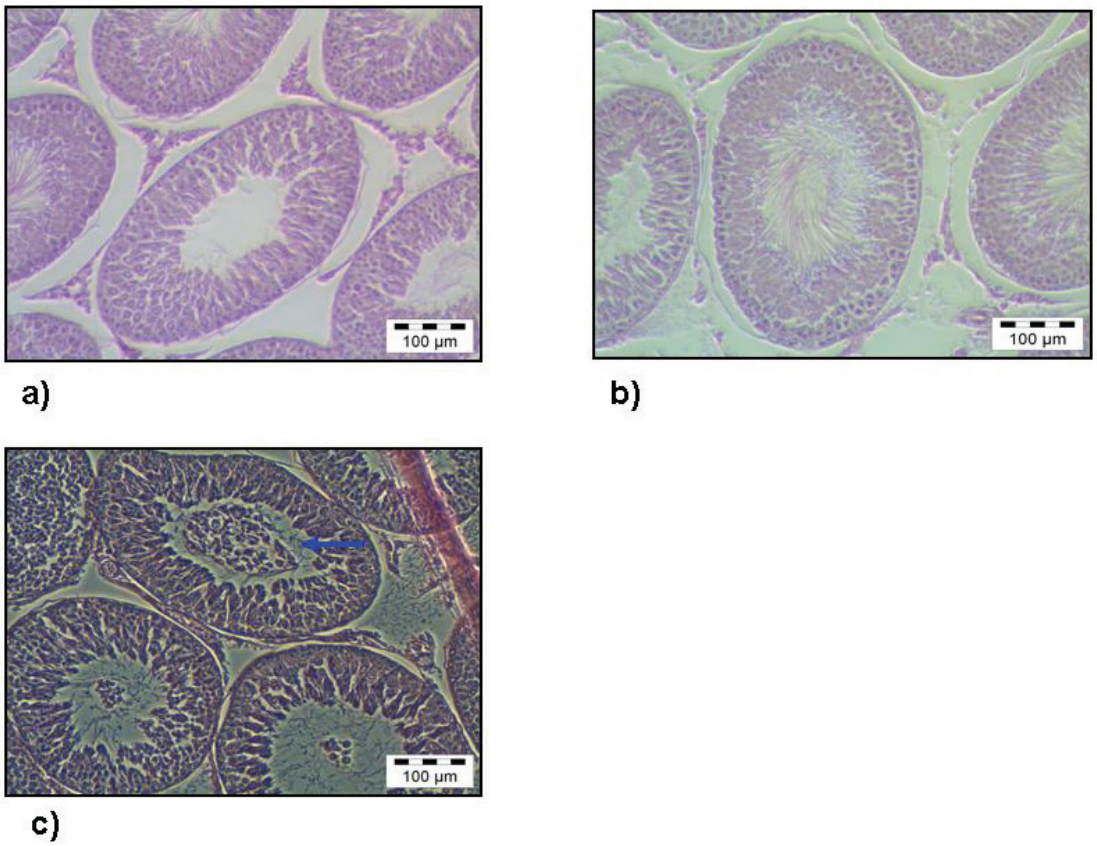

Figure 4.2. Testicular histology showing normal spermatogenesis in the seminiferous tubule of the control group 1 (a) and group 2 (b) compared to group 3 (c) where apical sloughing is present in the lumen.

Note the presence of apical sloughing of immature germ cells (indicated by the arrow) in (c)

Group 1: Control;

Group 2: Zilpateral (0.12 µg/kg), Diethylstilbestrol (DES) (0.24 µg/kg), α-Zearalanol (2.4 µg/kg);

Group 3: β-Trenbalone (12 µg/kg), Methyltestosterone (6 µg/kg);

Group 4: Zilpateral, Diethylstilbestrol (DES), α-Zearalanol; 3, β-Trenbalone, Methyltestosterone

4.3.4 F1 Females

Three out of 12 females in group 3 and 1 female out of 12 in group 4 did not fall pregnant.

4.3.5 Sex ratio – F2 pups

The Fisher's LSD test indicated no statistical significance between the control group and experimental group for the number of pups, number of males, number of females and ratio of males to females.

4.3.6 Body mass of F2 pups

The Wilcoxon rank sum test indicated a statistically significant difference in the mean mass for the male pups between the control group (53.567 g) and group 2 (47.657 g; $p = 0.000$), groups 2 and 3 (51.943 g; $p = 0.0105$), and groups 2 and 4 (53.386 g; $p = 0.000$).

The mean mass for the experimental groups were lower than the control group (53.567 g; 47.657 g; 51.943 g, 53.386 g), respectively. The inter group comparisons in the F2 males showed statistically significant differences between group 2 and 3 (p = 0.0105), and group 2 and 4 (p = 0.000) (Table 4.3).

The mass of the F2 female pups was also statistically significantly lower between the control group (53.473 g) and group 2 (47.246 g) (p = 0.000), groups 1 and 3 (50.167 g; p = 0.0189), groups 1 and 4 (49.908 g; p = 0.0119), and groups 2 and 4 (p = 0.0103) (Table 4.3).
Within the F2 female groups there was a statistically significant difference between groups 2 and 4 (Table 4.4).

Table 4.3: The effect of various mixtures on the mass of the F2 generation in the different treatment groups using the Wilcoxon rank sum test.

Variables	***Group 1***	***Group 2***	***1:2***	**Group 3**	***1:3***	***Group 4***	***1:4***
Weight (g) F2 Pups	***Mean***	***Mean***	***p-value Pair-wise***	***Mean***	***p-value Pair-wise***	***Mean***	***p-value Pair-wise***
Males	*53.567*	*47.657*	***0.000***	*51.943*	*0.1458*	*53.386*	*0.8902*
Females	*53.473*	*47.246*	***0.000***	*50.167*	***0.0189***	*49.908*	***0.0119***

Group 1: Control
Group 2: Zilpateral (0.12 µg/kg), Diethylstilbestrol (DES) (0.24 µg/kg), α-Zearalanol (2.4 µg/kg)
Group 3: β-Trenbalone (12 µg/kg), Methyltestosterone (6 µg/kg)
Group 4: Zilpateral, Diethylstilbestrol (DES), α-Zearalanol; 3, β-Trenbalone, Methyltestosterone
Statistically significant: $p < 0.05$

Table 4.4: Wilcoxon pair-wise results for the inter-group comparison of F2 males and females for the different treatment groups

	p- value	*p- value*	*p- value*
Gender	*2:3*	*2:4*	*3:4*
Males	***0.0105***	***0.0000***	*0.1610*
Females	*0.0626*	***0.0103***	*0.7944*

Group 2: Zilpateral (0.12 µg/kg), Diethylstilbestrol (DES) (0.24 µg/kg), α-Zearalanol (2.4 µg/kg)

Group 3: β-Trenbalone (12 µg/kg), Methyltestosterone (6 µg/kg)

Group 4: Zilpateral, Diethylstilbestrol (DES), α-Zearalanol; 3, β-Trenbalone, Methyltestosterone

Statistically significant: $p < 0.05$

4.4 DISCUSSION AND CONCLUSIONS

This study investigated the ED effects on *in utero,* lactation and direct exposure to the following veterinary compounds, zilpateral, diethylstilbestrol (DES), α-zearalanol, β-trenbolone and methyltestosterone on the reproductive parameters and thyroid functions of the F1 male rats.

In this study there were no statistically significant differences between the mean body mass of the control group and the experimental groups. However the mean masses of group 3 and 4 was lower than the control group. Similar results were found by Wason et al. (2003) in study looking at 17α-methyltestosterone at higher concentrations (10, 40 and 200 mg/kg), where the male rat's weights decreased by 23% in the 200 mg/kg group. In the NADA approval document for Finaplix®, Hunter found that trenbolone also had a similar effect on body mass (NADA, 1986).

Ano-genital distance is an established reproductive endpoint for rodent studies examining the effects of exposure to EDCs (Phillips and Foster, 2008). The ano-genital distance in males is usually twice the distance of females (Rhees et al., 1997). In mammals the masculinisation of the external male genitalia and longer ano-genital distance is regulated by dihydrotestosterone (DHT) (Clarke et al., 1990; Phillips and Foster, 2008). Mammals seem to possess a single androgen receptor (AR) that mediates the effects of endogenous androgens including, testosterone, androstenedione and DHT. As a result chemicals with either androgen or antiandrogen activity may interact with the AR, preventing endogenous hormonal action (Phillips and Foster, 2008). In group 3 (androgen group) the mean ADG was statistically significant shorter than the control group. Reduced male AGD is an indication of feminisation and has been observed after treatment with estrogenic compounds (Gray et al., 1994; Kelce et al., 1994; Kilian et al., 2007).

The shorter AGD in this study could be due to the fact that methyltestosterone is converted to 17α-methylestradiol which is a potent estrogen (Hornung et al., 2004; Kishner and Svec, 2008). Hornung et al. (2004) found that aromatisation of methyltestosterone to 17α-methylestradiol had estrogenic effects after exposure.

Rodent studies have indicated a physiological role for estrogens in prostate development (Prins et al., 2006). In contrast to humans, the rodent prostate is rudimentary at birth and undergoes extensive branching morphogenesis followed by functional differentiation during the first 15 days of life (Prins et al., 2006). Elevated levels of endogenous estrogens and/or estrogenic EDCs lead to permanent disturbances in prostate growth (Prins et al., 2006). A study by Ahlborg et al. (1992) found that post natal exposed rats to PCBs had a lowered prostate mass and also seminal vesicle mass (Ahlborg et al., 1992; Pfliegler-Bruss et al., 2004) and Prins et al. (2001) found that the seminal vesicle weight in DES exposed rats also had a lower seminal vesicle mass. Similarly in this study the mean seminal vesicle mass in group 4 (combination group) was statistically significantly lower compared to the control group. This was also the case for the mean prostate mass. The effect could be attributed to a synergistic estrogenic activity in group 4 as a result of the conversion of methyltestosterone to estradiol. The mean epididymal mass of the experimental groups did not differ statistically significantly from the control group, at histological level there may well be effects but this was not investigated.

The mean testicular mass across the experimental groups was higher than the control group, although not statistically significantly. Considering the cellular elements present in the testis and the fact that light microscopy was similar in general comparison to group 1, the possible explanation for the testicular mass could lie on a molecular level, particularly fluid homeostasis of the testis. Fluid secretion in the testis serves as an important function in the transport of oxygen and nutrients from blood to tubule via the interstitial fluid and of sperm and proteins from the rete testis via the seminiferous tubule fluid (Creasy, 2001). There was a marginal increase but not a statistically significant difference in the seminiferous tubule diameter and seminiferous epithelial thickness, stayed the same. The lumen diameter however, was statistically significantly larger in groups 3 ($p = 0.0455$) and 4 ($p = 0.0289$). Seminiferous tubule fluid is produced by the Sertoli cell and is androgen dependent; it is also regulated by the germ cell complement of the tubule, particularly by the presence of elongated spermatids (Creasy, 2001; Sharpe, 1989). The volume of the tubular fluid is a function of the rate of secretion, the rate of transport from the tubule, and the rate of reabsorption in the rete and epididymis (Creasy, 2001). Alteration in any of these functions may be reflected by the tubular lumen dilatation or contraction (Creasy 2001).

In this study dilatation was observed. In group 3, two rats showed apical sloughing of the immature germ cells (Figure 4.2) which is also a sign of Sertoli cell toxicity. In clinical andrology, the idiopathic oligo-astheno-teratozoospermia syndrome is the most common (De Jager et al., 1999). Estrogenic substances might be important in the pathophysiology of the syndrome (De Jager et al., 1999).

Low sperm counts have been associated with *in utero* and early life exposure to estrogenic chemicals (De Jager et al., 1999; Sharpe and Skakkebaek, 1993). In this study the total sperm count lowered across the groups but was statistically significantly lower in group 3. This can be due to the fact that the xenobiotic androgen receptor agonist and estrogenic compounds can cause a reduction in testosterone production from the testis, together with a reduced release of gonadotropins, lutenizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. Estradiol also exerts a negative feedback on FSH secretion. This can result in depressed spermatogenesis (Kilian et al., 2007; Wason et al., 2003).

The mean liver mass of the experimental groups did not statistically significant differ from the control group. But there was a trend in all experimental groups to be lower than the control group. The concentrations in this study may be too low for macroscopic effects in the liver, there may be effects at microscopic level, but this was not included in the study.

Thyroid hormones are essential for the regulation of several major functions in all vertebrates. These include metabolism, growth and development of the body systems. Therefore, normal thyroid functioning is crucial to fetal development; and the possible effects of EDCs are important. A study by Sinha et al. (1991) found that an organochlorine, endosulfan, resulted in elevated levels of circulating T4, coupled with decreased levels of T3 in freshwater catfish experimentally exposed. The suggested mechanism for this was that the pesticide blocks the extra-thyroidal conversion of T4 to T3. Hewitt et al. (2002) found a similar effect in alligators from an intermediately contaminated site in Florida, USA. In this study T4 was statistically significantly higher in group 2 and 3 compared to group 1. The increase may be due to the fact estrogens increase the thyroid-hormone-binding globulin (TBG) as indicated by Bisschop et al. (2006). The rise in TBG is parallel with a T4 increase (Bisschop et al., 2006). It must be noted that methyltestosterone can be converted to 17α-methylestradiol thereby eliciting an estrogenic response (Hornung et al., 2004). There was no difference between T3 in the control group and the experimental groups. There was also no statistically significant difference in T3/T4 ratio.

A single agent or chemical may be weakly estrogenic and have a low threshold of activity but in combination may produce noticeable effects (de Jager et al., 1999; Killian et al., 2007). Low doses of EDCs may exert more potent effects than higher doses, particularly if exposure occurs during a critical developmental window (Diamanti-Kandarakis et al., 2009; Sheenan et al., 1999). Current evidence suggests that mammals are more susceptible to EDCs during fetal and post-natal life than in adulthood (Sweeney, 2002). Many normal homeostatic endocrine feedback mechanisms and immune systems are not fully developed and there is the potential that low dose exposure could have adverse effects on the developing fetus (Crisp et al., 1998; Sweeney, 2002).

The data from this study indicates that veterinary growth stimulants had effects on reproductive and thyroid parameters in maternally and direct exposed male rats. This is cause for concern as essentially these compound mixtures end up in the aquatic system.

4.5 RECOMMENDATIONS

In order to elucidate some of these findings additional analyses on hormonal profiles is recommended, especially testosterone, estradiol, LH and FSH. A more comprehensive study of liver functions is also needed. The most applicable kit for thyroid profile analysis (i.e. the rodent thyroid radio-immunoassay kit) was not available in South Africa, therefore the canine kit was used and the sensitivity may have been compromised, resulting in some possible false negative outcomes.

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PHASE 5

Life Stages (Lifecycle) Exposure of *Clarias gariepinus* and *Oreochromis mossambicus* to Sub-Lethal Concentrations of Selected Growth Stimulants

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LIST OF ABBREVIATIONS

BNF	*Buffered Neutral Formalin*
DES	*Diethylstilbestrol*
EDCs	*Endocrine Disrupting Chemicals*
EE_2	*17β-Ethinyl Estradiol*
EPA	*Environmental Protection Agency*
GnRH	*Gonadotropin-Releasing Hormone*
GSI	*Gonadosomatic Index*
GtH	*Gonadotrophic Hormones*
MDHT	*Methyldihydrotestosterone*
MT	*Methyltestosterone*
OECD	*Organisation for Economic Co-operation and Development*
TBT	*Tributyltin*
VTG	*Vitellogenin*

5.1 INTRODUCTION AND OBJECTIVES

5.1.1 Introduction

Recently androgenic compounds have been identified in the runoff water from feedlots in the United Kingdom (Thomas et al., 2002); United States of America (Orlando et al., 2004; Durhan et al., 2006); Europe (Radl et al., 2005) and Australia (Williams et al., 2007). These compounds included Trenbolone and 17α-methyltestosterone that was also used in this study. Both these compounds are known to have endocrine disruptive effects in fish (Ankley et al., 2003; Hornung et al., 2004). This can have negative implications for fish populations similarly exposed to synthetic hormones and hormones (Orlando et al., 2004). A study done in Japan (Okinawa) has indicated that discharge from bovine/swine farms caused adverse health effects in exposed *Tilapia* species (Tashiro et al., 2003). Diethylstilbestrol (DES) and Zearalanol are estrogenic chemicals. DES was used as a growth hormone in the beef and poultry industry during the 1960s but removed in the late 1970s (Renu and Snedeker, 2000). Zearalanol is commonly used combined with other hormones or singly to lower the levels of follicle-stimulating hormone and leutinizing hormone in cattle (Schiffer et al., 2001) but it is not known if zearalanol can interact with Gonadotropin-releasing hormone (GnRH) or Gonadotrophic hormone (GtH) receptors in fish (Orlando et al., 2004).

In South Africa certain hormones and synthetic hormones are used as growth enhancers (veterinary compounds) at feedlots and could possibly be discharged into the aquatic environment. These growth enhancers are usually anabolic steroids and estrogenic chemicals and have properties that cause them to be Endocrine Disrupting Chemicals (EDCs). Although endocrine disruption has been identified in the late 1930s, it only recently emerged as a major issue in terms of science, public concern and regulatory policies in first world countries. Several scientific studies reported that Endocrine Disrupting Chemicals (EDCs) might be linked to reproductive, developmental and behavioural effects in humans and wildlife (Guillette LJ Jr et al., 1994; Bowerman et al., 1998; Colborn et al., 2000; Skakkebæk et al., 2001). Human male exposure to certain EDCs may explain the increasing incidences reported for cryptorchidism, hypospadias, testicular cancer and poor semen quality (Skakkebæk et al., 2001; Weber et al., 2002). Investigations on endocrine disruption reported imposex in mollusc species exposed to Tributyltin (TBT), an antifouling agent used in paint, and abnormal sexual development in alligators environmentally exposed to organochlorine pesticides. Intersex has been reported in fish exposed to effluents from municipal and industrial wastes. The occurrence of this accidental phenomenon has been reported in several fresh water and marine fish species from around the globe. In United Kingdom Rivers it has been reported in wild roach, *Rutilus rutilus*, (Jobling et al., 1998) and the gudgeon, *Gobio gobio* (Van Aerle et al., 2001), the three spined stickleback, *Gasterosteus aculeatus*, from Northeastern Germany (Gercken and Sordyl, 2002), the barbel, *Barbus plejebus*, Italy (Viganò et al., 2001) and in the shovelnose sturgeon, *Scaphirhynchus platyorynchus*, USA (Harshbarger, 2000).

Recently the first evidence of intersex in a South African fish species (*Clarias gariepinus)* has been reported (Barnhoorn et al., 2004).

The sharptooth catfish, *Clarias gariepinus,* and Mozambique Tilapia, *Oreochromis mossambicus,* are both indigenous species to South Africa. *C. gariepinus* is considered to be one of the most important tropical catfish species for aquaculture and has an almost Pan-African distribution (De Graaf and Janssen, 1996). In South Africa *C. gariepinus* are present in large dams and natural aquatic systems across the country. Both these species inhabit calm waters from lakes, streams, rivers, swamps to floodplains. *C. gariepinus* can survive cases where swamps and floodplains are subjected to seasonal drying, but the presence of the accessory air breathing organs allows the catfish to survive (Skelton, 1993). Both *C. gariepinus* and *O. mossambicus* have distinct uro-genital sexual papilla, located behind the anus, which are absent in females (De Graaf and Janssen, 1996) to facilitate sex allocation.

No research has been conducted of the possible effects that exposure to selected veterinary compounds can have on aquatic organisms in South Africa. *C. gariepinus* and *O. mossambicus* should be useful models to use as biomarkers of exposure to selected veterinary compounds possibly discharged in South African waters. *C. gariepinus* is the species of choice but both these species can be included in the study to make sure that results obtained can be used, should difficulties be experienced in the spawning and supply of fingerlings.

Fish, alligators, frogs and rodents have been used to evaluate *in vivo* reproductive toxicity effects of EDCs in laboratory experiments (Guillette LJ Jr et al., 1994; De Jager, 1994; Nimrod and Benson, 1996). The applicability of *in vivo* models enhances the ability to indicate the presence and physiological effects of veterinary compounds in the aquatic environment.

5.1.2 Partial lifecycle tests

The early life stages of fish are the most sensitive period of the cycle and include the embryo/larval period (Environmental Protection Agency (EPA), 2002; Roex et al., 2002). During most partial lifecycle tests the embryo/larval tests should be a more exact evaluation of the toxic effects of pollutants on fish. Most partial lifecycle tests are done over periods of between 7 and 60 days according to the species early lifecycle.

Chronic static renewal exposure toxicity studies evaluate not only mortality, but also investigate endpoints such as individual growth/growth rate, abnormal development (fry developmental abnormalities, including ocular malformations and retarded development of the brain, notochord, organs and body segmentation (Metcalfe et al., 2008), hatching time and success, reproduction (the total number of young individuals) and vitality of offspring, behaviour of individuals, physiological parameters and histology (EPA 1996).

Chronic static renewal life stage exposures will occur for 32 days, and will also be done in triplicate using prior selected chemicals and concentrations.

5.1.3 Different life stages of *C. gariepinus and O. mossambicus*

Different life stages of the *C. gariepinus* include eggs, eyed eggs, fry, fingerling and juveniles. The early life stages of *O. mossambicus* consist of an embryonic period of about 15 days where after the fish becomes juveniles. The different embryonic stages include the cleavage stage (± 1 day); embryonic Stage (± 3 days) and a free swimming embryonic stage (± 10 days) (Figure 5.1).

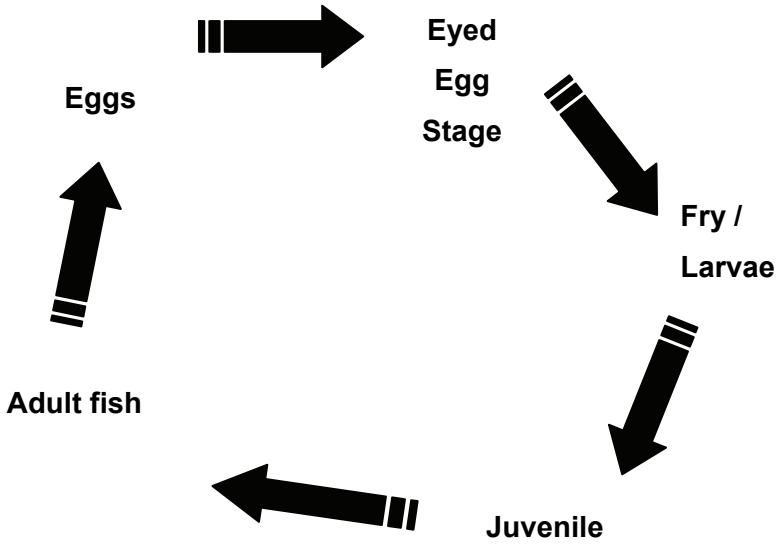

Figure 5.1 The lifecycle of *O. mossambicus* indicating the different life stages.

5.1.4 Original and adapted objectives Phase 5

Originally a toxicological study on sharptooth catfish, *Clarias gariepinus* and *Oreochromis mossambicus* in an aquarium after exposure to sub-lethal concentrations of veterinary compounds originating from livestock production units, investigating the Gonadosomatic Index (GSI), histology of the testes and papilla (*C. gariepinus* only) and gametogenesis in both species and to determine blood hormone levels of 11 ketotestosterone and estradiol and plasma VTG would have been done.

The new objectives were to expose the partial lifecycle of two species, *C. gariepinus* and *O. mossambicus* to selected concentrations of steroid hormones, estrogenic compounds and a mixture of these. The endpoints included (1) mortality, hatching time and success of the lifecycle stages (2) macroscopic observations and (3) histological analyses of the dead life stages. Juveniles will be investigated after three months grow for Gonadosomatic Index (GSI), histology of the testes and papilla and gametogenesis in both species and to determine blood hormone levels of 11 ketotestosterone and estradiol and plasma VTG.

5.2 MATERIALS AND METHODS

5.2.1 Breeding of *C. gariepinus*

Breeding was done according to a method used successfully by Viljoen (1999). Fish have been acclimatised beforehand for three months. Males and females have been kept separated to avoid fights between individuals, but tanks were connected via a biological filter to ensure the eventual exposure of male and female pheromones in all tanks. This enhances breeding rituals and stimulates egg production in female fish. After three months, two mature female catfish were injected with pituitary gland material (in this case aqua spawn) to ensure final maturation and ovulation. Simultaneously the male was injected with aqua spawn. After 12 hours the female was stripped and the eggs collected in a dry plastic container. The eggs will not be counted as a "ripe" female ovulates if a quantity of eggs that equals to 15-20% of her own body weight was produced. The males cannot be stripped and milt was collected after sacrificing the male. The male was killed, the testes dissected, cut into small pieces and the milt pressed over the eggs and mixed into milt with a feather. An equal amount of water was added to the eggs to ensure fertilization of the eggs. This produced a sticky mass. The sticky mass able the addition of the fertilized eggs to sieves in the same type of semi-static system in which eggs of *O. mossambicus* were exposed. Unfortunately after about 24 hours the no eggs hatched and the eggs were discarded.

This process was repeated three times without any success and the experiments were conducted using *O. mossambicus* instead.

5.2.2 Breeding and exposure of *O. mossambicus* partial lifecycle

Aquarium reared fish were divided into breeding pairs and kept in aquarium facilities for a few months. The water temperature was kept 26 ± 1°C and a day:night cycle of 12:12 were regulated. The fish were monitored and available eggs were removed from the mouth of the female after fertilization took place and placed in the containers of the static renewal exposure system.

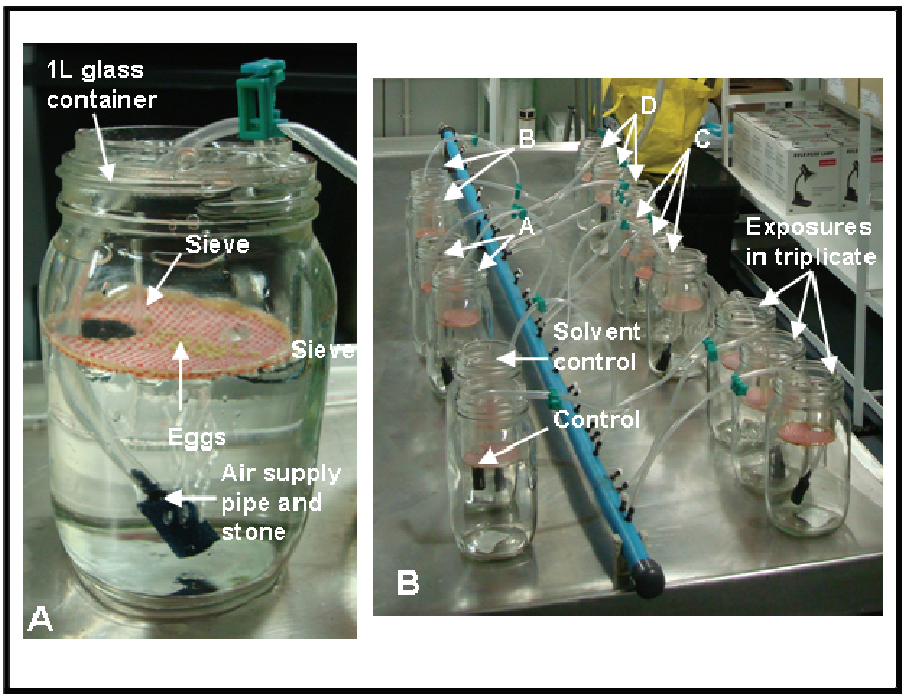

Figure 5.2: The exposure system adapted from Kruger (2002).

5.2.3 Static renewal exposure system

The static renewal exposure system was adapted from one designed and applied by Kruger 2002 (Figure 5.2). The exposure system consists of a 1 ℓ glass container with a sieve and an air supply pipe and stone (Figure 5.2A). A few of these glass containers were replicated to ensure that the three exposure studies could be done in triplicate and a control and solvent control at the same time (Figure 5.2B). The water containing the chemicals was decanted into the glass container and air bubbled through to ensure egg movement.

5.2.4 Chemicals

The partial life cycle of *O. mossambicus* was exposed to an estrogenic, androgenic and an estrogenic-androgenic mixture. The estrogenic mixture include *Trans*-Diethylstilbestrol (DES)(0.24 µg/ℓ) and α-Zearalanol (2.4 µg/ℓ) while the androgenic mixture consist of β-Trenbolone (12.0 µg/ℓ) and Methyltestosterone (MT) (6.0 µg/ℓ). The estrogenic-androgenic mixture consisted of a mixture of the androgens and estrogens listed above. For preparation of stock solutions, chemicals were dissolved in 96% ethanol. As a result of too little of the β-agonist zilpatrol it was not included in the estrogenic mixture. Unfortunately FDA laboratories only had certain amount which was already used up before the fish exposure study started.

5.2.5 Exposure procedures

Freshly released eggs were removed from the mouth of the breeding mother. The eggs were placed in 1 ℓ exposure containers containing the contaminant (thirty eggs per container). Thirty eggs were placed in a control container with no contaminant and another thirty eggs were placed in a container containing the solvent control. Exposure to each test concentration was done in triplicate with a control and solvent control (Figure 5.3).

Dead eggs and larvae were counted and removed daily, as was excess food. The hatching rate was also recorded daily. After the eggs have hatched the free swimming embryonic stage placed in a newly prepared contaminant container after 48 hours (required minimum according to the Organisation for Economic Co-operation and Development ((OECD), 1992 and 2006).

10 Free swimming larvae were fixed in 10% Buffered Neutral Formalin (BNF) for histological preparation for a microscopic evaluation of the development of exposed embryos. The rest of the free swimming larvae were moved to 9 ℓ tanks and the exposure continued until 28 days. After 32 days another 10 juveniles or that was still alive were fixed in 10% BNF for developmental evaluation while the rest of the living individuals will be left to grown into mature individuals (Lefebvre et al., 2004; Chen et al., 2006).

5.2.6 Histological preparation and evaluation

Fish for histological examination were fixed *in toto* in 10% Neutral Buffered Formalin (NBF).

The samples were processed and microscope slides prepared by the Pathology Department, Onderstepoort Veterinary Institute, University of Pretoria. The haematoxylin–eosin stained slides were examined by light microscopy to determine the development of the embryos.

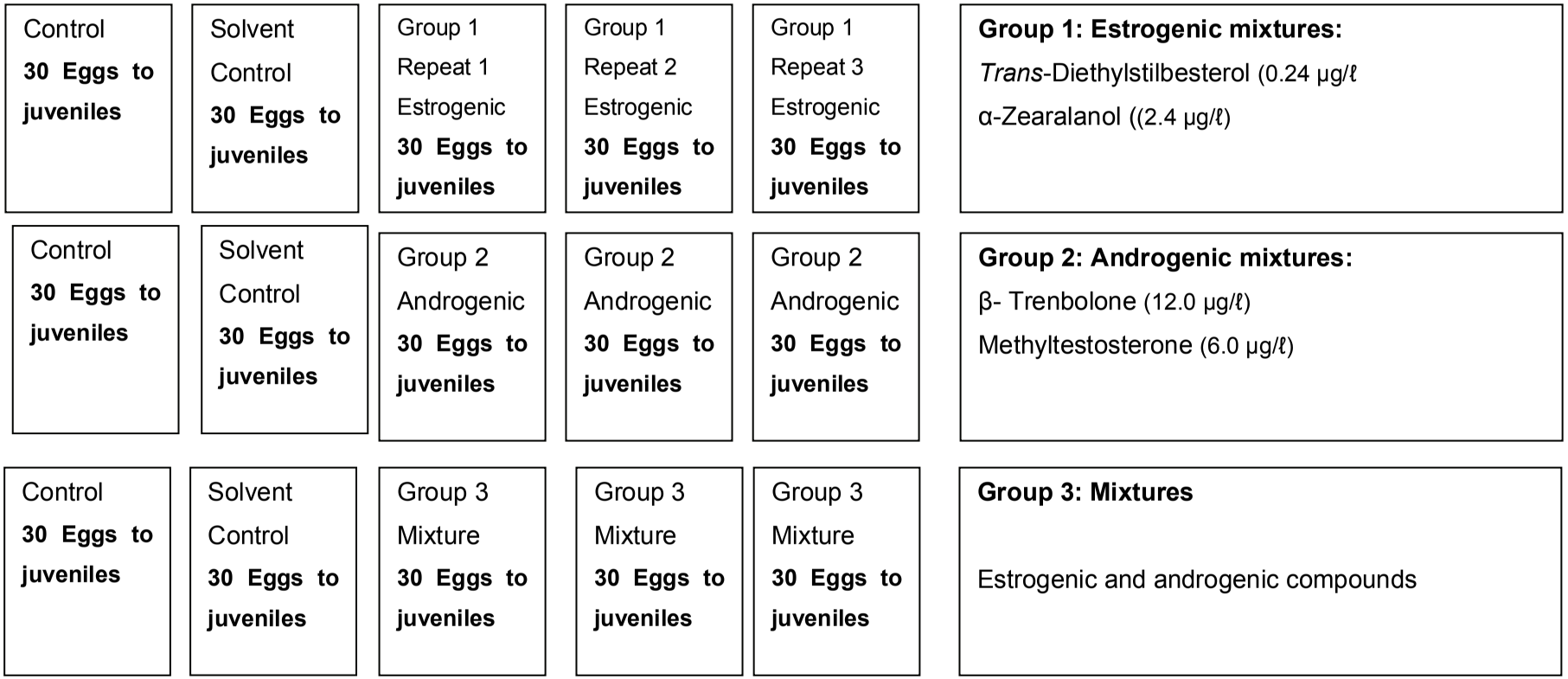

Figure 5.3: A schematic drawing of how O.mossambicus were exposed. The lifecycle of each from eggs to juveniles was monitored over a 28 day period in a static exposure system in triplicate including a control and solvent control

5.2.7 General water quality parameters

The general water quality were also measured every second day including pH and temperature. The oxygen was not measured in the 1 ℓ containers as the oxygen was the main supply of movement of water in the tanks and the amount of oxygen was sufficient. After the fish were transferred to the 9 ℓ tanks were oxygen measurement included.

5.3 RESULTS

5.3.1 Mortality, hatching time and success

The mortality rates are shown in Tables 5.1 to 5.3. The specific life stage that were dead, were collected and fixed. In the exposure groups all the eggs were in the egg-eyed stage after 48 hours. Then the androgenic group were all hatched to become free swimming larvae after 18 days while the estrogenic were all free swimming larvae after 17 days and the mixture group after 16 days of exposure. The free swimming larvae from the estrogenic and the mixture groups however much smaller and took a longer time to use the yolk and become juveniles.

Dead eggs were observed only on day 1 from the estrogenic exposure groups while the other egg mortalities occurred on day 4 and six from the mixture group. The dead eggs were also observed from the solvent control of both these exposures (Tables 5.1 to 5.3).

Mortalities occurred basically on a daily basis from the free swimming larvae stage from all the groups. Especially from the estrogenic and mixture exposures. From the androgenic group all the control juveniles died after 44 days while there were 22 exposed juveniles available after 44 days. However in the days and weeks to follow, deaths occurred and from the androgenic exposures there are a total of 5 fish left (Table 5.1).

From all the exposures there was no and not suitable numbers of fish that survived to become to the 3 months stage to do the planned biological analyses including investigating the gonadosomatic index (GSI), histology of the testes and papilla (*C. gariepinus* only) and gametogenesis in both species and to determine blood hormone levels of 11 ketotestosterone and estradiol and plasma VTG.

Table 5.1: Survival and dead count of fish from the androgenic exposed group.

Days	Control	Solvent control	Repeat 1	Repeat 2	Repeat 3
Start day 0	30	30	30	30	30
Day 20	-10	-10	-10	-10	-10
Day 22		-1	-1		
Day 24	-1	-1			
Day 32	-10	-10	-10	-10	-10
Day 41	-2			-2	
Day 44	-8	-4		-2	-3
Total	0	4	9	6	7

Table 5.2: Survival and dead count of fish from the estrogenic exposed group.

Days	Control	Solvent control	Repeat 1	Repeat 2	Repeat 3
Start day 0	30	30	30	30	30
Day 1		-2	-3	-2	-2
Day 7	-1	-1			
Day 11		-2 unnatural	-1		-1
Day 13		-2			-3
Day 14	-1		-5	-2	-1
Day 15		-1			
Day 16	-2		-2	-1	
Day 17	-10	-10	-10	-10	-10
Day 17		-2			-1
Day 20				-2	
Day 21			-2		-2
Day 25			-1	-1	-1
Day 26			-1	-3	
Day 32	-10	-10	-5	-9	-9
Total	6	1	0	0	0

5.3.2 Individual growth/growth rate, abnormal development

The androgenic exposure group juveniles had a better growing rate in general than the estrogenic and mixture exposure groups. This was observed macroscopically as well as histologically (Figure 5.2).

The most common abnormalities observed were (1) deformities of the spine (lordosis and scoliosis), (2) deformities of the fins (caudal, dorsal) dysplasia or aplasia and (3) deformities of the head (face, jaws, and operculi) dysplasiae (Figure 5.3).

Most of the juvenile fish from the estrogenic group had spine deformities followed by the mixture exposure group (Table 5.4). The head deformities were less obvious in the three different exposure groups. Three individual fish had torn yolk sacs where the developing internal organs bulged out (Figure 5.3E).

Table 5.3: Survival and dead count of fish from the mixture exposed group.

Days	Control	Solvent control	Repeat 1	Repeat 2	Repeat 3
Start day 0	30	30	30	30	30
Day 4		-2	-2		
Day 6			-1	-1	
Day 10		-2	-2		-2
Day 12	-2				-3
Day 14			-5		
Day 16 (fs)	-10	-10	-10	-10	-10
Day 17	-3		-1		
Day 18			-1		
Day 19			-1		
Day 20	-4				
Day 24	-6			-2	-2
Day 25		-1		-1	-1
Day 32	-8	-10	-7	-10	-10
Total left	0	5	0	6	2
Day 38				-4	

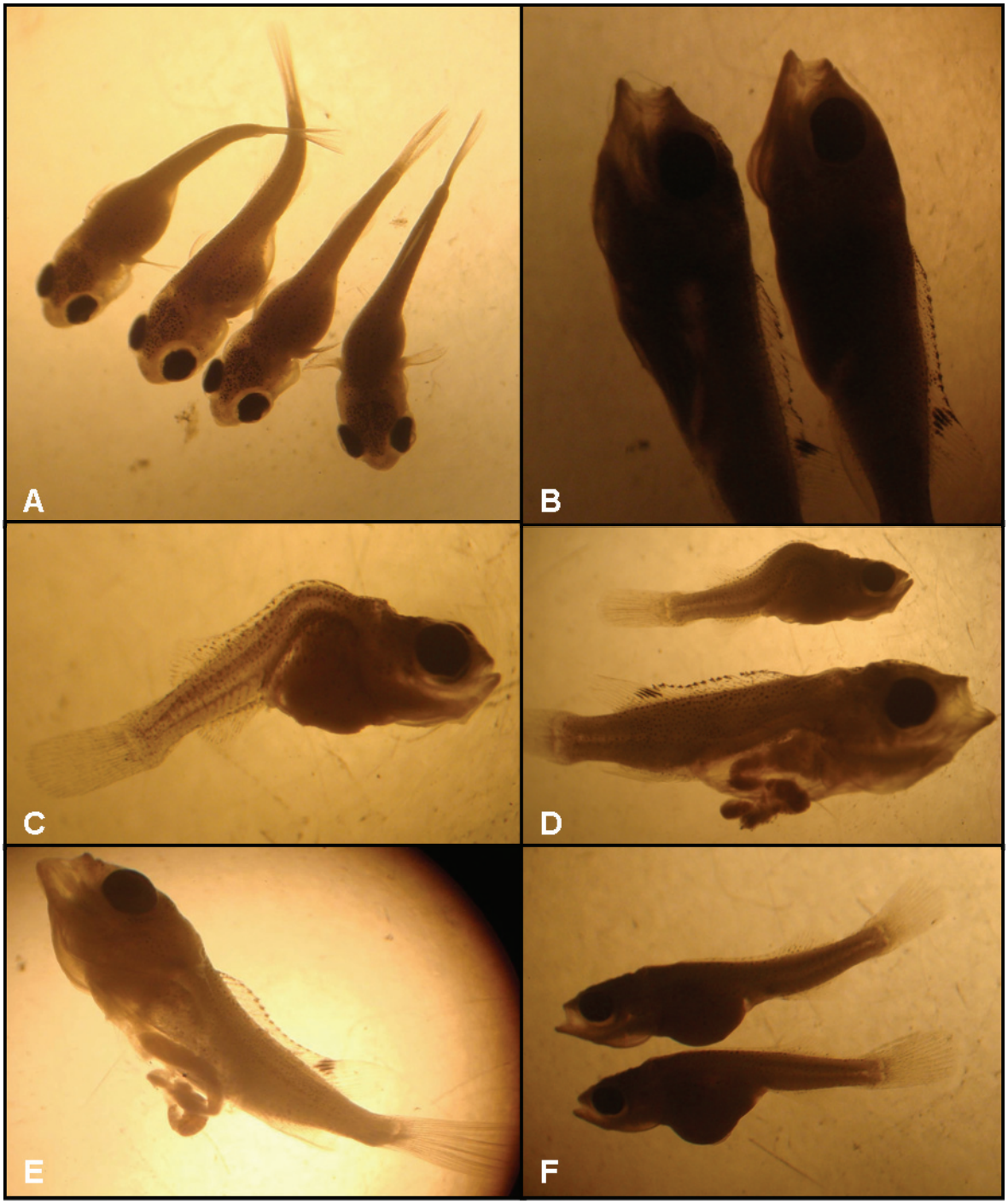

Figure 5.4: **The different deformities found in the exposed fish (A, C, D and F), deformities of the spine (C and D), deformities of the head as well as retarded growth rate in the estrogenic group (D) and fin abnormalities (F).**

Table 5.4: Macroscopic morphological deformities observed in each exposure group.

Deformity	*Control*	*Solvent control*	*Androgenic group*	*Estrogenic group*	*Mixture group*
Spinal	*4*	*2*	*1*	*23*	*17*
Head (mouth)	*0*	*0*		*1*	*1*
Extreme Growth			*1*	*1*	
Breaking of yolk sac			*1*	*1*	*1*
Total deformities	*4*	*2*	*3*	*25*	*18*

5.3.3 Histological evaluation

Histological evaluation confirmed the smaller sizes of the estrogen and mixture exposures compared to the androgen exposed group as well as the deformed spines in the estrogenic and mixture exposures (Figure 5.5A and B). The androgen exposed group had more individuals with no yolk left and the organs such as the liver and intestine were developed during the beginning of the free swimming larvae stages/beginning of juveniles (Figure 5.5C and D). A bigger yolk concentration were presence in the free swimming juveniles of the estrogen and mixture exposure group at the same time of development compared to the androgen exposed group.

The free swimming larvae/juveniles that were collected after they had died over the exposure time had mostly spine deformities (Figure 5.5A).

Histological evaluation of the day 28 juveniles indicated development of the different organs in all the exposure groups although the estrogenic group had smaller individuals. The livers of the estrogenic and mixture exposure groups were in general more vacuolated and bigger in size (Figure 5.5E) than those of the controls and the androgenic exposures. Most of the fish showed normal eye and mouth development (Figure 5.5F).

No sex could be allocated using the histological slides of the free swimming larvae and that of the 32 days old juveniles.

5.3.4 General water quality variables

The pH was between 7.6 and 8.1, the temperature between 23 and 25°C and a day:night cycle of 12:12 was provided. After transfer of the fish to the 9 ℓ tanks the dissolved oxygen concentration remained above 70% of the air saturation value throughout the experiment.

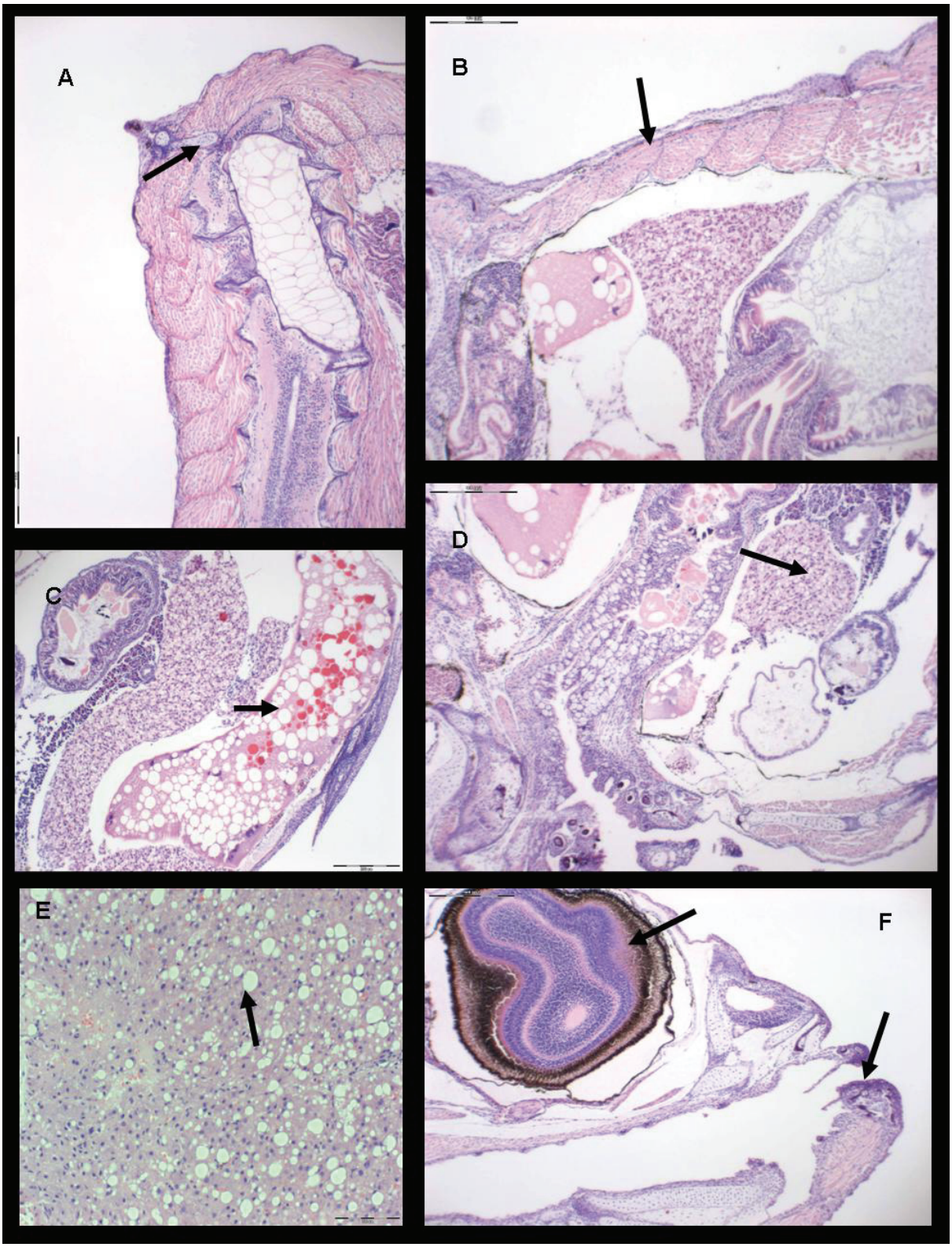

Figure 5.5: General histological evaluation of the exposed fish indicating: A) The severely bend spine B) a normal spine, C) yolk sac in the free swimming larvae/juveniles, D) same age fish as in C but yolk incorporated (arrow indicates liver), E) vacuolated and enlarged liver and F) normal development of eye and mouth.

5.4 DISCUSSION

During all the exposures and as well as in the control/solvent control eggs/free swimming larvae/juveniles died on a daily basis and some of these deaths could be as result on natural causes. In every natural breeding cycle of this species there seems to be a variation in the speed of growth/development in the same and in different clutches as well as a number of non-survival juveniles/eggs (Morrison et al., 2001). Apart from the toxicological factors most developing aquatic organisms have predisposing factors that can cause deformities and abnormal growth/development. These include environmental factors, hereditary material, trauma and infections (Ariav, 2007). The only dead eggs were however observed during day one of the estrogenic exposure whereas the only other early deaths were observed in the mixture exposure.

The androgenic exposure group day 28 juveniles were bigger than the rest of the surviving fish at the same age from the different exposure groups. The androgenic exposure consisted of β-trenbolone (12.0 µg/ℓ) and MT (6.0 µg/ℓ) from which trenbolone is a steroid used by feedlots/veterinarians on livestock to increase muscle growth and appetite (Roche and Quirke, 1986). Trenbolone has a high affinity for binding to the androgen receptor and once this drug is metabolized it stimulates the uptake of nitrogen in the muscle tissue and consequently an increase in protein synthesis, therefore an increase in fish size. Although methyltestosterone is not as potent androgen as the synthetic steroid methyldihydrotestosterone (MDHT) (Galbreath et al., 2003) it also enhances muscle growth and masculine behaviour. During this study no signs of spinal deformities were observed in the androgenic exposure. Previous studies by Seki et al. (2004) and Galbreath et al. (2003) also found no signs of spinal deformations exposing early-life stages of fish to the androgens MT and MDHT respectively while a study conducted by Bogers et al. (2006) found that in total up to 30% of the fish developed deformation of the spine at 0.32 and 1.0 MDHT µg/ℓ exposures. Usually when fish are exposed to androgens such as MT it biased a greater male population (Gale et al., 1999) but this was impossible to evaluate during this study as there were only 3 fish left (Control). In female fish however exposed to trenbolone has resulted in reduced plasma steroids and VTG protein levels and it caused masculinisation of secondary sex characteristics (Ankley et al., 2003; Seki et al., 2006). Trenbolone effects could therefore be argued as positive and therapeutic in fish with regards to growth and production objectives found during these exposures.

The anatomical abnormalities and deformities were mainly observed in the estrogenic (28%) and mixture (20%) exposures. The estrogenic mixture that includes *Trans*-diethylstilbestrol (DES) (0.24 µg/ℓ) and α-zearalanol (2.4 µg/ℓ) seemed to be quite potent to the developing fish. The most prominent finding in this exposed group was spine deformities and the slow growth rate of the different life stages. DES is known to cause spinal deformities in fish species including Zebra fish (Kishida et al., 2001) and Chinese rare minnow (1 and 5 µg/ℓ DES exposure) (Zhong et al., 2005) yet this experiment used 240 (0.24 mg/ℓ) which represents a significantly higher exposure. DES in very low concentrations can therefore causes adverse effects in developing fish.

Another similar synthetic hormone EE_2 causes growth reduction in fish species (Lange et al., 2001; Belt et al., 2003). The mortality rate of the juveniles from all the groups especially those of the estrogenic and mixture exposures could also have been as a result feeding difficulty due to developmental abnormalities of the oesophagus leading to starvation.

5.5 CONCLUSIONS

It is known that variables such as the characteristics of the chemicals, test conditions or the mechanical apparatus may cause variation (EPA, 1996). It is prominent that the mortalities in the estrogenic and mixture exposures were earlier in development. Furthermore the number of fish with spinal deformities and delayed growth were also much higher in these groups than in the androgenic group exposure and compared to the controls. During the androgenic exposure it was evident that some of the juveniles grew faster. Therefore it is a possibility that trenbolone can be positive and therapeutic with regards to growth in juvenile *O. mossambicus.*

Recommendations are proposed to future exposure studies in this regard:
Firstly, to refine the methodology used in the exposure system to ensure optimal conditions for the exposed fish to grow. This is necessary as most of the control fish died before the markers such as histology, VTG and other reproductive endpoints could be measured. Secondly, it is necessary to expose clutches with higher number of eggs to ensure more juveniles for comparable analyses. Also, there would be a higher number of 3 month old fish available for further analyses such as the presence of intersex, VTG and other reproductive endpoints.
Thirdly, more endpoints of the dead exposed juveniles should be measured before future preparation.

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PHASE 6

Possible Effects of Cattle Feedlot Activity and the Use of Growth Stimulating Hormones on Aquatic Ecosystems

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LIST OF ABBREVIATIONS

AEV	Acute Effect Values
CEA	Cellular Energy Allocation
Ec	Cellular Respiration Rate
COD	Chemical Oxygen Demand
CEV	Chronic Effect Values
EDH	Endocrine Disruptive Hormones
Ea	Energy Reserve Fractions
FFGs	Functional Feeding Groups
IHI	Habitat Integrity Index
ICP-OEP	Inductively Coupled Plasma Optical Emission Spectrometry
IHAS	Invertebrate Habitat Assessment Score
MIRAI	Macro-invertebrate Response Assessment Index
PES	Present Ecological State
PCA	Principle Component Analysis
RHI	River Health Indices
TWQR	Target Water Quality Range
OM	Total solids

6.1 INTRODUCTION AND OBJECTIVES

6.1.1 Feedlot activities

This phase of the project is principally concerned with the possible effects of selected growth hormone residues on aquatic freshwater systems. Most of the growth hormones entering the water systems are from the faecal and urine matter of cattle. Feedlots, in general, contain large numbers of cattle concentrated on a relatively small space. The environmental implications of this can only be speculated upon. The effect of organic, abiotic and hormone metabolites needs to be taken into consideration, when the potential contamination of a natural stream by effluent from a cattle feedlot is studied. To establish whether any contamination of the natural streams associated with feedlots does occur, the study was done at sites in three different river catchments after careful selection to include operations that ranged in size. Site information is treated as confidential and therefore no co-ordinates or site descriptions are given.

6.1.2 Present Ecological State (PES)

River Health Indices (RHI) has been used to determine in Present Ecological State (PES) of the rivers next to the feedlots with the possibility to receive effluent from the feedlot (River Health Programme, 2005; Kleynhans et al., 2005). The main aim of the indices is to assess and categorise a current state for any given aquatic system at a selected site. This, in turn, fits into a national River Health Programme (RHP) (River Health Programme, 2005). The main objective for the RHP is to measure, assess and report on the ecological state of aquatic ecosystems.

The RHI provides a measure of relative stress on community level. In other words, any alteration in abiotic driving conditions (water quality) would reflect in the invertebrate and fish community composition. Additional to that, supporting habitat assessment indices have also been developed.

RHI typically implemented in the RHP, have been used in this study to ascertain the PES of both upstream and downstream sites in rivers associated with different cattle feedlot activities. By applying an upstream-downstream assessment, a location of possible pollution can be determined, indicating point source pollution, as well as the relative intensity thereof.

6.1.3 Invertebrate community composition

Landscape-level investigations of invertebrate communities show that species diversity, abundance and composition are largely dependent on the environmental conditions to which the communities are exposed (Wally 1998; Ruse 2000; Probst et al., 2004). Effluent from feedlot activity could introduce soil, salts, organic matter, manure, urine, metals, as well as pharmaceuticals and their metabolites into associated aquatic systems (Cooper, 1993; Matthiessen et al., 2006; Whitehurst, 1991). The feedlot effluent could therefore change the environmental conditions of the receiving aquatic systems, requiring the associated aquatic biota to adapt (Hilsenhoff, 1982; Plafkin et al., 1989).

Feedlots are relatively small surface areas, with intensive cattle feeding activities that result in the disappearance of plant cover. This phenomenon causes sedimentation that is an important abiotic driver of invertebrate community responses. An increase in sedimentation leads to a reduction in habitat integrity (Zweig and Rabeni, 2001). Sediment yields are often higher in fields that drain agricultural land (Allan et al., 1997). Soil disturbance accompanying land clearing and soil integrity loss will further intensify the rate of erosion and downstream sedimentation (Dallas and Day, 2004). It has been indicated that even small increases in sediment deposition may alter invertebrate community compositions and cause changes in dominance from the *Ephemeroptera*, *Plecoptera* and *Trichoptera* taxa to *Oligochaeta* (Lenat and Crawford, 1994; Quinn et al., 1997). In addition to this Bo et al. (2007) also suggests that a significant decrease in taxa number and abundance occur with increased sedimentation.

Large amounts of salts could also be deposited into associated aquatic systems (Erickson, 2003) through runoff, or indirectly through manure application to agricultural fields (Dodds and Welch, 2000). An Australian study indicated increased salinity, in general receiving aquatic environment, as one of the main contributors to changes in macro-invertebrate community structures (Kay et al., 2001). The study showed that invertebrate communities, exposed to saline conditions, reflected uniform structures, consisting of species with a high tolerance to saline conditions. Thus, urinal and faecal salt influxes associated with feedlot activity will alter water quality and act as a driver for macro-invertebrate responses.

The effects of nutrient enrichment due to organic matter from feedlot activities on associated aquatic systems have not been well documented (Wang et al., 2007). The potential effect of a nutrient increase will result in an alteration in the primary production structure. Studies that have been undertaken in this regard states that an influx of nutrients will influence light availability and temperature and this may augment in-stream primary production, resulting in changes in the trophic structure of benthic communities (Johnson et al., 1997; Sponseller et al., 2001). Camargo et al. (2004) represents new data on nitrate toxicity to the freshwater invertebrates *Eulimnogammarus toletanus, Echinogammarus echinosetosus* and *Hydropsyche exocellata*. From this it is clear that aquatic macro-invertebrates will respond to increase concentrations of nutrients, not just chronically but also acutely. Controlled experiments have shown that macro-invertebrate and periphyton abundance increased with higher nutrient availability (Dudley et al., 1986). Changes in the makeup and abundance of periphyton algae have also been found to reduce macro-invertebrate drift (Kerans, 1996). Increased periphyton abundances can also affect the survival of sensitive macro-invertebrates by depleting oxygen through nocturnal respiration (Wang et al., 2001).

Because macro-invertebrates integrate natural and anthropogenic effects temporarily, they have been used widely to characterise catchment and water quality conditions related to land use (Rosenberg and Resh, 1993). It also takes the physiochemical variables of water and sediment composition into consideration.

Invertebrates are known to be good indicators of river health (Gerber and Gabriel, 2002). The variation in community structures will be correlated with the physiochemical characteristics of the respective sites. There are numerous ways of assessing freshwater ecosystem disturbance via the use of macro-invertebrate community structure. Diversity indices such as the Shannon-Weiner index (Wilhm and Dorris, 1968), Margalef's species richness and Pielou's evenness (Pielou, 1966) and biotic indices such as the South African Scoring System Version 5 (SASS5) (Dickens and Graham, 2002) and the Macro-Invertebrate Response Assessment Index (MIRAI) (Thirion, 2007) have been widely used in South Africa. A less utilised approach to gauge ecosystem disturbance is the use of macro-invertebrate Functional Feeding Groups (FFGs) which can be used to address the functional role that invertebrates play in the food base of a stream ecosystem.

6.1.4 Biomarkers

Exposure to a variety of anthropogenic compounds has been shown to interfere with normal development, physiology and reproduction of organisms. The complex nature of the endocrine system makes it hard to establish the cause of possible stressors. As a result of this, a vast number of chemicals used commercially have not adequately been tested for effects on the physiological functioning of aquatic organisms. There is, therefore, a need to establish a biomarker that is capable of indicating stress induced by growth promoters (De Coen et al., 2001). This study was therefore done to investigate the possible use of metabolomics and cellular energy allocation as biomarkers for the effect of the selected growth stimulants at the same concentration used in other exposure experiments in this project with fish and small mammals.

Metabolomics is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind (Daviss, 2005). The metabolomics represents the collection of all metabolites in a biological organism, which are the end products of its gene expression (Daviss, 2005). It is expected that, by using metabolomic profiling, the results will indicate the physiological differences between exposed and non-exposed cells of the respective organisms. Metabolomics has been the subject of numerous reviews in recent years (Bino et al., 2004; Fernie et al., 2004; Fiehn, 2002; Goodacre et al., 2004; Lindon et al., 1999; Nicholson et al., 1999; Weckwerth, 2003) and a volume on metabolic profiling was published in 2003 (Harrigan and Goodacre, 2003). Even within the specific arena of toxicological applications of metabolomics a great deal has been published (Griffin, 2003; Lindon et al., 1999; Nicholson et al., 1999; Robertson and Bulera, 2000; Shockcor and Holmes, 2002).

"Metabolomics is a comprehensive analysis in which all metabolites of an organism are identified and quantified" (Bino et al., 2004). It can be considered the systematic study of the unique fingerprinting that cellular processes leave behind (Davis, 2005). In other words, it can be considered as a productive, complimentary and functional tool in genomics. Nicholson et al. (1999) defines metabolomics as the quantitative measurement of the dynamic multiparametric metabolic response of living systems to patho-physiological stimuli or genetic modification.

Metabolomics, on the other hand refers to the complete set of small molecule metabolites to be found within a biological sample. Metabolites are the intermediates and end products of metabolism.

Responses at molecular and cellular levels can be used as markers of biological effects to environmental exposure (Stegeman et al., 1992). One of these recently developed markers evaluates the energy metabolism of organisms. The energy metabolism is under hormonal control and is thus susceptible to endocrine disruption. Changes in the energy metabolism will influence life characteristics such as growth and reproduction on organism level (Verslycke and Janssen, 2001). This will later be amplified and observed in the total fertility of the exposed population. Organisms exposed to suboptimal environments acquire a cost of dealing with stress in terms of metabolic resources.
The total amount of energy available for maintenance growth and reproduction can be calculated, based on biochemical analysis of the energy budget (Orlando et al., 2004). This analysis is called Cellular Energy Allocation (CEA).

The method of CEA was developed as a biomarker technique to assess the effects of toxic stress on the energy budget of exposed organisms (De Coen and Jansen, 1997a; De Coen and Jansen, 1997b; De Coen and Jansen 2003). It can be considered a short term biochemical assessment that combines the variation in the energy reserve (lipids, protein and sugar) with energy consumption (amount of oxygen consumed) to form an indicator of physiological stress. Organisms exposed to a stress inducing environment shows higher energy consumption per unit time than organisms that are not exposed to a stressful environment.

6.2 EXPERIMENTAL

6.2.1 Site selection

Due to the aim of this phase to determine the possible implications of feedlot activity on associated aquatic systems, three feedlots were selected, each in close proximity to a perennial system: Feedlot A (Gauteng) with 85 000 cattle, Feedlot B (North West Province) with 20 000 cattle, and Feedlot C (Mpumalanga) with 45 000 cattle. An associated upstream and downstream site was selected at each feedlot. Sites were accessible from and located in relatively close proximity to the feedlots. Biomonitoring results from downstream sites were interpreted in relation to those from upstream sites for each feedlot respectively. River reaches associated with each site were representative of the river unit.

6.2.2 Water quality

The following Surface water quality variables were measured on site for both the high flow and the low flow survey respectively: pH, conductivity, water temperature, dissolved oxygen and oxygen saturation using a Eutech Cyber Scan Series 600 portable water quality meter.

During the high flow and the low flow survey one water sample per site were collected. Water samples were taken in 500 mℓ polypropylene bottles and kept on ice until their return to the laboratory where they were frozen until analysis (DWAF 1996). Water samples were thawed and prepared for chemical and metal analyses. Standard sample preparation techniques were followed before measuring the concentration of each nutrient (DWAF 1996). Sample were analysed for the following chemical variables: turbidity (NTU), chloride (Cl), orthophosphate (PO_4), sulphate (SO_4), nitrite (NO_2) and nitrate (NO_3). Analysis was conducted using the Merck Spectroquant Spectrophotometer.

Filtered metals contents of Aluminium (Al), Cadmium (Cd), Chromium (Cr), Copper (Cu), Iron (Fe), Nickel (Ni), Zink (Zn) and Lead (Pb), were ascertained by filtering 250 mℓ of each sample through 0.45 µm filters. The filter was then dissolved in 33% HNO_3 acid solution. A sample of 0.05 mℓ of dissolved filter solution was made up to 10 mℓ solution and read through the inductively coupled plasma optical emission spectrometry (ICP-OES).

To each sample of 10 mℓ of filtered water 0.05 mℓ of HNO_3 was added and analysed using the ICP-OES in order to determine the suspended metal content. The values obtained for metals in the digested filter samples and in the filtered water of each sample were added to give the total metal concentration in each water sample.

Primer version 6 statistical suite was used on water quality data for sample sites at high and low flow. Temperature data was excluded after an initial statistical analysis due to the contribution of the variation in temperature between seasons to skewness in the data.

Selected water quality data variables (Conductivity, pH, NO_2, NO_3, COD, NH_3 and NH_4) were Log (V) transformed due to the skewness in data indicated by the scatter plot for variable pairs (Draftsman Plot). Principal Component Analysis (PCA) was carried out on normalised data.

6.2.3 Sediment

Bottom sediment was collected at each side and was subjected to the following laboratory analysis (Anon, 1995):

- Percentage grain size
- Organic content

The following methods were used:

For the percentage grain size was determined by taking a sediment sample of every site and sieving it for ten minutes. A 4000 µm, 2000 µm, 500 µm, 212 µm and a 53 µm sieve was used. The weight of the grain in each sieve was determined and worked out as a percentage of the whole sample.

Determining the organic content of the sediment was done in duplex. Two crucibles were filled three quarters of the way for each sample and weighed.
The crucibles were placed in an incinerator at 600ºC for 6 hours and left to cool down for 24 hours. After incinerating the sediment was weighed again to determine the weight of the organic content (Duft et al., 2002).

6.2.4 Habitat assessment

A habitat assessment was conducted on site using Invertebrate Habitat Assessment Score (IHAS) Version 2 (McMillan, 1998) to enrich macro-invertebrate data obtained. IHAS measures the availability and integrity of each biotope (Stones, Vegetation and Gravel, Sand and Mud) as well as physical stream integrity.

The Index of Habitat Integrity (Kleynhans, 1996) was applied on a site basis and aims to assess the number and severity of anthropogenic disturbances on a river and the changes they potentially cause on the habitat integrity of the system. These disturbances include abiotic factors and biotic factors. Aspects considered in the assessment comprise those in-stream and riparian zone perturbations regarded as primary causes of degradation of a river ecosystem. The severity of each of these impacts is assessed, using scores as a measure of impact.

6.2.5 Invertebrate community composition

The SASS5 methodology (Dickens and Graham, 2002) was used at the selected sites to assess the benthic macro-invertebrate community integrity.
It should be noted that SASS5 complies with international accreditation protocols and a SASS5-accredited practitioner has undertaken this assessment. Invertebrates were preserved in 10% neutrally buffered formalin with Rose Bengal as a colorant.
Upon arrival at the lab, invertebrates were washed and stored in 70% ethanol until identification. Identification of the organisms was done to family level (Davies and Day, 1998; Dickens and Graham, 2002; Gerber and Gabriel, 2002).

Primer version 6 was used to construct Bray-Curtis similarity matrices from log (V) transformed macro-invertebrate species abundance data recorded for each site at high and low flow occasions. Similarity matrices were subjected to group averaged hierarchical clustering (CLUSTER) and ordination by NMDS to summarise patterns in species composition. Factors were assigned to Bray-Curtis resemblance matrices based on groupings from the CLUSTER analysis and NMDS ordination. K-dominance plots were included to indicate sites that have an increased dominance of species relative to the other samples and flow periods.

6.2.6 Fish Assemblage Integrity Index assessment (FAII)

The species intolerance ratings used in the calculation of the FAII was taken from Kleynhans (2007). Four components are taken into account in estimating the intolerance of the relevant fish species, namely habitat preferences and specialisation (HS), food preference and specialisation

(TS), requirements for flowing water during different life-stages (FW) and water quality requirements (WQ) (Nguyen, 1997).

Each of these aspects are scored for a species according to low requirement/specialisation (rating = 1), moderate requirement/specialisation (rating = 3) and high requirement/specialisation (rating = 5). The total intolerance (IT) of a fish species is estimated as follows:

IT = (HS + TS + FW + WQ)/4

The percentage of fish with externally evident disease or other abnormalities are used to score this metric. The following procedure is used to score the health of individual species:

Frequency of affected fish > 5%, score = 1

Frequency of affected fish 2-5%, score = 3

Frequency of affected fish < 2%, score = 5

The expected health for a species living under unperturbed conditions is assumed to be unimpaired and would score 5.

The *expected index score* [FAII (exp.)] per segment:

FAII (exp.) = Σ (TxH)

Where: T = Tolerance rating for individual species

H = Expected health rating for individual species.

The *observed index score* [FAII (obs)] is calculated on a similar basis but is based on the information collected during the survey:

FAII (obs) = Σ (TxH).

The observed fish assemblage index score for a segment is expressed as a percentage of the expected total FAII score to arrive at a relative FAII rating:

FAII (obs)/FAII (exp.) x 100

6.2.7 Flow through system

Four separate 1000 ℓ flow through systems where used to expose test organism *Clarias gariepinus* (Sharptooth Catfish). 1000 ℓ tanks were set at a flow through rate of 100 mℓ per minute. Environmental room conditions were set at 25ºC and pH, conductivity, temperature and oxygen saturation were monitored daily (results not shown). Two different exposures comprising of 15 fish respectively were done. Because the dose response curve for Trenbolone and Diethylstilbestrol is not known, fish were exposed with temporal intervals of 5 days for 15 days.

Exposure concentrations were done at detectable limit + 20% which relate back to:

- Trenbolone acetate = 12.00 µg/ℓ
- Diethylstilbestrol = 0.24 µg/ℓ

6.3 EXPERIMENTAL PROCEDURES

6.3.1 Cellular Energy Allocation

Energy available (De Coen and Janssen, 1997)

Total lipids were extracted following Bligh and Dyer (1959).

Liver samples were homogenised in 5 mℓ homogenising buffer after which chloroform (spectrofotometric grade) and 500 µℓ methanol were added. After centrifugation the top phase was removed and 500 µℓ H_2SO_4 were added to 100 µℓ lipid extract and charred for 15 minutes at 200ºC. The total lipid content was determined by measuring the absorbance at 370 nm using tripalmitin as a standard. To determine total protein and carbohydrate content, fish livers were homogenised in 200 µℓ homogenizing buffer after which 15% Trichloroacetic Acid (TCA) was added and incubated at 20ºC for 10 minutes. After centrifugation, the pellet was washed with 5%. TCA and both supernatant fractions were combined and used for the total carbohydrate analysis. The remaining pellet was re-suspended in NaOH, incubated at 60ºC for 30 minutes and neutralised with HCl. Total protein content was then determined using Bradford's reagent. The absorbance was measured at 590 nm using bovine serum albumin as a standard. As a third fraction of the *E*a parameter, total carbohydrate content of the supernatant fraction was quantified by adding 5% phenol and H_2SO_4 to the extract. After 30 min incubation at 20ºC, the absorbance was measured at 492 nm using glucose as a standard.

Energy consumed (De Coen and Janssen, 1997)

The electron transport activity was measured following King and Packard (1975) with major modifications as described hereunder. The fish liver were homogenised on ice using a motor-driven teflon pestle in homogenising buffer (0.1 M Tris-HCl pH 8.5, 15% (w/v) Poly Vinyl Pyrrolidone, 153 µM $MgSO_4$ of 0.2% (w/v) Triton X-100). After centrifugation (4ºC, 3000 g during 10 minutes), 50 µℓ from each extract were added to 150 µℓ buffered substrate solution (BSS; 0.13 M Tris HCl, 0.3% (w/v) Triton X-100, pH 8.5) and 50 µℓ NAD(P)H solution (1.7 mM NADH and 250_M NADPH).

The reaction was started by adding 100 µℓ INT (p-IodoNitroTetrazolium; 8 mM) and the absorbance measured kinetically at 20ºC for 10 minutes. The amount of formazan formed was calculated.

CEA calculation (De Coen and Janssen, 1997)

The different energy reserve fractions (*E*a) for the individual organisms were transformed into energetic equivalents using the energy of combustion: 17500 mJ/mg glycogen, 24000 mJ/mg protein and 39500 mJ/mg lipid. The cellular respiration rate (*E*c) was determined, using the ETS data, based on the theoretical stoichiometrical relationship that for each 2 µmol of formazan formed, 1 µmol of O_2 was consumed in the ETS system. The quantity of oxygen consumed per fish liver unit was transformed into energetic equivalents using the specific oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture of 484 kJ/mol O2. The *E*a value was calculated by integrating the change in the different energy reserve fractions over the

15 day exposure period. Similarly, the *E*c value was obtained by integrating the change in energy consumption over the exposure period. The total net energy budget was then calculated.

6.3.2 Metabolomics

Plasma samples were analysed on a group basis. Plasma (400 µℓ) and D_2O were mixed and transferred to 5 mm NMR micro tubes.

Samples were analysed by H-NMR spectroscopy within 2 hours of sample preparation. Buffering the plasma sample did not reduce variability and were thus not applied (Duran et al., 2003).

H-NMR spectra were measured at 300 MHz on a Varian Inova 300 MHz spectrometer equipped with 300 MHz cold probe. Tuning and gradient shimming was performed for each sample. H-NMR chemical shifts were referenced internally to the methyl doublet of valine at 1.042 ppm. All spectra were Fourier transformed followed by 1st order phasing and drift correction. H-NMR spectra of fish were required at 25ºC with a standard sequence using a 90º pulse and a relaxation period of 2.0 s. Suppression of the large water resonance was achieved by pre-saturation during 1.5 s of the relaxation delay and determining the saturation frequency for each individual sample (preset spectra) (Duran et al., 2003).

H-NMR spectra were integrated and divided into chemical shift buckets using a custom-written programme. The total sum of all bucket integrals in each spectrum was set at 90, encompassing 0.04 ppm.

Data were imported into Conoco multi variable statistical programme. Principal Component Analysis (PCA), (Duran et al., 2003) was performed to create an overview of trends, groupings and outliers in data.

6.4 RESULTS, TREATMENT OF RESULTS AND DISCUSSION

6.4.1 Present Ecological State (PES) determination

6.4.1.1 Water quality

This section describes the water quality variables that are divided into *in situ* variables, Nutrients and Metals for each site respectively. The descriptions are designed in such a manner that both inter-site and inter feedlot comparisons can be made, at the same time incorporating both low flow and high flow variability. The following site abbreviations are used:

- K = Feedlot A
- T = Feedlot B
- B = Feedlot C
- U = Upstream
- D = Downstream

The following time abbreviations are used:

- L = Low flow
- H = High flow

KUL therefore translates to: Feedlot A, Upstream, Low flow.

6.4.1.1.1 In situ variables

Table 6.1 indicated the *in situ* and nutrient variables for the low flow survey, while Table 6.2 indicated the same variables for the high flow survey. Comparisons between values measured at each site and the 95'Th percentile values monitored at nearby monitoring stations for the last 5 years (Table 6.3) as well as comparisons to Target Water Quality Range (DWAF, 1996; DWAF, 2002) for ecological functioning (Table 6.4), was made.

The amount of oxygen available in downstream sites associated with Feedlot A is higher than that of the upstream sites at 7.81 mg/ℓ in the low flow survey and 4.4 mg/ℓ in the high flow survey. Sites in the river near Feedlot A shows oxygen saturation levels below that of the Target Water Quality Range (DWAF, 2002). A similar trend is visible when referring to conductivity, which is higher downstream (1882 µs/cm, 724 µs/cm) than upstream (549 µs/cm, 207 µs/cm) for both seasons sampled, but less so in the high flow. In addition to this, pH for the downstream site is also higher for both sampling efforts, upstream is 7.24 and 5.95 respectively, while downstream pH is, 8.68 and 7.5 respectively.

In situ variables for Feedlot B show less variability between sites and between flow surveys. Concentration of oxygen is similar for the upstream and downstream site, with the amount of oxygen available being marginally higher in the downstream site during both high flow and low flow field assessments. The conductivity during the low flow survey was higher at Feedlot B downstream (1786 µs/cm) compared to upstream sites (720 µs/cm) during the same sampling time. In the high flow survey the difference reduced slightly but downstream was still higher (409 µs/cm) than upstream (211 µs/cm).

The *in situ* variables for Feedlot C reflect differently when compared to the other two feedlots. Oxygen availability is higher for the upstream site for both low flow and high flow surveys (9.79 mg/ℓ and 4.6 mg/ℓ respectively) compared with downstream site (7.29 mg/ℓ and 3.2 mg/ℓ respectively). During the low flow survey the upstream conductivity was higher (728 µs/cm) than the downstream site conductivity (395 µs/cm). This however, changed for the high flow survey where the upstream site conductivity was measured at 211 µs/cm and downstream was measured at 409 µs/cm. pH measurements were highest for both upstream low flow survey (8.45) and for upstream high flow survey (8.10).

6.4.1.1.2 Nutrients

Nutrient content of water samples associated with Feedlot A does have distinct differences. Cl^- for upstream sites during both survey times were lower (4.36 mg/ℓ and 1.5 mg/ℓ respectively), than downstream sites (10.5 mg/ℓ and 22 mg/ℓ respectively). Chemical Oxygen Demand (COD) was the highest at the downstream site during high flow field survey and lowest at the upstream site

during the high flow survey. On both occasions the COD was substantially higher downstream from the feedlot than upstream. During the low flow survey there was little difference between PO_4 when comparing the upstream site with that of the downstream site.
However, the PO_4 for the downstream site during the high flow survey is higher (1.87 mg/ℓ) compared to upstream site (0.18 mg/ℓ) during the same surveying period. PO_4 levels downstream for Feedlot A, during the high flow survey show concentrations far greater than that of the 95th percentile value (Table 6.3). A similar trend is visible when comparing SO_4 concentrations measured at Feedlot A. The concentrations were 52 mg/ℓ during the low flow survey at the upstream site and 34 mg/ℓ at the downstream site. The high flow situation differs, with the downstream site at 306 mg/ℓ and the upstream site at 10 mg/ℓ The SO_4 concentration for the downstream site associated with Feedlot A is higher than the TWQR (Table 6.4) during the high flow survey. NH_4 concentrations for Feedlot A is similar for both sites during the low flow (0.09 and 0.08 mg/ℓ respectively) survey but exponentially higher for the downstream site (0.16 mg/ℓ) during the high flow survey. NH_4 concentrations for both upstream and downstream sites associated with Feedlot A during both low flow and high flow surveys are far above the TWQR. NO_2 concentrations are higher at downstream sites (0.14 and 0.20 mg/ℓ) for both low flow and high flow surveys compared to upstream sites (0.07 and 0.02 mg/ℓ).

Turbidity is lowest at the downstream site (2.5 NTU) during the low flow survey and highest at the upstream site (7 NTU) during the high flow survey. NO_3 does not show the same trend as NO_2. NO_3 concentration was highest during the low flow survey for both upstream (9.2 mg/ℓ) and downstream sites (3.85 mg/ℓ). While being much lower and similar during the high flow survey.

The nutrient content of samples from the stream associated to Feedlot B shows a downward trend for most of the nutrients. With differences more related to seasonality than feedlot activity. Cl^- concentration for samples from the stream at Feedlot B is highest for the upstream site during the low flow (11 mg/ℓ) survey. However, it is similar for both sites during the high flow survey (7.5 mg/ℓ and 7.25 mg/ℓ respectively).

COD is highest for the upstream site during low flow and high flow surveys. While PO_4 is lower for the downstream site for both low flow (0.92 mg/ℓ) and high flow (0.51 mg/ℓ) survey, compared to the upstream site during the low flow (1.57 mg/ℓ) and high flow (0.81 mg/ℓ) survey. PO_4 concentrations for both upstream and downstream sites are higher than the 95th percentile values obtained from a nearby monitoring station (Table 6.3). SO_4 concentration is highest for the site located up from the feedlot during the low flow survey (26 mg/ℓ) and the same for the upstream and downstream sites during the high flow survey (16 mg/ℓ). NH_4 is higher for the upstream site during both low flow (0.05 mg/ℓ) and high flow surveys (0.07 mg/ℓ) compared to the downstream sites during the low flow (0.03 mg/ℓ) and high flow surveys (0.05 mg/ℓ). NO_3 shows a similar trend, being higher for the upstream site during the low flow survey (4.3 mg/ℓ) and the high flow (2.28 mg/ℓ) when compared to the downstream site during the low flow and high flow surveys at 0.65 mg/ℓ and 1.8 mg/ℓ respectively. There is little or no variation in NO_2 concentration measured

at upstream and downstream sites, furthermore there is also no difference between low flow and high flow surveys. Turbidity for both upstream and downstream was the same during the low flow survey and lower for both sites during the high flow survey.

Nutrients concentrations at Feedlot C does show some fundamental differences between upstream and downstream sites, as well as differences in concentration between low flow and high flow surveys.

Cl^- concentrations is highest at the downstream site during the high flow survey (8 mg/ℓ) and lowest at the upstream site during the low flow survey (4 mg/ℓ). COD is much higher at the upstream site during the high flow survey. While PO_4 concentrations are similar for all sites during low flow and high flow surveys. SO_4, during the low flow survey is highest at the upstream site (15.67 mg/ℓ) and during the high flow survey highest at the downstream site (13 mg/ℓ). NH_4 concentrations show a seasonal trend being higher upstream (0.1 mg/ℓ) and downstream (0.07 mg/ℓ) during the high flow survey, compared to the 0.03 mg/ℓ and 0.05 mg/ℓ for upstream and downstream sites respectively, during the low flow survey.

NH_4 concentrations at the upstream site associated with Feedlot C feedlot during the low flow survey are far above the TWQR. NO_3 concentrations increase downstream from Feedlot C with both downstream surveys yielding higher values than the upstream site. However, the NO_3 concentrations also show a general decrease in concentration during the high flow survey. NO_2 concentrations show little or no change between sites or between seasons. Turbidity decreases downstream form Feedlot C, as the upstream site showed a higher turbidity during low flow (5.5 NTU) and high flow (4 NTU) surveys.

Figure 6. shows the Principal Component Analysis (PCA) ordination for nutrients and *in situ* variables for sites associated with Feedlot A. Both high flow and low flow data sets are being represented. The two dimensional PCA bi-plot describes 96.1% of the variation in the data. 89.6% of the variation is represented in the first axis while the rest (6.6%) is represented by the second axis. Regardless of seemingly higher concentrations of nutrients present at Feedlot A downstream during the high flow survey, the PCA indicates seasonal similarities in the data sets. Conductivity contributes the most towards the low flow similarities between upstream and downstream sites. Sulphate (SO_4), Cl^- and PO_4 contribute towards the dissimilarities observed in the results from the downstream site near Feedlot A during the high flow survey. A site ordination is also visible, regardless of season. Both low flow and high flow data sets for the upstream site group together. The similar grouping can be seen for the downstream site, but to a lesser extent.

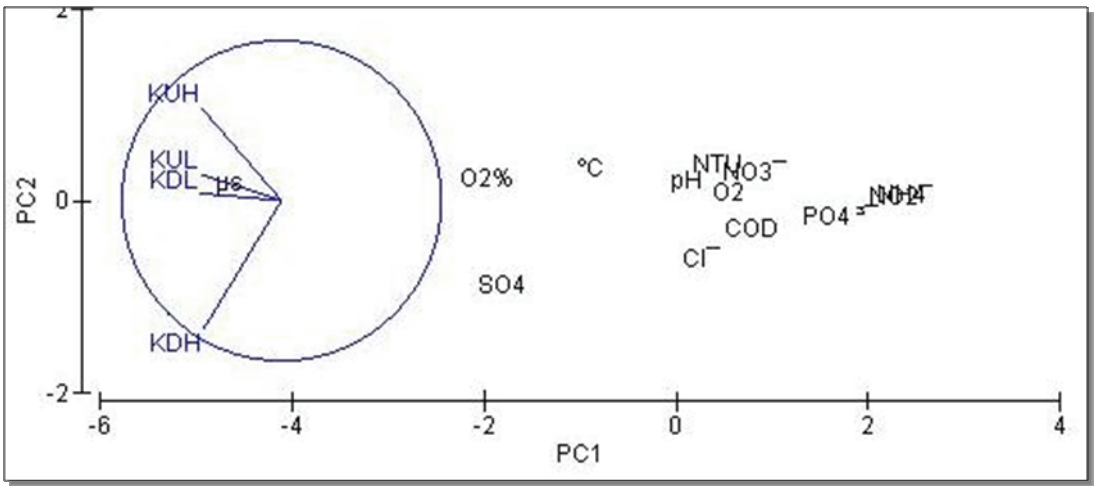


Figure 6.1: PCA bi-plot of water quality variables showing (dis)similarities for upstream and downstream sites associated with Feedlot A during low flow and high flow surveys. KUH = Feedlot A, upstream, high flow; KUL = Feedlot A, upstream, low flow; KDL = Feedlot A, downstream, low flow; KDH = Feedlot A, downstream, high flow.

Table 6.1: Water quality data including in situ variables and nutrients for upstream and downstream sites associated with each feedlot, during the low flow survey (November, 2007). KUL = Feedlot A, upstream, low flow; KDL = Feedlot A, downstream, low flow; TUL = Feedlot B, upstream low flow; TDL = Feedlot B, downstream, low flow; BUL, Feedlot C, upstream, low flow; BDL = Feedlot C, downstream, low flow.

	O_2 (mg/ℓ)	O_2 (%)	Temperature °C	Conductivity (µs)	pH	NH_4 (mg/ℓ)	Cl (mg/ℓ)	COD (O_2/mg/ℓ)	NO_3 (mg/ℓ)	NO_2 mg/ℓ)	PO_4 (mg/ℓ)	SO_4 (mg/ℓ)	Turbidity (NTU)
KUL	5.64	62.60	20.60	549.00	7.24	0.09	4.36	2.22	9.20	0.07	1.13	52.00	6.00
KDL	7.81	92.70	22.40	1,882.00	8.68	0.08	10.50	5.29	3.85	0.14	1.11	34.00	2.50
TUL	8.35	99.60	23.50	720.00	8.45	0.05	11.00	1.50	4.30	0.02	1.57	26.00	4.50
TDL	9.75	116.50	23.60	1,786.00	9.16	0.03	6.50	0.00	0.65	0.01	0.92	19.00	4.50
BUL	9.79	129.00	28.20	728.00	9.25	0.03	4.00	2.01	2.05	0.01	0.04	15.67	5.50
BDL	7.29	88.90	26.00	395.00	8.10	0.05	6.00	3.03	3.50	0.04	0.02	8.67	2.00

Table 6.2: Water quality data including in situ variables and nutrients for upstream and downstream sites associated with each feedlot, during the low flow survey (November, 2007). KUH = Feedlot A, upstream, high flow; KDH = Feedlot A, downstream, high flow; TUH = Feedlot B, upstream, high flow; TDH = Feedlot B, downstream, high flow; BUH = Feedlot C, upstream, high flow; BDH = Feedlot C, downstream, high flow.

	O_2 (mg/ℓ)	O_2 (%)	Temperature °C	Conductivity (µs)	pH	NH_4 (mg/ℓ)	Cl (mg/ℓ)	COD (O2/mg/ℓ)	NO_3 (mg/ℓ)	NO_2 (mg/ℓ)	PO_4 (mg/ℓ)	SO_4 (mg/ℓ)	Turbidity (NTU)
KUH	2.20	27.00	15.30	207.00	5.95	0.03	1.50	1.50	1.50	0.02	0.18	10.00	7.00
KDH	4.40	53.00	17.20	724.00	7.50	0.16	22.00	8.00	1.75	0.20	1.87	306.00	5.00
TUH	8.48	92.51	15.80	794.30	9.00	0.07	7.50	7.75	2.28	0.02	0.81	16.00	4.25
TDH	9.46	96.10	15.50	806.60	9.28	0.05	7.25	0.75	1.80	0.02	0.51	16.00	3.75
BUH	9.14	110.76	14.50	211.00	7.09	0.10	4.00	14.00	0.25	0.02	0.05	6.00	4.00
BDH	8.38	92.50	14.60	409.00	7.50	0.07	8.00	1.50	2.95	0.02	0.09	13.00	3.00

Table 6.3: 95'th Percentile values for Suikerbosrand River Uitvlught monitoring station, from 1996-2000 (DWAF)

	pH	NH_4 (mg/ℓ)	Cl (mg/ℓ)	NO_3 (mg/ℓ)	PO_4 (mg/ℓ)	SO_4 (mg/ℓ)
C2H004Q1 (Suikerbosrand)	8.5	0.26	199.14	0.97	0.322	1061.17
C1H017Q01 (Vaal River)	8.64	0.091	28.68	0.786	0.09	45.57

Table 6.4: Target Water Quality Range (TWQR) for in situ and nutrient variables (South African Water Quality Guidelines, 1996)

	O_2 (%)	pH	NH_4 (mg/ℓ)	Cl (mg/ℓ)	COD (O_2/mg/ℓ)	NO_3 (mg/ℓ)	NO_2 (mg/ℓ)	PO_4 (mg/ℓ)	SO_4 (mg/ℓ)	Turbidity (NTU)
TWQR	80-120	< 5% of background pH	0.007	0-100		0-100	0-10	< 15% of background concentration	0-200	< 15 from normal cycle of water body

Figure 6.2 shows a similar PCA bi-plot for sites associated with Feedlot B, also including low flow and high flow data sets. The two dimensional bi-plot accounts for 98.5% of the variation in the data, 66.9% of the variation is represented on the first axis, while 31.5% is represented on the second axis. Greater similarities can be observed during season, with high flow sites grouping together and low flow sites sharing similarities in turbidity, SO_4 and PO_4 concentrations. Similarly there is also a site ordination. Downstream sites are principally driven by a higher conductivity, oxygen and pH values, while NO_3 and COD contribute more to the similarity of the upstream sites.

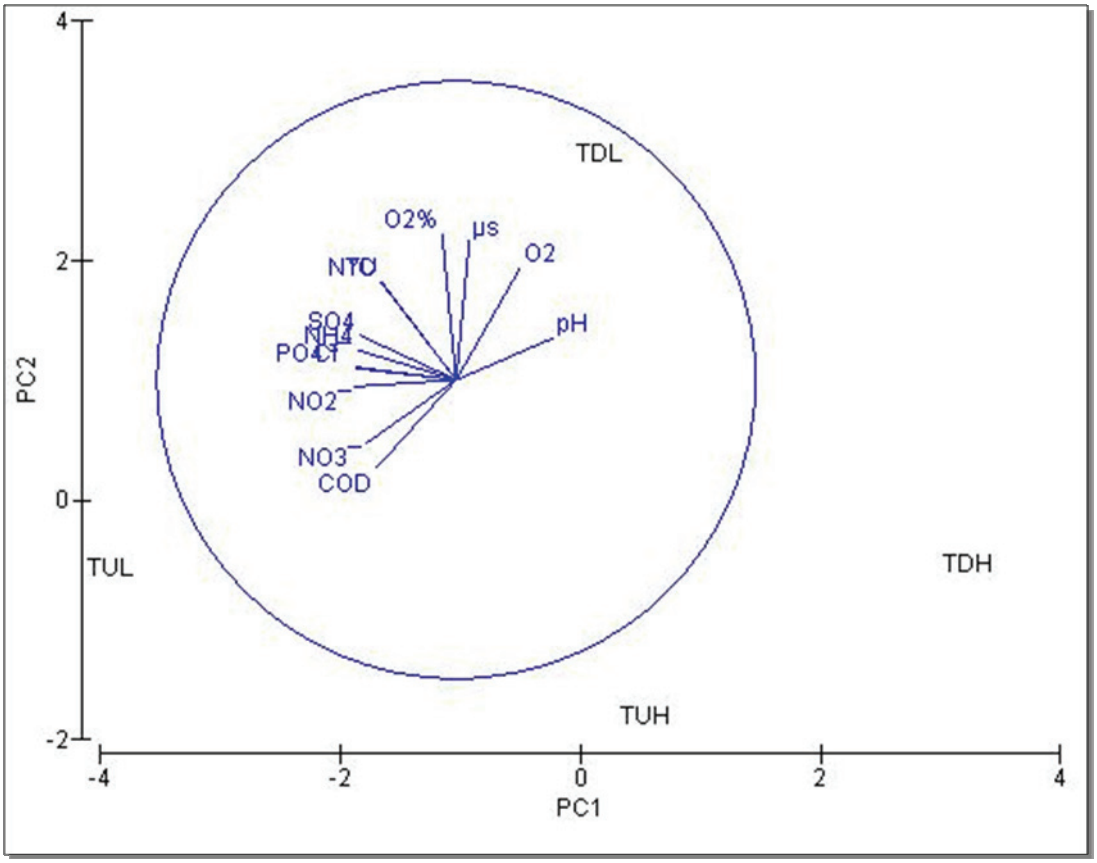


Figure 6.2: PCA bi-plot of water quality variables showing (dis)similarities for upstream and downstream sites associated with Feedlot B during low flow and high flow surveys. TUL = Feedlot B, upstream low flow; TDL = Feedlot B, downstream, low flow; TUH = Feedlot B, upstream, high flow; TDH = Feedlot B, downstream, high flow.

The PCA for sites associated with Feedlot C (Figure 6.3) indicate similarities in season and site ordination. The two dimensional bi-plot accounts for 84% of the variation in the data presented. The first axis represents 49.2% of the variation while the rest (34.8%) is represented by the second axis. A strong seasonal similarity can be observed, especially during the high flow survey. Sites, during the high flow survey, show similarities in NH_4 and PO_4 concentrations. COD, Cl^- and NO_3 contribute towards the low flow ordination. Despite the seasonal grouping of sites, an upstream downstream grouping can also be observed. Driving similarities of the downstream site, during both surveys are NO_2 and NH_4, while turbidity and oxygen concentration contributes to the similarities of the upstream site associated with Feedlot C.

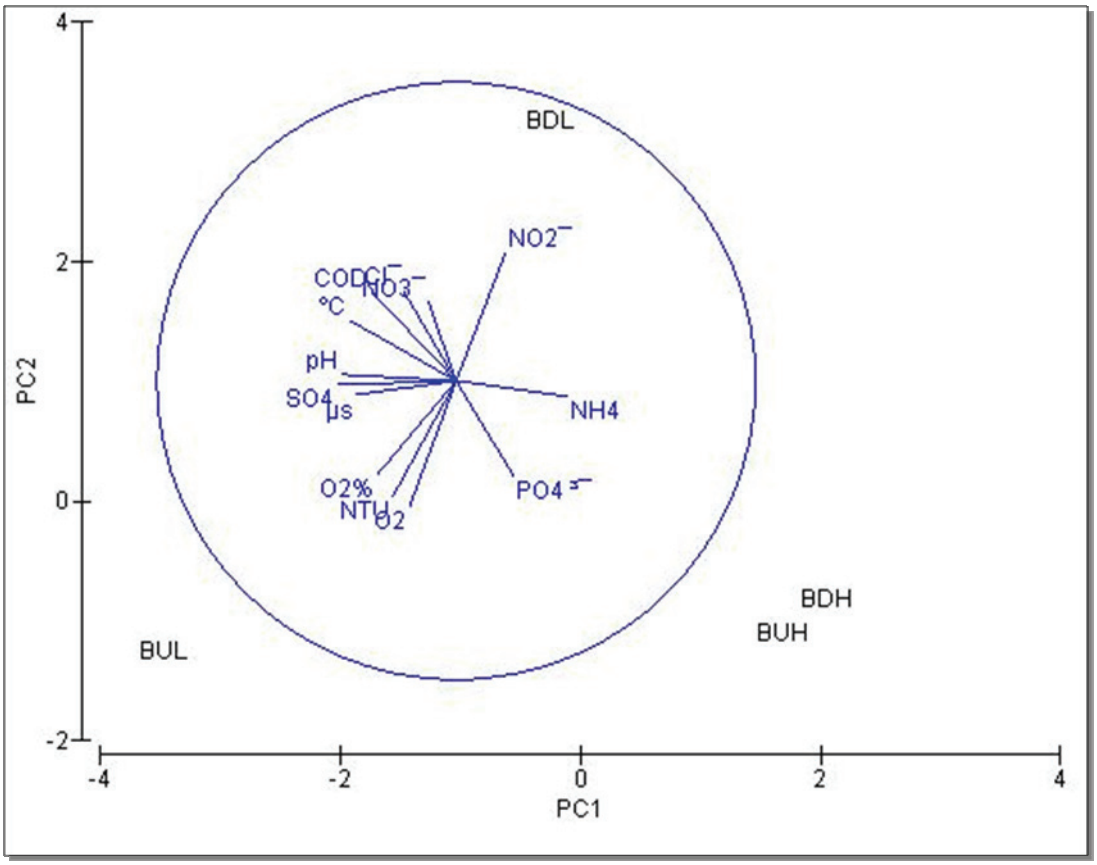


Figure 6.3: PCA bi-plot of water quality variables showing (dis)similarities for upstream and downstream sites associated with Feedlot C during low flow and high flow surveys. BUL, Feedlot C, upstream, low flow; BDL = Feedlot C, downstream, low flow; BUH = Feedlot C, upstream, high flow; BDH = Feedlot C, downstream, high flow.

6.4.1.1.3 Metals

Data representation shows inter site comparison as well as a comparison between feedlots. Data represented reflects the filtered metal values ascertained for each site during each survey period. Together with TWQR, Chronic Effect values (CEV) and Acute Effect Values (AEV) are also represented in Table 6.5.

Aluminium (Al) concentrations (Figure 6.4) obtained for all feedlots show no apparent difference between upstream and downstream sites. It also does not show any correlation with seasonal variation. Al concentration was the highest at Feedlot C downstream (1001 µg/ℓ) during the low flow survey. Feedlot C also shows on average a higher concentration of Al when compared to that of Feedlots A and B. Both Feedlot A and Feedlot B showed their highest concentration of Al at the downstream sites (50.5 µg/ℓ and 332.5 µg/ℓ respectively) associated with each, but again with no seasonal preference.

Al concentrations at all the sites and during both low flow and high flow surveys indicate concentrations far above the TWQR (Table 6.5). Both Feedlot A downstream and Feedlot C downstream have Al levels exceeding the AEV (levels in excesses of 150 µg/ℓ).

Cadmium (Cd) concentrations (Figure 6.5) much like Al concentrations do not show consistent differences between upstream and downstream sites associated with each feedlot. However, sample sites near Feedlot A and Feedlot B, on average have a higher Cd concentration than those near Feedlot C. At Feedlot C a decline in Cd concentration during the high flow survey were observed for both upstream and downstream sites at 33.5 µg/ℓ and 28.5 µg/ℓ respectively. The highest Cd concentration was detected at the (101.5 µg/ℓ) at the downstream site during the low flow survey near Feedlot C. Cd levels for all sites are above the TWQR and also far exceed the AEC (Table 6.5).

Chromium (Cr) (Figure 6.6) does not show obvious differences between upstream and downstream sites. However, it does show a seasonal correlation, with Cr levels being higher in the streams at all three feedlots during the high flow survey. Feedlot C shows the highest Cr levels, measured at the downstream site during the high flow survey (210.5 µg/ℓ), while Feedlot C upstream site shows the lowest Cr levels during the low flow survey (173 µg/ℓ). Cr concentrations for all sites and during both low flow and high flow surveys are above TWQR, while sites associated with Feedlot C show Cr levels in excess of 200 µg/ℓ. This value is higher than the AEV (Table 6.5).

Copper (Cu) concentration (Figure 6.7) ranges between 172.5 µg/ℓ and 220 µg/ℓ, with the highest levels being upstream from Feedlot B, during the low flow survey. Sites associated with Feedlot C on average have lower levels of Cu compared to the sample sites associated with Feedlot A or Feedlot B. There is now site or season differences between Cu concentrations. Cu concentrations far exceed the TWQR as well as AEV (in excess of 7.5 µg/ℓ) (Table 6.5).

On average sites associated with Feedlot A shows higher levels of Iron (Fe) than those associated with Feedlots C and B (Figure 6.8). Fe levels are the lowest in the water at the downstream sites at all three feedlots during the low flow survey, which suggests some form of seasonality. This trend repeats itself during the high flow survey, where Fe levels for both upstream and downstream sites are higher on average when compared to the low flow Fe levels. TWQR for Fe dictates variation of smaller than 10% when compared to background concentrations. No background concentration could be established. Therefore Fe concentration cannot be compared.

Manganese (Mn) levels (Figure 6.9) in the water sampled in the stream associated with Feedlot A are higher upstream than downstream during both low flow and high flow surveys. Feedlot C indicated higher Mn levels during the high flow survey at both upstream and downstream sites.
Mn levels obtained from sites associated with Feedlot B feedlot do not show any differences between upstream and downstream sites, however, Mn levels in the stream associated with Feedlot B were lower during the high flow survey than the low flow survey. The Mn concentration measured at all the sites are below the TWQR (Table 6.5).

The water samples during the low flow survey downstream of feedlot C, obtained the highest Nickel (Ni) concentrations (Figure 6.10) (167 µg/ℓ). Feedlot A also shows elevated levels of Ni during the low flow survey for both upstream and downstream sites, while Ni concentrations for Feedlot B was higher during the low flow assessment for both upstream and downstream sites. Ni does not show distinct differences between sites or between seasons. Ni concentrations for all the sites are below TWQR (Table 6.5).

Zinc (Zn) levels (Figure 6.11) are very similar in the water samples at all the feedlots. The stream associated with Feedlot A and Feedlot B show slightly higher concentrations of Zn, especially during the high flow survey, while samples in the stream associated with Feedlot C show slightly elevated Zn levels during the low flow survey. Zn concentrations in the streams at all the sites are much higher than TWQR and the AEV (in excess of 36 µg/ℓ) (Table 6.5).

With the exception of Feedlot C upstream, during the low flow survey with a Lead (Pb) concentration of 819 µg/ℓ, Pb concentrations (Figure 6.12) show a definite site preference, with downstream sites at both Feedlot A and Feedlot B indicating elevated levels of Pb during both low flow and high flow surveys. Downstream from Feedlot C shows a similar trend but only for the high flow survey. Pb levels at all the sites far exceed the TWQR for aquatic ecosystems as well as the AEV (in excess of 13 µg/ ℓ) (Table 6.5).

Table 6.5: Indicated the TWQR, CEV and AEV for metals assessed in this study (DWAF, 1996).

	Al µg/ℓ	**Cd µg/ℓ**	**Cr µg/ℓ**	**Cu µg/ℓ**	**Fe µg/ℓ**	**Mn µg/ℓ**	**Ni µg/ℓ**	**Zn µg/ℓ**	**Pb µg/ℓ**
TWQR	10	0.35	7	1.2	< 10 variation on background concentration	180	0-1000	2	1
Chronic Effect Value (CEV)	20	0.7	14	2.4	NA	370	NA	3.6	2
Acute Effect Value (AEV)	150	10	200	7.5	NA	1300	NA	36	13

NA = Not Available.

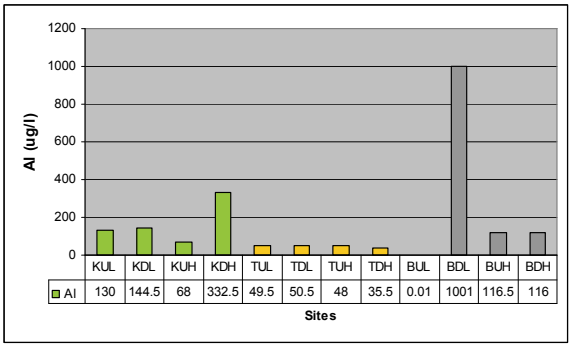


Figure 6.4: Aluminium concentrations for upstream and downstream sites, associated with three feedlots respectively.

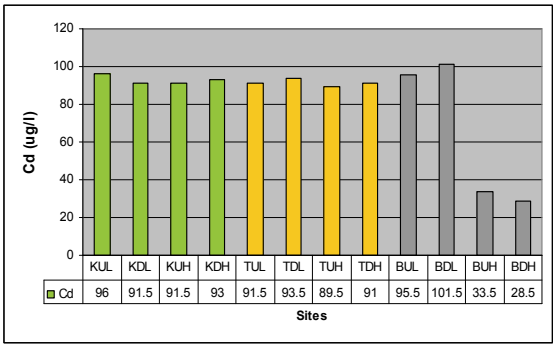


Figure 6.5: Cadmium concentrations for upstream and downstream sites, associated with three feedlots respectively.

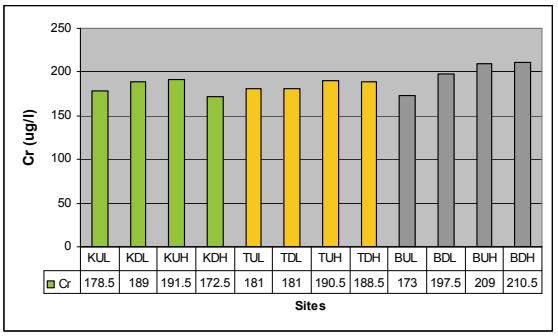


Figure 6.6: Chromium concentrations for upstream and downstream sites, associated with three feedlots respectively.

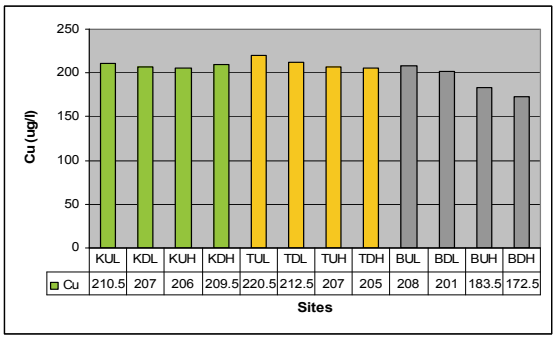


Figure 6.7: Copper concentrations for upstream and downstream sites, associated with three feedlots respectively.

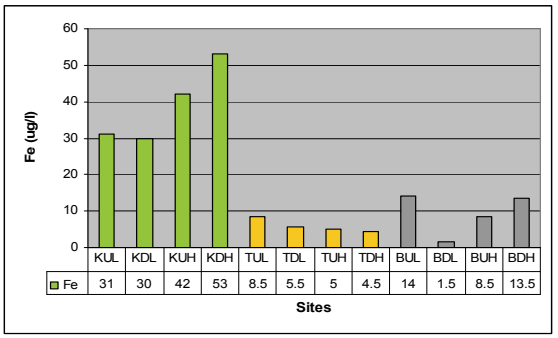


Figure 6.8: Iron concentrations for upstream and downstream sites, associated with three feedlots respectively.

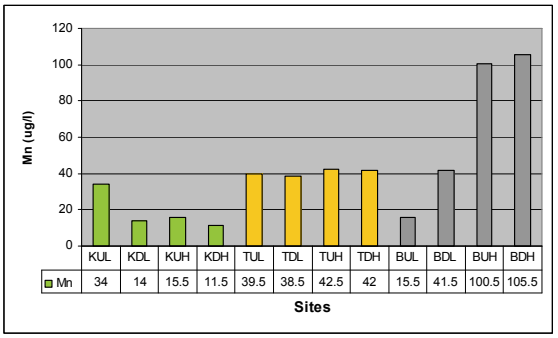


Figure 6.9: Manganese concentrations for upstream and downstream sites, associated with three feedlots respectively.

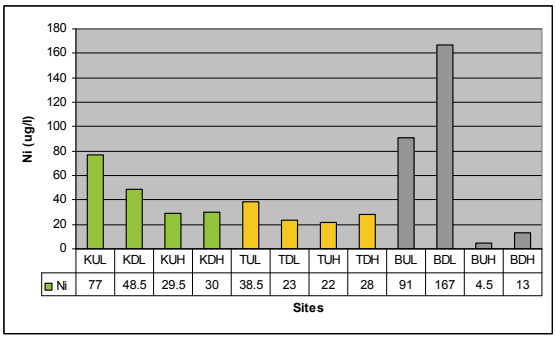


Figure 6.10: Nickel concentrations for upstream and downstream sites, associated with three feedlots respectively.

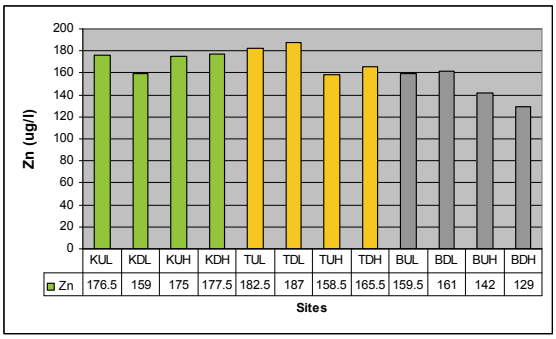


Figure 6.11: Zinc concentrations for upstream and downstream sites, associated with three feedlots respectively.

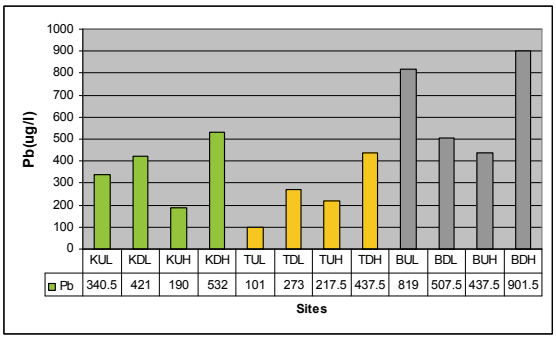
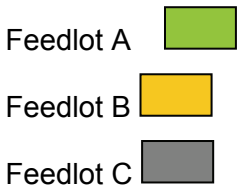


Figure 6.12: Lead concentrations for upstream and downstream sites, associated with three feedlots respectively.



6.4.1.2 Sediment grain size and organic composition

6.4.1.2.1 Grain size

Sediment is an important factor to consider in any toxicological study. Soil texture and structure relate back to the soil's ability to accommodate the movement of nutrients and water. Grain size analysis will indicate the amount of small clay particles present at each site. Clay, in turn, shows high affinity for organic matter which acts as storing bin for pollutants and toxins. Variation in grain size composition will also indicate alteration in hydrology.

Figure 6.4 to 6.15 shows spider diagrams indicating the percentage grain size of sediment samples associated with each feedlot during low flow and high flow field assessments. Figure 6.4 shows that the sediment grain size composition of samples taken upstream of Feedlot A during the low flow survey is sandier. During the high flow survey it turns slightly silt-like in composition. Neither upstream nor downstream sites associated with Feedlot A consist of high clay content. However, the sediment sampled downstream of Feedlot A during the high flow survey consisted of more than 40% silt in soil composition. Soil composition of sediment in the stream associated with Feedlot B (Figure 6.5) shows a similar texture to that of sediment samples from the stream near Feedlot A. Feedlot B upstream low flow sample has the highest silt content, while Feedlot B downstream high flow shows a more courser soil texture. Overall soil texture for both upstream and downstream sites associated with Feedlot B shows a loamy sand grain size composition.

Figure 6.15 shows the grain size composition for sediment sampled in the stream associated with Feedlot C. Feedlot C upstream low flow shows a silty soil composition while the downstream site during the same survey is composed of a less courser grain size. The downstream high flow sample near Feedlot C shows a soil composition that is consistent with a silty clay loam soil, with more than 60% of the grain size composition being smaller than 53 µm.

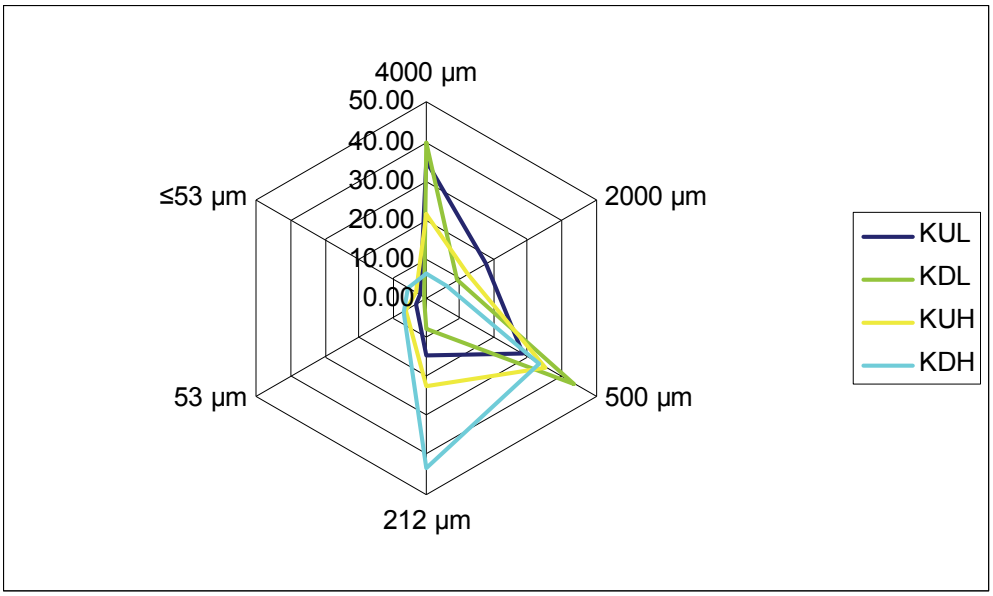


Figure 6.13: Spider diagram representing the percentage grain size composition of both upstream and downstream sites associated with Feedlot A. KUH = Feedlot A, upstream, high flow;

KUL = Feedlot A, upstream, low flow; KDL = Feedlot A, downstream, low flow; KDH = Feedlot A, downstream, high flow.

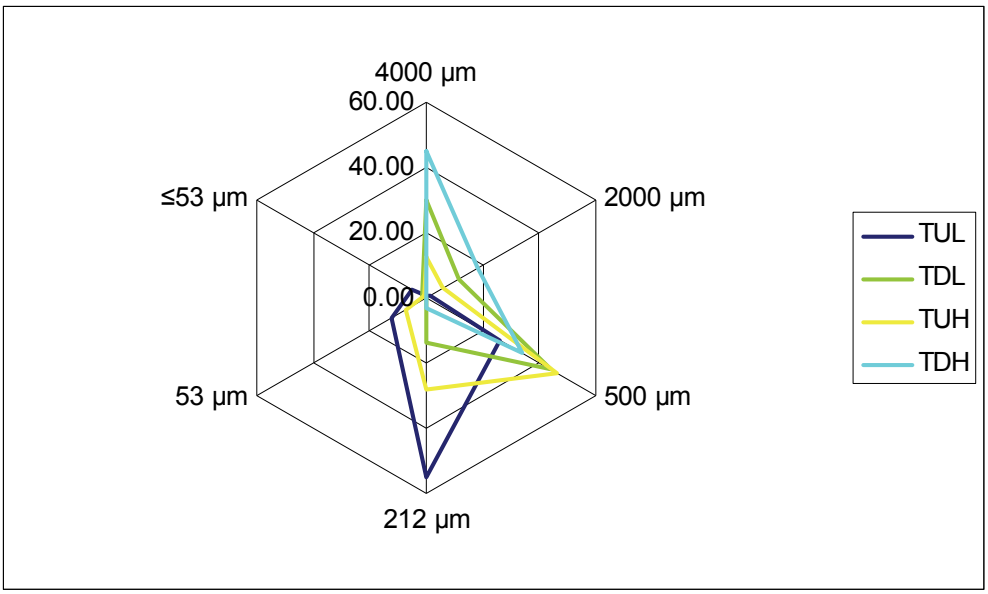


Figure 6.14: Spider diagram representing the percentage grain size composition of both upstream and downstream sites associated with Feedlot B. TUL = Feedlot B, upstream low flow; TDL = Feedlot B, downstream, low flow; TUH = Feedlot B, upstream, high flow; TDH = Feedlot B, downstream, high flow.

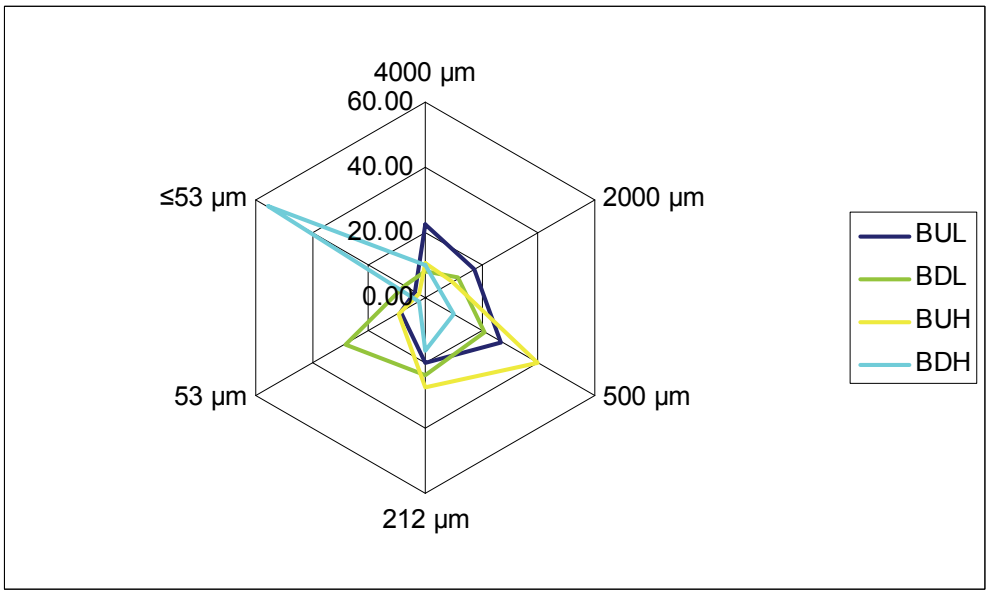


Figure 6.15: Spider diagram representing the percentage grain size composition of both upstream and downstream sites associated with Feedlot C. BUL, Feedlot C, upstream, low flow; BDL = Feedlot C, downstream, low flow; BUH = Feedlot C, upstream, high flow; BDH = Feedlot C, downstream, high flow.

6.4.1.2.2 Organic matter

The amount of organic matter present in the soil will indicate the ability of each site to assimilate toxicants. Organic matter in soil also plays a very important role in soil formation as well as

nutrient and gas cycling. Here, the percentage organic matter present at each upstream and downstream sites associated with each feedlot, is represented in a spider diagram.
Feedlot A upstream shows a higher organic content during both low flow and high flow surveys, when compared to the downstream site during the same surveying times.
The organic content for sediment associated with Feedlot A upstream site is almost 3% during low flow and high flow surveys, compared to a much lower 1% at the downstream site.

Soil organic content for sediment sampled in the stream near Feedlot B is higher for the downstream sites, consistently during the low flow and high flow surveys. Overall sediment samples in the stream associated with Feedlot B have on average less organic matter per unit soil than near Feedlot A.

Organic content of the sediment sampled in the stream near Feedlot C is the highest during the low flow survey, for both upstream and downstream sites. Feedlot C downstream shows the highest percentage organic matter present in sediment at 8%.

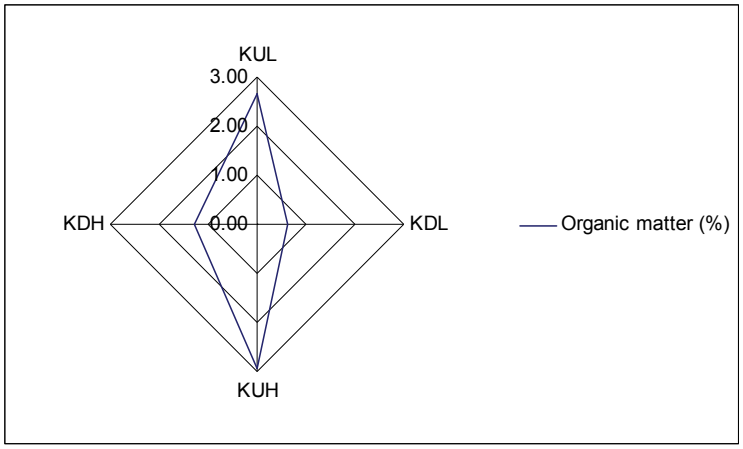


Figure 6.16: Spider diagram representing the percentage organic matter present at both upstream and downstream sites associated with Feedlot A. KUH = Feedlot A, upstream, high flow; KUL = Feedlot A, upstream, low flow; KDL = Feedlot A, downstream, low flow; KDH = Feedlot A, downstream, high flow.

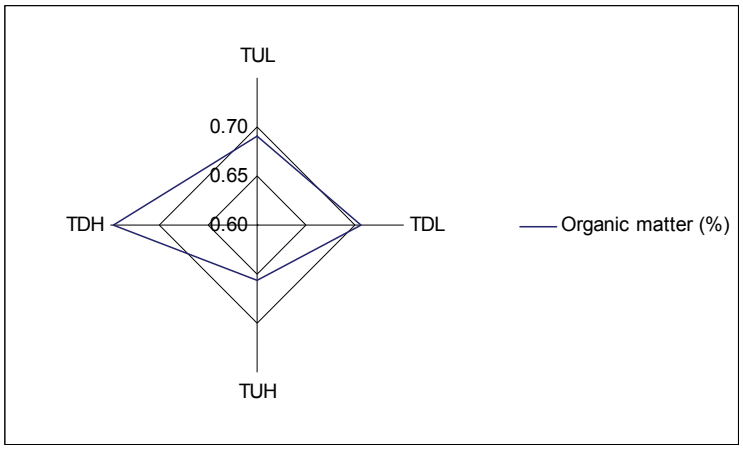


Figure 6.17: Spider diagram representing the percentage organic matter present at both upstream and downstream sites associated with Feedlot B. TUL = Feedlot B, upstream low flow; TDL = Feedlot B, downstream, low flow; TUH = Feedlot B, upstream, high flow; TDH = Feedlot B, downstream, high flow.

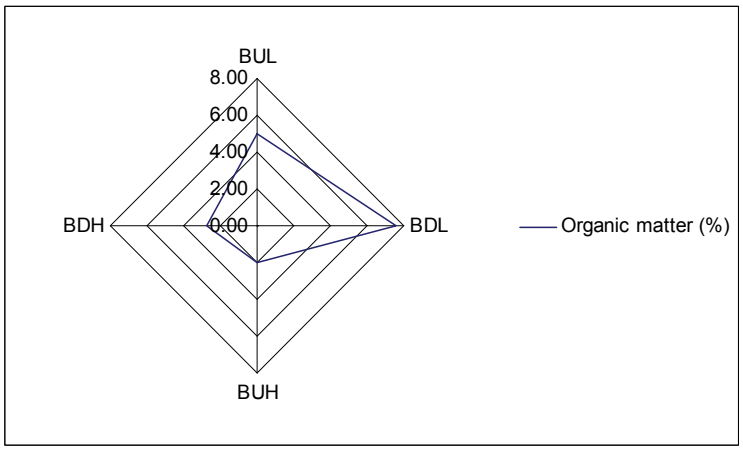


Figure 6.18: Spider diagram representing the percentage organic matter present at both upstream and downstream sites associated with Feedlot C. BUL, Feedlot C, upstream, low flow; BDL = Feedlot C, downstream, low flow; BUH = Feedlot C, upstream, high flow; BDH = Feedlot C, downstream, high flow.

6.4.1.3 Habitat assessment (IHAS, IHI)

6.4.1.3.1 IHAS

IHAS is a measure of the SASS biotopes sampled. Biotopes sampled include stones vegetation and other biotopes (McMillan, 1998).

All available habitats were sampled, including stones in and out of current, vegetation in and out of current, gravel between rocks, as well as sand and mud in the deeper pools.

Table 6.6: IHAS scores for stream associated with Feedlot A.

	Feedlot A	Upstream
IHAS	Stone in-current score	20
	Vegetation	13
	Other habitats	16
	Stream Condition	29
	IHAS Score	78
	State category	**C**
		Downstream
IHAS	Stone in-current score	21
	Vegetation	10
	Other habitats	8
	Stream Condition	37
	IHAS Score	76
	State category	**C**

Habitat integrity in terms of availability and the general state of the habitat that is available, for both upstream and downstream sites near Feedlot A falls with a C-class. This class relates to moderately modified.

Feedlot B is located next to a bigger river system (Vaal River). Both of the upstream and the downstream sites related to this feedlot fall within a similar ecological category. A number of different habitat biotopes are present at both sites, with no obvious differences between them. Both the upstream and the downstream sites are classified in a B-class which relates to largely natural state.

Table 6.7: IHAS scores for stream associated with Feedlot B.

	Feedlot B	Upstream
IHAS	Stone in-current score	20
	Vegetation	15
	Other habitats	18
	Stream Condition	32
	IHAS Score	85
	State category	**B**
		Downstream
IHAS	Stone in-current score	20
	Vegetation	15
	Other habitats	18
	Stream Condition	32
	IHAS Score	85
	State category	**B**

The stream associated with Feedlot C (Table 6.8) is the only stream that shows obvious differences in habitat availability for the purpose of invertebrate colonisation. The upstream site in this stream falls with a C-class ecological category, which relates to moderately modified state of habitat. The downstream site associated with Feedlot B falls in a D- class and related to seriously modified state.

Table 6.8: IHAS scores for stream associated with Feedlot C.

	Feedlot C	**Upstream**
IHAS	Stone in-current score	19
	Vegetation	14
	Other habitats	19
	Stream Condition	24
	IHAS Score	76
	State category	**C**
		Downstream
IHAS	Stone in-current score	0
	Vegetation	13
	Other habitats	10
	Stream Condition	23
	IHAS Score	46
	State category	**D**

6.4.1.3.2 IHI

The Habitat Integrity Index (IHI) rates impacts on a scale between 0 (none) to 25 (extreme). It subdivides impacts that impacts on in-stream habitat and riparian habitat. When comparing the habitat integrity of upstream and downstream sites, near each feedlot respectively, not much difference is evident (Table 6.9 to Table 6.11).

Table 6.9: IHI scores for the stream associated with the Feedlot A.

	Feedlot B	**Upstream**
IHI	Primary IHI	36
	Secondary IHI	28
	Riparian Zone Habitat Integrity	72
	Total (425)	136
	Percentage	68%
	Category	**C**
		Downstream
IHI	Primary IHI	49
	Secondary IHI	20
	Riparian Zone Habitat Integrity	89
	Total (425)	158
	Percentage	62.8%
	Category	**C**

Both sites in the streams near Feedlots A and B fall within a C-class. The downstream site in the river associated with Feedlot C shows more of a decline in habitat integrity and is classed in a D-class.

Table 6.10: IHI scores for the stream associated with the Feedlot B.

	Feedlot B	**Upstream**
IHI	Primary IHI	38
	Secondary IHI	20
	Riparian Zone Habitat Integrity	54
	Total (425)	112
	Percentage	73.6%
	Category	**C**
		Downstream
IHI	Primary IHI	32
	Secondary IHI	20
	Riparian Zone Habitat Integrity	51
	Total (425)	103
	Percentage	75.8%
	Category	**C**

Table 6.11: IHI scores for the stream associated with the Feedlot C.

	Feedlot C	Upstream
IHI	Primary IHI	43
	Secondary IHI	0
	Riparian Zone Habitat Integrity	50
	Total (425)	93
	Percentage	78%
	Category	**C**
		Downstream
IHI	Primary IHI	62
	Secondary IHI	18
	Riparian Zone Habitat Integrity	96
	Total (425)	58.6%
	Percentage	**D**
	Category	

6.4.1.4 Aquatic macro-invertebrate assessment (SASS5)

Aquatic macro-invertebrates have long been used to assess the biological integrity of river ecosystems more commonly than any other biological group. The reason for this is that they are relatively sedentary and enable the detection of localised disturbances.

Their relatively long life histories (± 1 year) allow for the integration of pollution effects over time. The methodology is relatively simple and since the communities are heterogeneous and several phyla are usually represented, response to environmental impacts is normally detectable in terms of the community as a whole.

SASS5 scores obtained during the low flow, November 2007, show little variation between invertebrate abundances and sensitivity when comparing sites in the rivers above and below feedlots (Table 6.12). However, there are observable difference for the high flow, survey (Table 6.13).

During the low flow assessment the highest sensitivity score obtained was 5.6 ASPT for the site upstream of Feedlot B with a taxon diversity of 25 families, while the lowest was obtained for the site upstream form Feedlot A (3.8 ASPT) with a total taxon diversity of 13 families. Overall, there is a smaller diversity presence at the downstream sites

Table 6.12: Aquatic macro-invertebrate data: SASS 5 Scores, ASPT's and respective SASS5 biotope scores for upstream and downstream of each Feedlot, respectively for the low flow assessment.

SASS5 Score		No. Taxa	ASPT	SASS5 Score per biotope		
				Stones	Vegetation	GSM
Feedlot A	Upstream	13	3.8	3.9	3.4	3.8
	Downstream	13	4.2	4.0	4.5	3.2
Feedlot B	Upstream	25	5.6	6.2	5.5	3.4
	Downstream	15	5.1	4.8	5.3	0
Feedlot C	Upstream	19	4.4	3.7	3.5	5.2
	Downstream	16	4.6	0	4.2	3.7

GSM – Gravel Sand and Mud habitat; ASPT – Average Score per taxon

Table 6.13 shows the invertebrate diversity and sensitivity scores for all the feedlots during the high flow assessment.

The highest sensitivity score was obtained upstream from Feedlot A (6.6 ASPT), while the lowest was obtained for Feedlot C downstream. The SASS5 scores do show a general decline during the high flow survey when comparing the upstream sites with that of the downstream sites. Upstream sites for Feedlot B and Feedlot C yielded the highest taxon diversity with 21 and 27 families respectively.

The biggest discrepancy in terms of SASS5 scores was observed between upstream and downstream sites associated with Feedlot C.

Table 6.13: Aquatic macro invertebrate data: SASS 5 Scores, ASPT's and respective SASS5 biotope scores for upstream and downstream of each Feedlot, respectively for the high flow assessment.

SASS5 Score		No. Taxa	ASPT	SASS5 Score per biotope		
				Stones	Vegetation	GSM
Feedlot A	Upstream	16	6.6	6.7	4.2	5.3
	Downstream	10	5.1	4.8	5.6	3.2
Feedlot B	Upstream	21	5.6	5.5	5.3	4.4
	Downstream	19	5.1	6	4.9	4.3
Feedlot C	Upstream	27	5.4	4.9	3.1	4.6
	Downstream	10	4.1	0	5.1	4.0

GSM – Gravel Sand and Mud habitat; ASPT – Average Score per taxon

6.4.1.5 Fish Assemblage Integrity Index Assessment (FAII)

The FAII aims to measure the biological integrity of a river as based on the attributes of the fish assemblage's native to the river. Alien species (introduced indigenous and exotic species) are not included as metrics in the FAII. Their presence and distribution are noted but interpreted as possible causes for a decline in the FAII score.

A desktop review pertaining to distribution and habitat preference of ichthyofauna (Kleynhans, 2007) was done to ascertain what fish species may find potential refuge in associated systems (Table 6.14). The fish distribution list represents the potential fish species at all three feedlots.
Both *Labeobarbus kimberleyensis* and *Austroglanus sclateri* have conservation status. A number of exotic fish species also share the same distribution range, these include: *Gambusia affinis, Lepomis macrochirus* and *Micropterus salmoides*.

Table 6.14: Expected fish occurrence for all six sites assessed. Expected occurrence list includes alien species. Particular occurrence list excludes historical distribution.

Scientific Name	Common name
Austroglanus sclateri (Boulenger, 1901)	Rock Catfish
Labeobarbus aeneus (Burchell, 1822)	Smallmouth Yellowfish
Barbus anoplus (Weber, 1897)	Chubbyhead Barb
Labeobarbus kimberleyensis (Gilchrist and Thompson, 1913)	Largemouth Yellowfish
Barbus neefi (Greenwood, 1962)	Sidespot Barb
Barbus pallidus (Smith, 1841)	Goldie Barb
Barbus trimaculatus (Peters, 1852)	Threespot Barb
Clarias gariepinus (Burchell, 1822)	Sharptooth Catfish
Gambusia affinis (Baird and Girard, 1853)	Mosquitofish (Ex)
Labeo capensis (Smith, 1841)	Orange River Labeo
Lepomis macrochirus (Rafinesque, 1819)	Bluegill Sunfish (Ex)
Labeo umbratus (Smith, 1841)	Moggel
Micropterus salmoides (Lacepède, 1802)	Largemouth Bass (Ex)
Pseudocrenilabrus philander (Weber, 1897)	Southern Mouthbrooder
Tilapia sparrmanii (Smith, 1840)	Banded Tilapia

Fish assemblage integrity classes for all sites surveyed during the low flow survey are shown in Table 6.15. No obvious differences are visible. It must however be noted that sampling efforts had a time constraint on them. Subsequently, shocking was applied at all sites for the same amount of time in order to obtained comparable data.

Table 6.15: Ecological categories for Fish Assemblage Integrity are shown, for both upstream and downstream sites associated with each feedlot respectively.

Site	Relative Fish Assemblage	Fish Assemblage Integrity Ecological Category
KU	47.5	D
KD	39.8	E
TU	32.6	E
TD	34.9	E
BU	22	E
BD	9.5	F

6.4.2 Invertebrate community composition

6.4.2.1 Aquatic macro-invertebrate diversity and dominance

Figure 6. shows a K-dominance plot for macro-invertebrate communities for upstream and downstream sites associated with Feedlot A. K-dominance plot is a measure of disturbance. Impacted sites are characterised by high abundances and low diversity of macro-invertebrates. Results on the invertebrate community in the river downstream from Feedlot A during the low flow survey show the highest disturbance with 55% of the invertebrates counted belonging to a single taxon. While the same site, during the low flow survey, showed less disturbance with 28% of invertebrates counted belonging to one taxon. However the invertebrate community response is connected to seasonality, more than what it is connected to the feedlot activity.

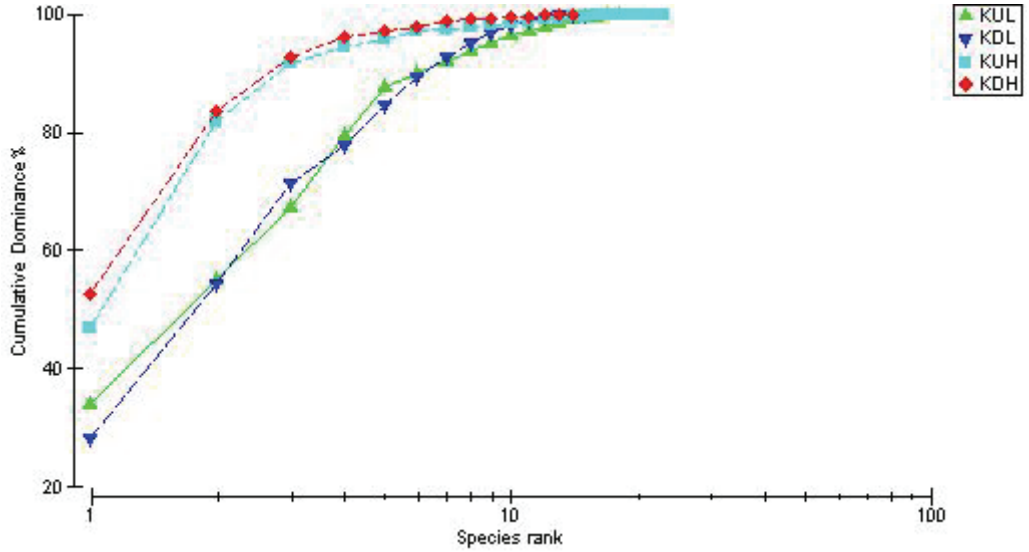


Figure 6.19: Ranked species K-dominance plot for macro-invertebrate communities collected at upstream and downstream sites associated with Feedlot A feedlot during low flow and high flow field surveys. KUH = Feedlot A, upstream, high flow; KUL = Feedlot A, upstream, low flow; KDL = Feedlot A, downstream, low flow; KDH = Feedlot A, downstream, high flow.

Figure 6.20 represents a K-dominance plot for macro-invertebrates community composition responses in the river associated with Feedlot B. Feedlot B downstream, during the high flow survey, shows the biggest disturbance with 68% of invertebrates counted belonging to a single taxon. Upstream from Feedlot B, during the low flow survey, shows healthier distribution of invertebrate diversity and abundances.

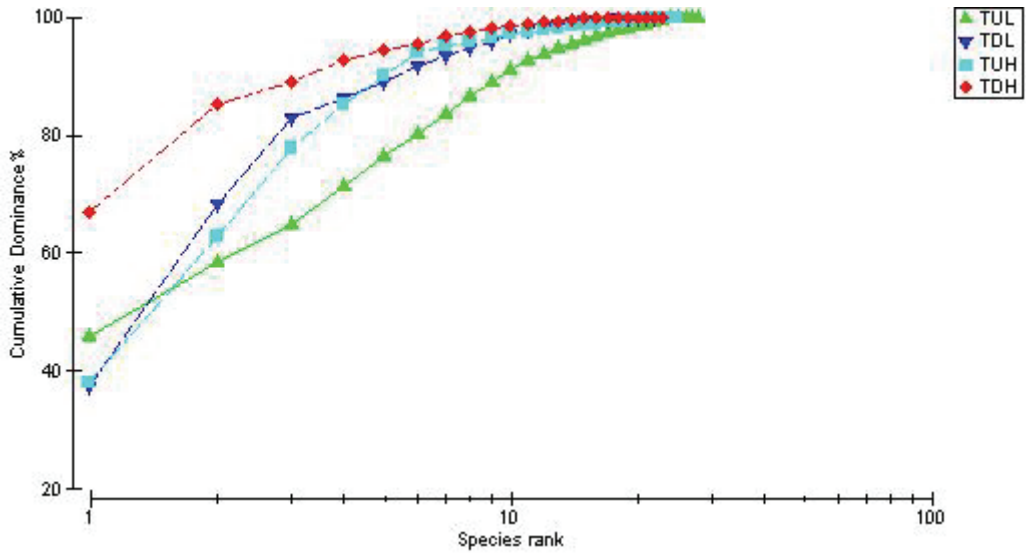


Figure 6.20: Ranked species K-dominance plot for macro-invertebrate communities collected at upstream and downstream sites associated with Feedlot B feedlot during low flow and high flow field surveys. TUL = Feedlot B, upstream low flow; TDL = Feedlot B, downstream, low flow; TUH = Feedlot B, upstream, high flow; TDH = Feedlot B, downstream, high flow.

Figure 6.21 represents the diversity and abundance distribution for sample sites associated with Feedlot C. Analysis of samples collected downstream from Feedlot C, during the high flow survey, shows better macro-invertebrate abundance distributions than that of the upstream site. This however does not remain the same during the low flow survey, with nearly 50% of invertebrates belonging to a single taxon. The low flow survey for both upstream and downstream sample sites yielded a similar diversity and abundance relationship.

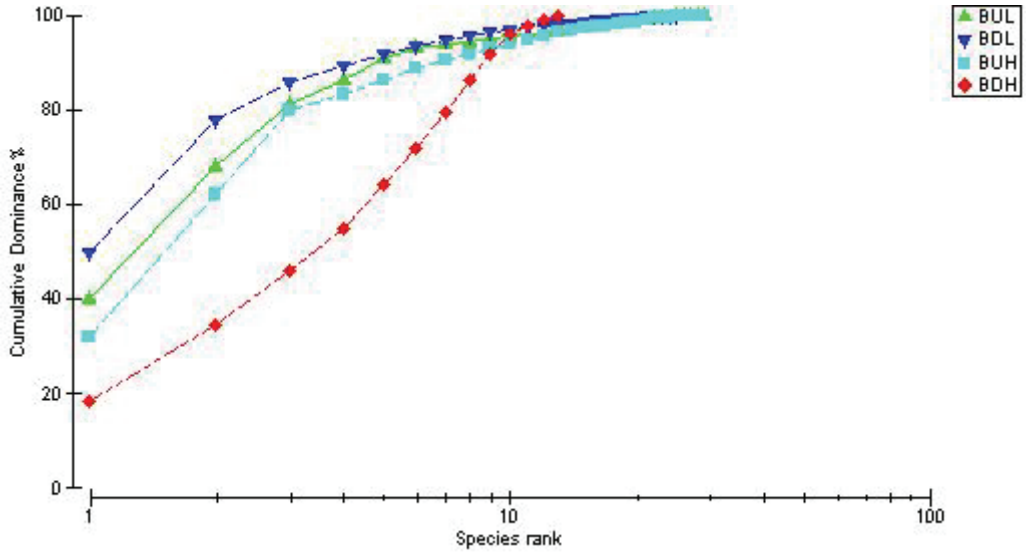


Figure 6.21: Ranked species K-dominance plot for macro-invertebrates communities collected at upstream and downstream sites associated with Feedlot C feedlot during low flow and high flow field surveys. BUL, Feedlot C, upstream, low flow; BDL = Feedlot C, downstream, low flow; BUH = Feedlot C, upstream, high flow; BDH = Feedlot C, downstream, high flow.

6.4.2.2 Aquatic macro-invertebrate community structure

The PCA for sites associated with Feedlot A (Figure 6.22) indicates a site ordination based on invertebrate diversity and abundances as well as the water quality variables influencing that ordination. Here, a clear site grouping can be observed, with both downstream sites grouping together as well as both upstream sites. The invertebrates responsible for the ordination of the downstream site are Physidae, Ancylidae, Planaria and Corbiculidae. The sites downstream from Feedlot A, during the high flow survey, shows elevated levels of nutrients and pH, relative to the upstream site which is predominantly influenced by turbidity. There is also a larger difference between the macro-invertebrate diversity and abundances of the upstream site during low flow and high flow assessment, indicating a seasonal influence.

Figure 6.23 represents a PCA for the sites associated with Feedlot B: here again, a site ordination is visible. Downstream site for both low and high flow surveys group together below the x-axis while the upstream site, for both surveys, group above the x-axis. pH, Oxygen, conductivity and turbidity are the driving factors at the downstream site during both high flow and low flow surveys. PO_4, Cl^- and SO_4 are more prominent at the upstream site during the low flow, while COD, NH_4 and NO_3 contribute toward the similarity between upstream sites regardless of season. The downstream site macro-invertebrate community structure mostly composes of Simuliidae, Muscidae and Corbicullidae.

The PCA for results from the sites near Feedlot C (Figure 6.24) indicates a definite macro-invertebrate community structure difference between upstream and downstream sites associated with Feedlot C.

Furthermore the differences are dissimilar with regards to seasonality and site ordination. Subsequently, the water quality driving forces associated with this invertebrate community composition for the upstream site are O_2, Turbidity, SO_4, pH and COD, while the downstream site is characterised by higher NO_2 and NO_3 concentrations.

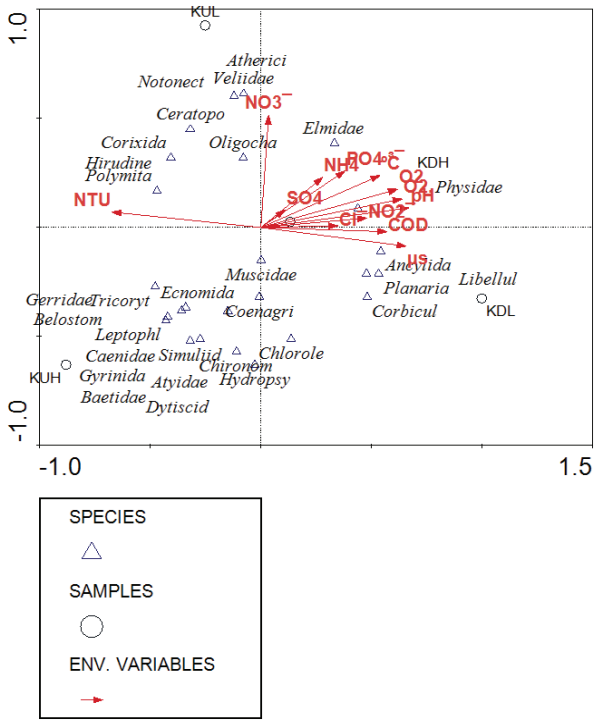


Figure 6.22: Principle Component Analysis (PCA) indicating Site and Season ordination based on (a) invertebrate abundances and diversity and (b) water quality variables, for both upstream and downstream sites associated with Feedlot A feedlot over low and high flow field surveys. K=Feedlot A, D=Downstream, U=Upstream, H= High flow, L= Low flow.

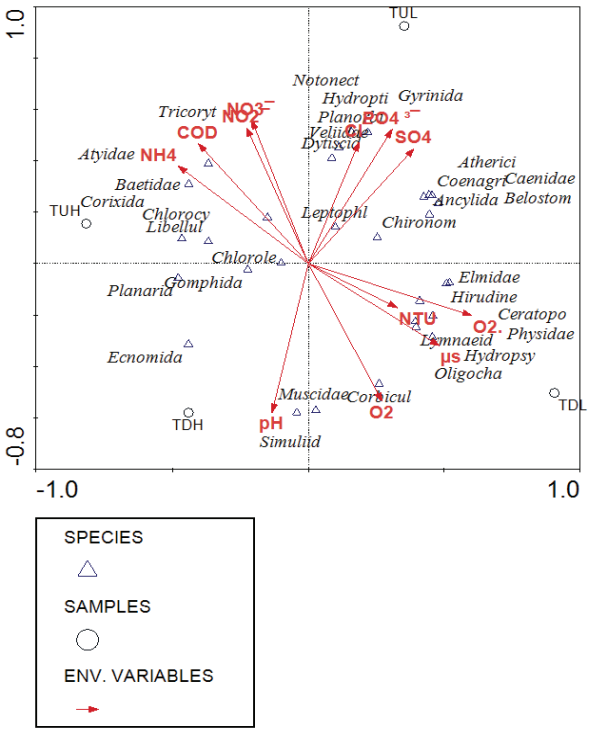


Figure 6.23: Principle Component Analysis (PCA) indicating Site and Season ordination based on (a) invertebrate abundances and diversity and (b) water quality variables, for both upstream and downstream sites associated with Feedlot B feedlot over low and high flow field surveys. T=Feedlot B, D=Downstream, U=Upstream, H= High flow, L= Low flow.

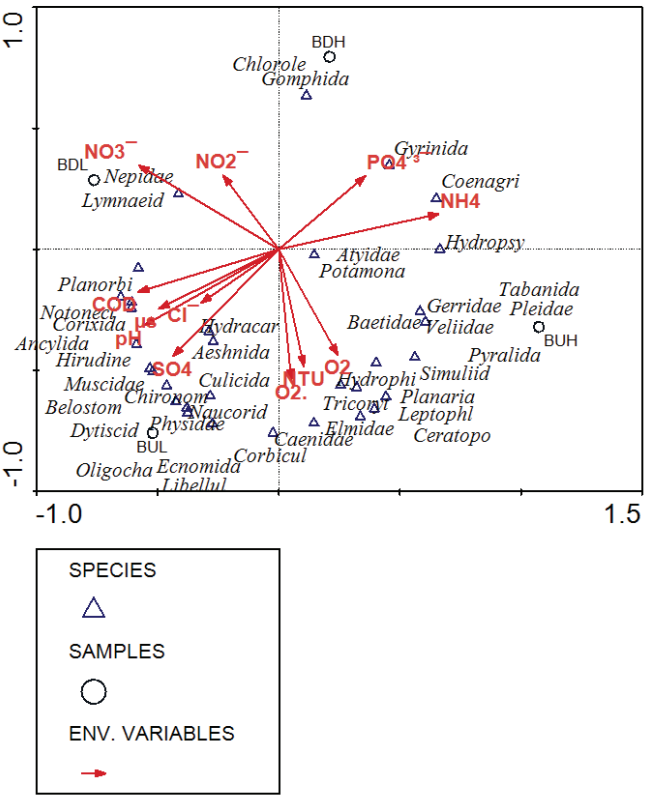


Figure 6.24: Principle Component Analysis (PCA) indicating Site and Season ordination based on (a) invertebrate abundances and diversity and (b) water quality variables, for both upstream and downstream sites associated with Feedlot C feedlot over low and high flow field surveys. B=Feedlot C, D=Downstream, U=Upstream, H= High flow, L= Low flow.

6.4.3 Biomarkers

6.4.3.1 Cellular Energy Allocation (CEA)

CEA is a cellular measure of energy consumption. Figure 6.25 shows the quantity of glucose represented by three different exposure groups over a 15 day period with 5 day intervals. Diethylstilbestrol shows a decrease in glucose concentration after 10 days of exposure. However these levels return to “normal” after 15 days of exposure. Trenbolone shows the smallest standard deviation and a similar trend to that of the oestrogen exposure.

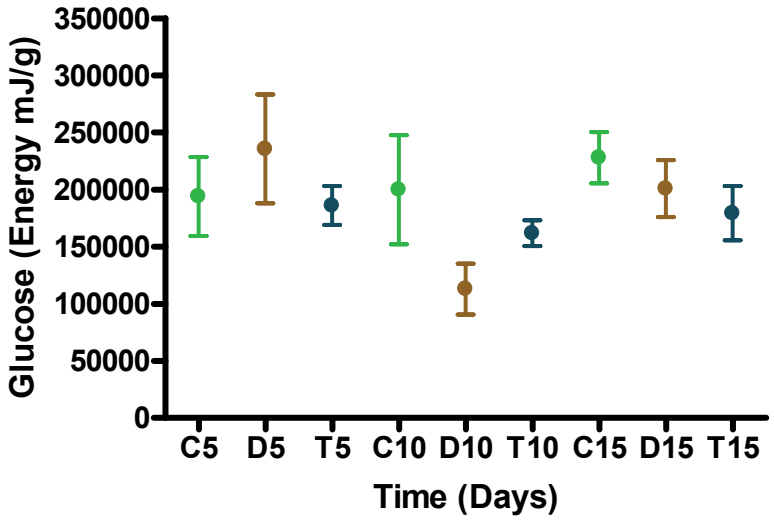


Figure 6.25: Representation of variation in glucose concentration within liver tissue of (C) Control, (D) Diethylstilbestrol and (T) Trenbolone acetate exposures groups. Readings represent 5 day exposures for 5, 10 and 15 days respectively.

With regards to protein availability (Figure 6.26), the Diethylstilbestrol exposure group shows a constant increase in protein concentration over the 15 day exposure time. At the same time the Trenbolone exposure group indicates a decline in energy availability from protein.

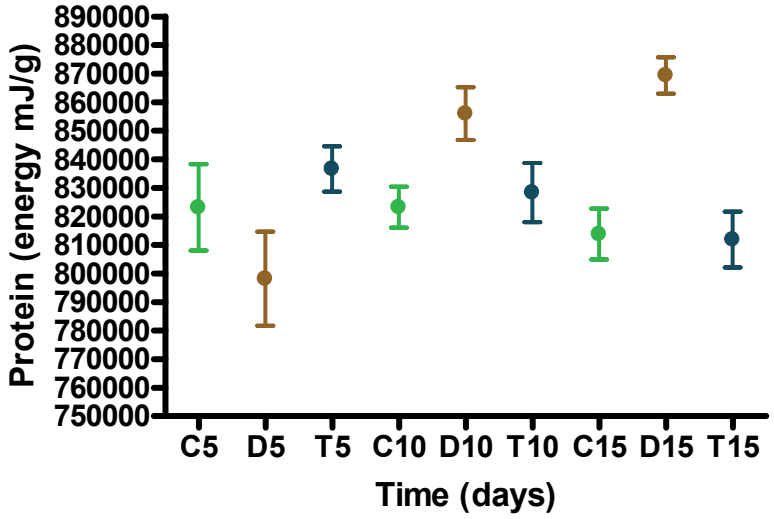


Figure 6.26: Representation of variation in protein concentration within liver tissue of (C) Control, (D) Diethylstilbestrol and (T) Trenbolone acetate exposures groups. Readings represent 5 day exposures for 5, 10 and 15 days respectively.

The energy available from lipids (Figure 6.27) shows a slight increase (5days), then a decrease (10 days) followed by a dramatic increase (15 days) for the Diethylstilbestrol exposure. The Trenbolone exposure indicates a constant decrease, in energy available for lipids, over the 15 day exposure period.

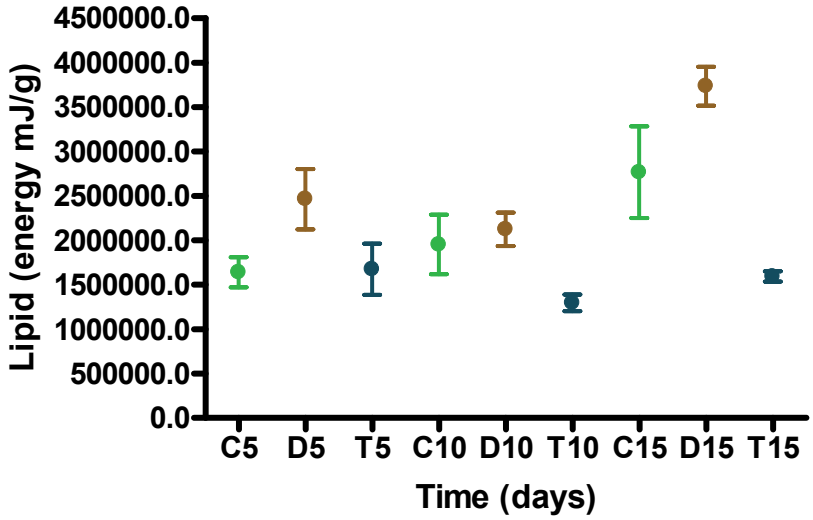


Figure 6.27: Representation of variation in lipid concentration within liver tissue of (C) Control, (D) Diethylstilbestrol and (T) Trenbolone acetate exposures groups. Readings represent 5 day exposures for 5, 10 and 15 days respectively.

Subsequently the energy available from glucose, protein and lipids relates to the Energy allocation for exposed organisms (Figure 6.28).

From this it is clear that the energy available for the Diethylstilbestrol group is higher than that of the Trenbolone group, which shows a dramatic decrease in energy available over the 15 day exposure period.

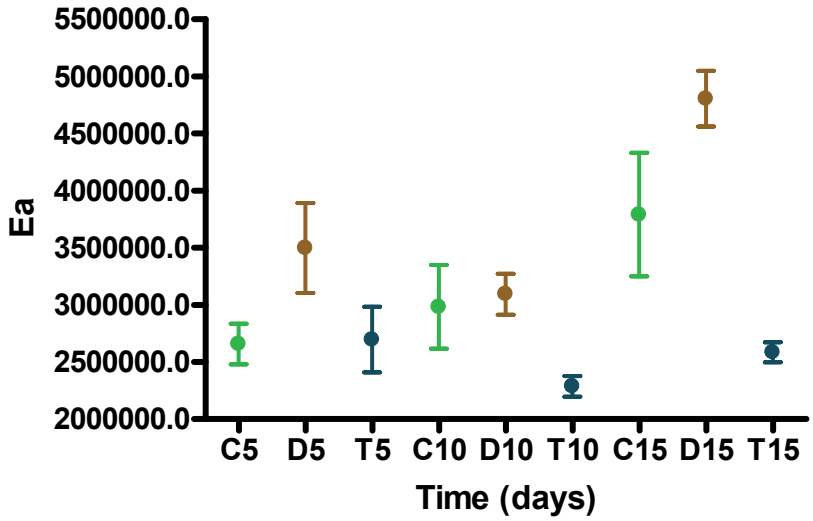


Figure 6.28: Representation of variation in Energy allocation within liver tissue of (C) Control, (D) Diethylstilbestrol and (T) Trenbolone acetate exposures groups. Readings represent 5 day exposures for 5, 10 and 15 days respectively.

The assumption with CEA is that “stressed” organisms consume more energy in the process of dealing with the external stress factor. Thus less energy will be available to deal with normal biological processes like growth and reproduction.

Keeping this in mind and referring to Figure 6.29 and Figure 6.30 it is clear that the exposure to Trenbolone caused much higher energy consumption when compared to the control and the Diethylstilbestrol group. Furthermore it can be seen that the energy consumed, for Trenbolone systematically increases over the 15 day exposure period. In contrast with the effects of the Trenbolone exposure, Diethylstilbestrol exposure shows an increase in CEA (Figure 6.30) over the 15 day exposure period. Indicating the additional production of lipids and protein, that is expected from an oestrogen or oestrogen-mimicking compound.

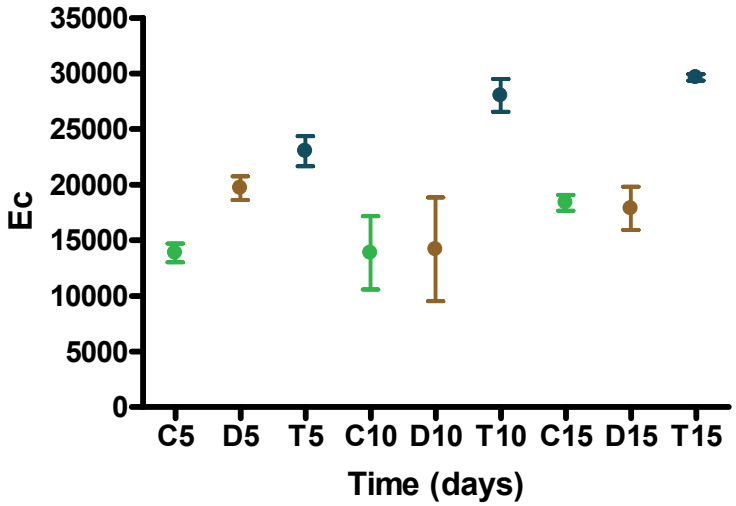


Figure 6.29: Representation of variation in Energy consumption within liver tissue of (C) Control, (D) Diethylstilbestrol and (T) Trenbolone acetate exposures groups. Readings represent 5 day exposures for 5, 10 and 15 days respectively.

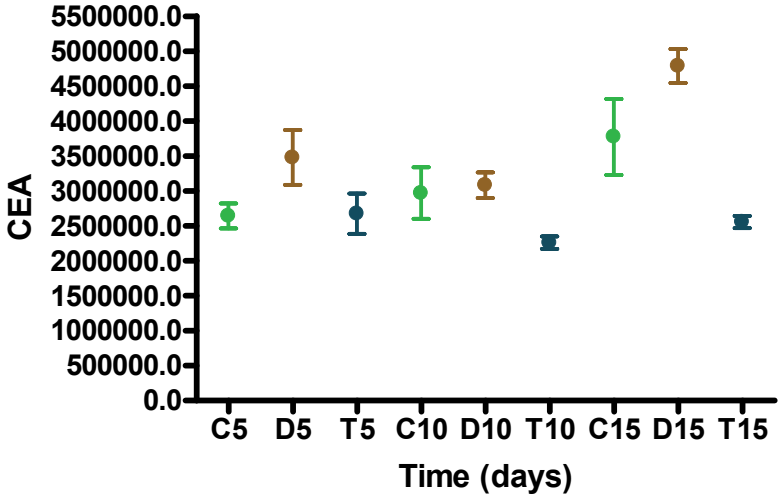


Figure 6.30: Representation of variation Cellular Energy Allocation within liver tissue of (C) Control, (D) Diethylstilbestrol and (T) Trenbolone acetate exposures groups. Readings represent 5 day exposures for 5, 10 and 15 days respectively

6.4.3.2 Metabolomics

Figure 6.31 shows a PCA for the blood plasma metabolite expression for three different exposure groups after a 5 day exposure. Data was log (V) transformed and normalised. No conclusive differences can be observed in metabolic expression of the blood plasma after 5 days of exposure. With the exception of fish C1 and TBA4, whose blood plasma metabolic composition shows similarities, the other fish from different exposure groups, ordinates together.

Figure 6.32 shows a similar PCA (10 days) than that of the 5 day exposure. With no observable differences in blood plasma metabolic composition of three exposed groups.

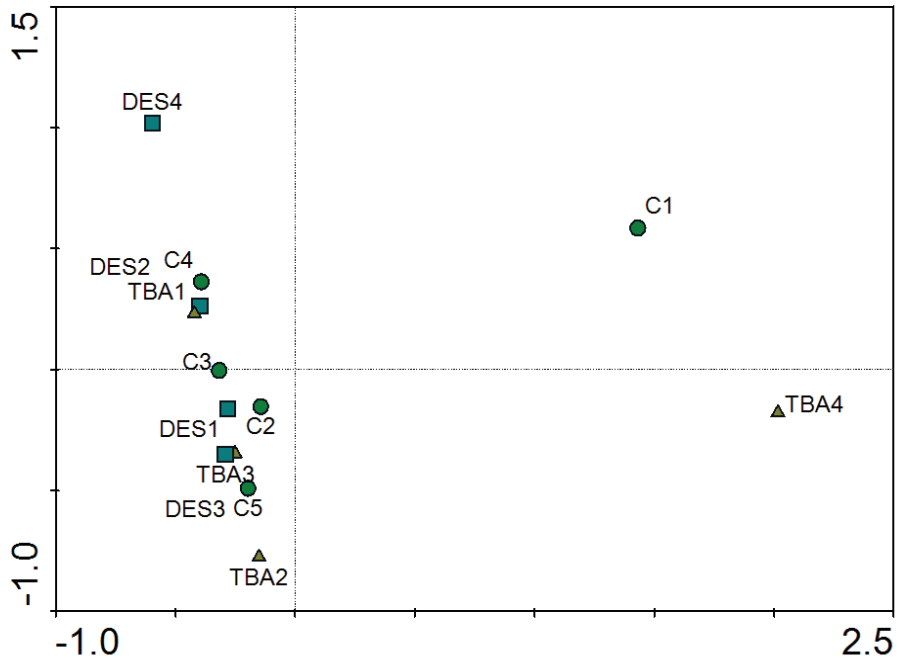


Figure 6.31: PCA representing the ordination of test organism based on (dis)similarities of metabolite excretion in blood plasma, of (C) Control, (DES) Diethylstilbestrol and (TBA) Trenbolone acetate exposure groups after a 10 day exposure period. ○ = Control, Δ = TBA, □ = DES

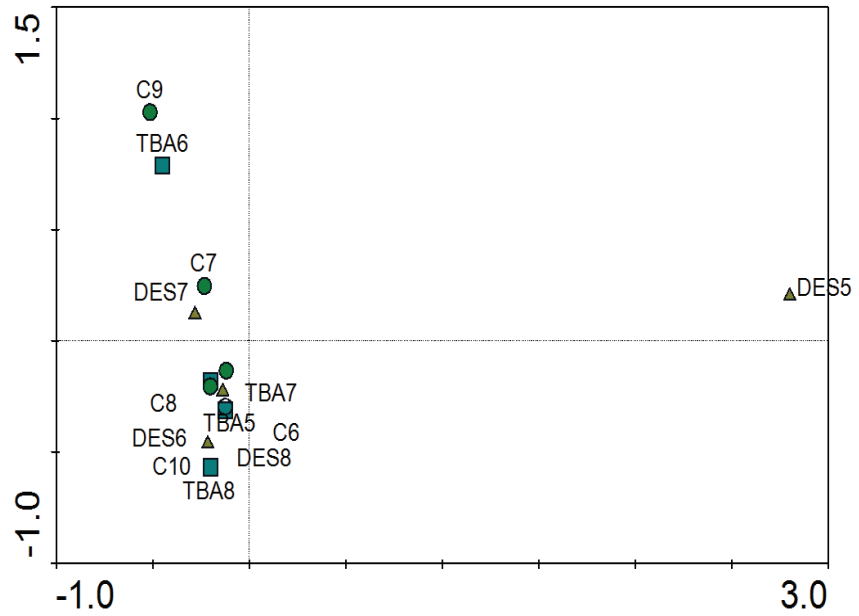


Figure 6.32: PCA representing the ordination of test organism based on (dis)similarities of metabolite expression in blood plasma, of (C) Control, (DES) Diethylstilbestrol and (TBA) Trenbolone acetate exposure groups after a 10 day exposure period. ○ = Control, Δ = TBA, □ = DES

This, however, is not the case after an exposure period of 15 days, where distinct similarities in metabolic expression can be seen for the Trenbolone exposure. The metabolites responsible for this ordination will still have to be identified and will be available in the final report.

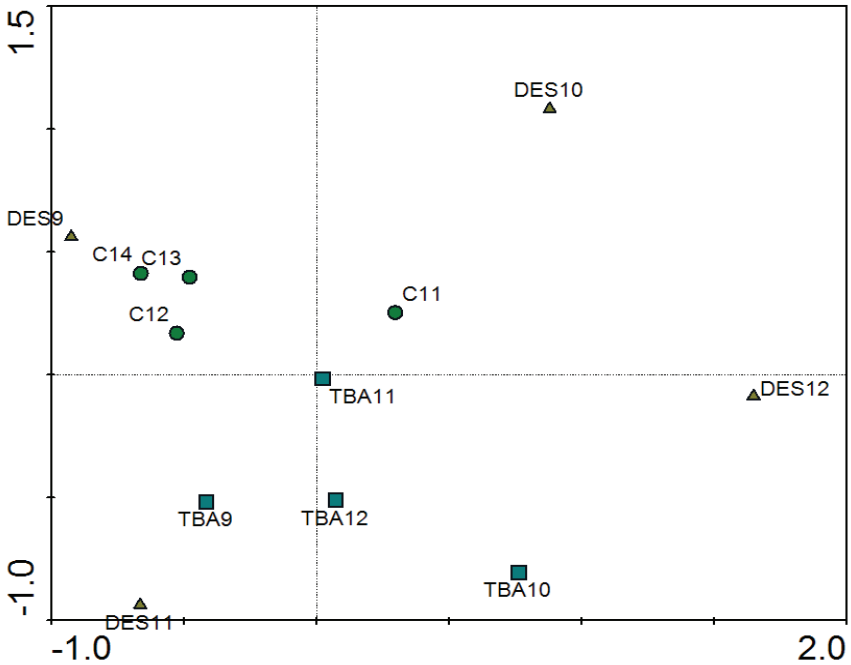


Figure 6.33: PCA representing the ordination of test organism based on (dis)similarities of metabolite expression in blood plasma, of (C) Control, (DES) Diethylstilbestrol and (TBA) Trenbolone acetate exposure groups after a 15 day exposure period. ○ = Control, Δ = TBA, □ = DES

6.5 CONCLUSIONS

6.5.1 Present Ecological State (PES)

With regards to the PES of upstream and downstream sites the following conclusions can be made:

- The periodical increases in the conductivity and pH of downstream sites associated with feedlots are probably caused by effluent from the feedlots. The most marked changes are observed in the river associated with Feedlot A. These observed changes show increases during the high flow survey, indicating a mobilisation of nutrients and not a dilution. No other potential sources of effluent were observed in the area that could affect the water quality of the streams associated with the feedlots.
- Metal concentration, with the exception of Pb, does not indicate seasonal or site perseverance and is subsequently not the result of feedlot activity. Pb concentrations, however, are periodically higher downstream from feedlot activity than upstream. With the exception of Mn, all metal concentrations are at levels of concern and much higher than the TWQR for aquatic ecosystems.
- SASS5 scores do show some changes at downstream sites with a smaller diversity and lower sensitivity scores. This was especially true during the high flow assessment.

6.5.2 Macro-invertebrate Community Structure

With regards to the macro-invertebrate community structure and composition the following conclusions can be drawn:

- Macro-invertebrate community structures (diversity and abundances) do differ at upstream and downstream sites associated with feedlot activities.
- At the same time clear seasonal differences can also be observed.
- Further interpretation is needed to ascertain which macro-invertebrate community structures are prominent downstream and which water quality variables are driving the compositions.

6.5.3 Biomarkers

As far as the biomarkers, for growth hormone exposure, are concerned. The following can be concluded:

- There is a significant difference in the CEA of the group exposed to Trenbolone acetate, compared to that of the Diethylstilbestrol exposure group. Trenbolone acetate exposed fish utilised more of their allocated energy suggesting a negative stress response.
- The CEA of the Diethylstilbestrol exposure group increases over the time of the exposure, indicating increased lipid and protein production in these fish. Suggesting a typical oestrogen exposure response.
- The application of metabolomics as a functional biomarker was done with some success. A dose response was obtained after exposure to a single concentration of both growth stimulants at set intervals.

The exposure to Diethylstilbesterol caused greater response both after 5 and 10 days of exposure. This was not the case with the Trenbolone acetate group, which reflected a slight sub-cellular response after 5 days of exposure.

- Furthermore the application of metabolomics aided in determining which metabolites are responsible for the sub-cellular response observed between exposed organisms. An increase in the exposure concentrations of Diethylstilbesterol and Trenbolone acetate would probably provide more marked effects.

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APPENDIX A: QUESTIONNAIRE FOR THE FEEDLOTS

Questionnaire for cattle feedlot owners

Introduction

The Onderstepoort Veterinary Institute, University of Pretoria and the University of Johannesburg joined forces in order to study the use of growth hormones in cattle feedlots and its effect on surface and groundwater. This is a confidential study in terms of the feedlots that participate. It is being undertaken since very little is known about the environmental effects of these substances. The South African Feedlot Association has requested Dr Johann van Niekerk to assist with field work since he is an experienced cattle feedlot environmental practitioner and his assistance will allow the least interference during fieldwork. Before filling out the questionnaire it is important to mention shortly what the questionnaire does not want to achieve and indeed what it wants to achieve:

What it does not want to achieve

- this study does not want to go into so many details as to reveal the specific administering programme in each feedlot and neither will this information be exposed to other feedlots.
- it is certainly not aimed at stopping the use of growth hormones and
- neither will feedlots be prescribed what practises to follow in the future

What does this study want to achieve

- to conduct a scientific study that is not a public participation process
- and eventually to safeguard meat safety and environmental water quality

The spinoff of this impact survey will certainly be the outcome of best management practises for the cattle feedlot industry. In the light of this introduction you are requested to fill out the questionnaire with the aid of our fieldworker and you are assured that your participation will be kept confidential.

Questions

1. Name of the feedlot
2. Province
3. Do you only keep cattle in feedlots?
4. When was the feedlot established?
5. Initial number of cattle standing?
6. Current number of cattle standing?
7. What % of cattle receives growth hormones?

8. How was the pen floor covered when the pens were constructed (e.g. do you have compaction or do you maintain a compacted layer of manure on the surface, etc.?)
9. Is the ground below the pens clay, sand or loam?
10. How many wells are in and around the pens (not further than 200 meters from the pens)?
11. Please assist us to draw the relative position and distance of wells, manure dams, clean water dams, rivers and wetlands in and around the feedlot on the separate paper that is provided?
12. Were there any geological or geohydrological studies conducted at your feedlot?
13. With how many percentage points does the feedlot slope from high to low ground and does the slope orientate towards a natural water body (describe)? One % is one meter drop for every 100 meters.
14. What was the ratio between male and female animals during the past 5 years?
15. Name the hormonal implants currently being administered?
16. Which of the following hormones were used in the feedlot and also indicate the period?

Hormone	*Year beginning (e.g. 1985)*	*Year ending (e.g. 1991 / to present)*
diethylstilbestrol		
17ß-estradoil		
Trenbolone		
Zeranol		
Zilpaterol		

17. What primary animal drugs are being administered at processing and pen hospitals?

Name of drug	Quantities per month	Number of months per year

18. Fill out the following table if antibiotics and Zilmax are being used in feed in your feedlot?

Name of antibiotic / substance in which antibiotic is mixed	% Of daily intake (relative to total daily intake volume) or quantities per animal per day	Number of days per administered per animal (e.g. 30, 60 or 120 days)

19. Do you implement an environmental management system such as ISO 14001 or EUREPGAP?

20. Provide the annual mean rainfall for your area?

21. If you possess holding ponds (or manure dams) for manure what is the total capacity?

22. What percentage of your runoff water is being irrigated onto pastures per annum?

23. What area is being planted by pastures?

24. What percentage of this area is irrigated with clean water and what % is being irrigated with effluent water?

25. What water source is being used to provide cattle drinking water?

26. If any, describe the water pre-treatment process that your effluent water undergoes (be it a series of connected ponds or chemically). Mention what percentage of annual effluent is treated. Name the chemicals that are used for water treatment (chlorine, copper sulphate or aluminium sulphate, etc.)?

APPENDIX B: SURFACE & GROUNDWATER SUSCEPTIBILITY TO CONTAMINATION: GEOGRAPHICAL INFORMATION SYSTEM ASSESSMENT